Host-parasite interactions: Snails of the genus *Bulinus* and *Schistosoma margrebowiei*

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ABSTRACT

In Africa the schistosomes that belong to the haematobium group are transmitted in a highly species specific manner by snails of the genus Bulinus. Hence the miracidial larvae of a given schistosome will develop in a compatible snail but upon entering an incompatible snail an immune response will be elicited which destroys the trematode.

The factors governing such interactions were investigated using the following host/parasite combination; Bulinus natalensis and B. nasutus with the parasite S. margrebowiei. This schistosome develops in B. natalensis but not in B. nasutus.

The immune defence system of snails consists of cells (haemocytes) and haemolymph factors. The haemocytes from the two snail species were compared on both a quantitative and qualitative level. Cell numbers were compared between species before and following infection with S. margrebowiei. The existence of sub-populations of haemocytes was examined using both functional tests and differential binding by lectins.

The triggering of the immune response requires that the invasive pathogen be recognised as foreign. Research in other fields of invertebrate immunology indicate that this may be mediated by factors, such as agglutinins, present in the haemolymph. In snails and schistosomes it is postulated that these factors recognise molecules on the surface of the larval schistosomes and the binding of factor to larvae elicits the immune response. The cell-free haemolymph of the snails was

investigated for the presence of haemagglutinins. A protein capable of agglutinating vertebrate red blood cells was found in the haemolymph of B. nasutus snails.

The surface carbohydrate and protein profiles of the different larval stages, miracidium, sporocyst and cercaria, were analysed using techniques of lectin binding and biotinylation of proteins followed by subsequent visualisation with labelled avidin. For these purposes the miracidia were transformed into sporocysts in vitro to ensure they were free of acquired snail proteins and sugars. The larvae were also investigated for possible interactions with the snail haemagglutinin. B. nasutus agglutinin was found to bind to the surface of S. margrebowiei miracidia but not to other larval forms.

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CONTENTS

FIGURES

TABLES

INTRODUCTION

Schistosomiasis is regarded as one of the most serious parasitic diseases known to man. It is endemic in 74 countries of the world and affects some 200 million people as well as their domestic livestock. (World Health Organisation report, 1987). This chronic debilitating disease is caused by digenetic trematodes belonging to the genus Schistosoma.

The adult flukes live in the blood vessels of their vertebrate (definitive) hosts, however their life-cycle is a complicated one and involves an intermediate host where asexual reproduction takes place. For all species of schistosome the intermediate hosts are aquatic or semi-aquatic gastropod molluscs and water is the medium required for the transmission to both the definitive and the snail hosts.

In developing countries where this disease is prevalent, the economies are largely agriculturally based. The people most at risk are farm labourers and villagers whose livelihood depends on almost daily contact with contaminated water. In recent years the disease has spread due to the construction of dams and irrigation channels that have provided new breeding grounds for the snails.

Control of the disease requires a co-ordinated approach encompassing four main areas:- water management, to provide piped water and sanitation; the development of drugs and vaccines; health education; the control of the intermediate hosts through molluscicides. This fourth area requires the detailed knowledge of not only the ecology of the snail but also its interaction with the parasite. It is this

FIGURE 1

The Life-cycle of trematode parasites

belonging to the genus Schistosoma

A Eggs. Schistosme eggs are voided from the vertebrate host in either the faeces or the urine. The eggs shown are representative of the three major schistosome groups.

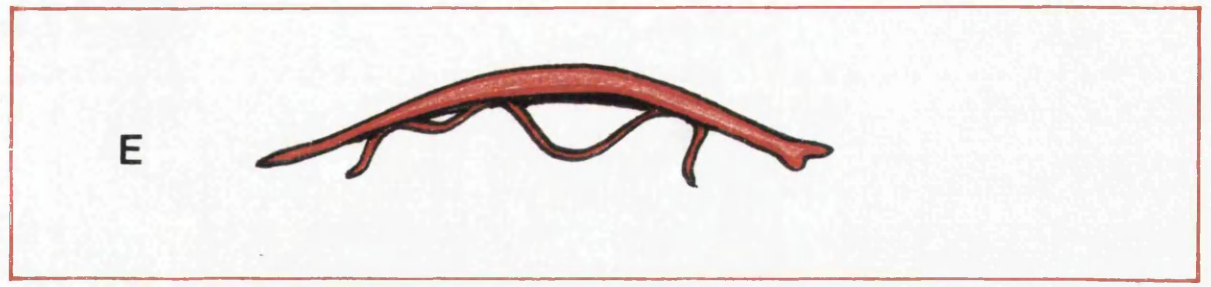
B Miracidium. In fresh water the eggs hatch into a free-living larval form, the miracidium. The miracidium is the infective form for the molluscan, intermediate, host.

C Snails. The miracidia enter the snails and undergo rounds of asexual reproduction. The three major groups of schistosomes use snails of different genera as their intermediate hosts.

D Cercariae. After multiplying within the snail the schistosomes produce a second free living larvae, the cercariae. They are released from the snail into the water and are the infective form for the vertebrate host.

E Mature schistosomes. On entering the vertebrate host the larvae transform into schistosomulae. Each schistosomulum develops into an adult, either male or female. The worms pair with the male holding the female in a longitudinal groove, the gynaecophoric canal. Different species of schistosome infect different vertebrates including man, cattle, wild antelope and rats.

Drawings not to scale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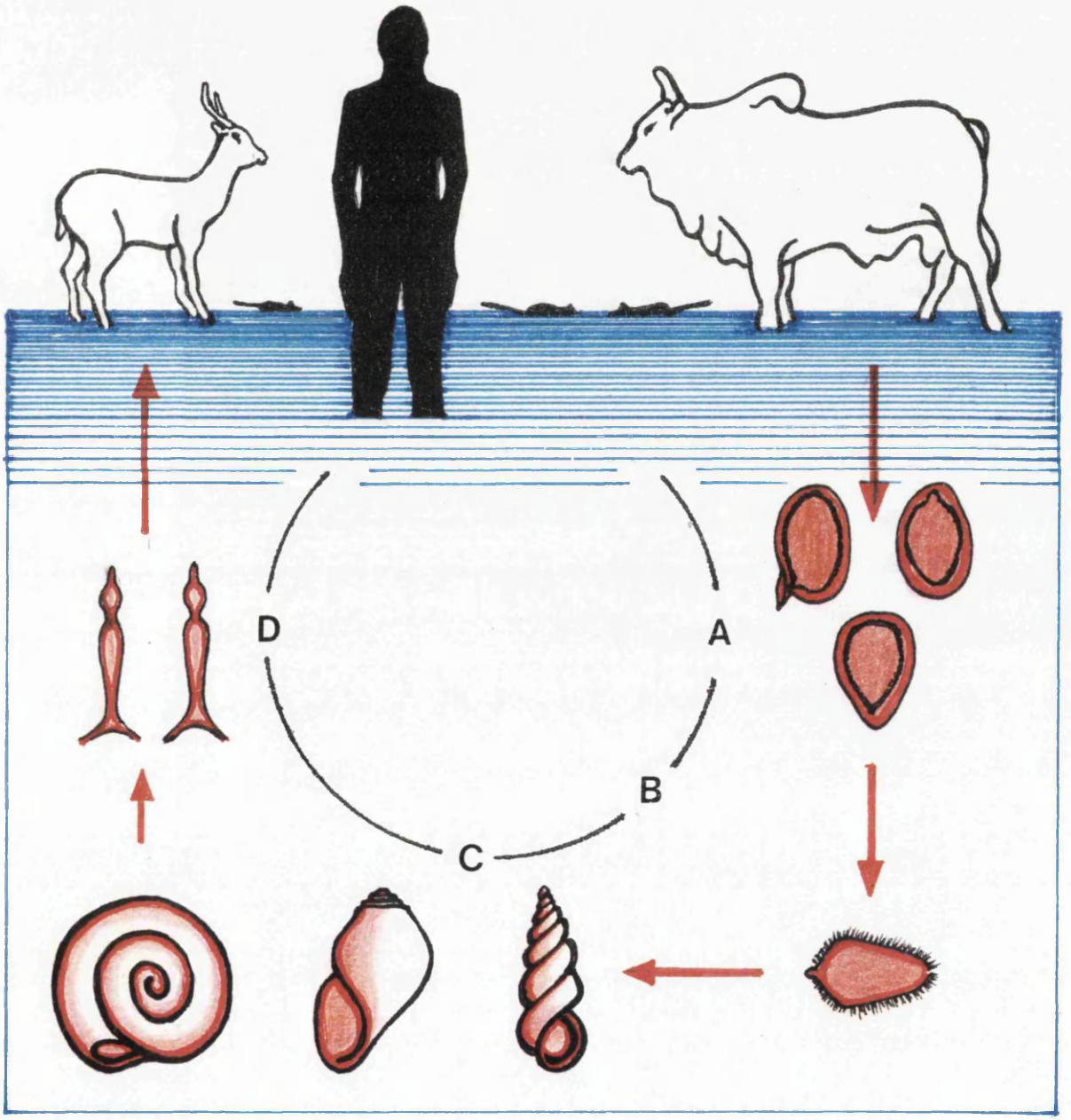
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snail/schistosome interaction that forms the basis of this dissertation.

The economic importance of schistosomiasis has resulted in much research covering the different aspects of the disease. The current status of which is summarised below.

1 Schistosome Life Cycle

The details of the life cycle differ between the different species of schistosome but all follow the same general plan (figure 1). The eggs (figure 2a) are fully embryonated and hatch quickly on reaching fresh water. The egg membranes are permeable to hydroxyl ions and the influx of water causes the egg to swell and these membranes rupture (Kusel, 1970). Following rupture of the egg membrane the miracidium is released. The miracidium (figure 2b) is the first of the larval stages and the infective form for the molluscan host.

Miracidia vary in size depending on species but are, on average, about 150μm long and 50μm wide. They are covered in cilia and the specialised ciliated epidermal cells are arranged in tiers along the length of the miracidium. Between each tier is a non-ciliated layer, the intercellular ridge, where sensory receptors and excretory ducts emerge (Southgate and Knowles, 1977). The anterior end bears an apical papilla which consists of highly folded non-ciliated epidermal membrane rich in sensory organelles and the site where the penetration glands open (Jourdane and Théron, 1987). The miracidium is equipped with various glands whose secretions contain histolytic enzymes that aid penetration. A nervous

FIGURE 2

Stages in the life-cycle of Schistosoma margrebowiei

A Egg with terminal spine

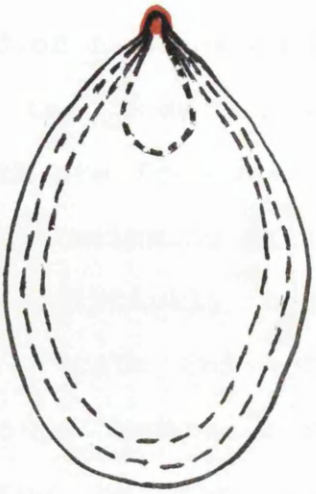
B Miracidium, diagram to show ciliated epidermis and the position of the intercellular ridges.

C Sporocyst. On transformation the cilia are lost, the early sporocyst is oval and germinal cells are visible towards the posterior end of the sac.

D Cercaria. The body and tail of the cercaria are covered in spines.

E Adult worms. The female worm (red) is held within the gynaecophoric canal of the male (blue).

A



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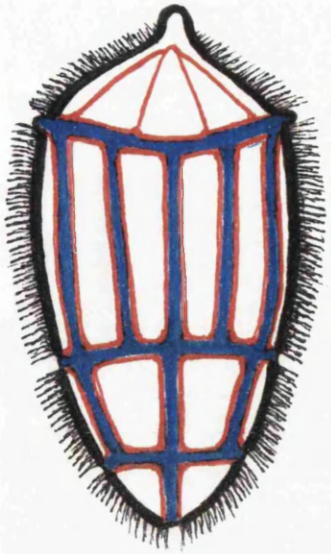

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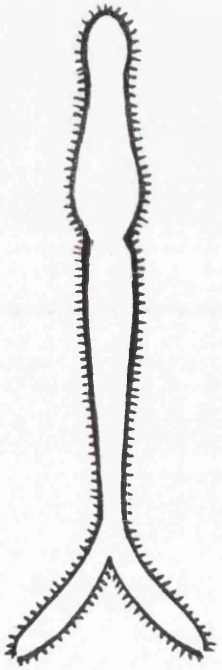

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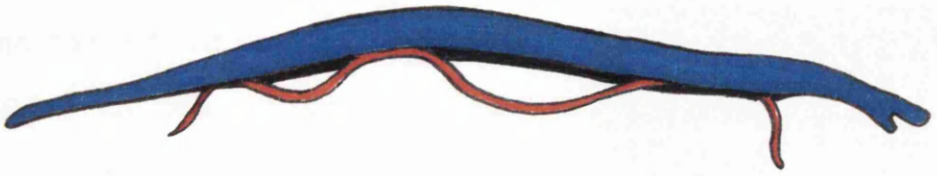


system is present which consists of a neural mass which innervates the sensory receptors. The excretory system is composed of flame cells and excretory ducts and the posterior end of the body contains embryonic germinal cells. The miracidia are free living larval forms, which are active and capable of swimming at rates of 2mm s^{-1} (Smyth, 1966). However they are relatively short lived (8-15 h) and during this time have to locate and enter their snail hosts. Correct host location is important since each species of schistosome is restricted by the species of snail in which it can successfully develop. In order to achieve this, the miracidia have developed behaviour patterns that correspond to the ecology of their hosts. For example, one of the molluscan hosts of Schistosoma mansoni is the snail Biomphalaria glabrata. This snail spends most of its time at the surface of the water attached to floating vegetation. The miracidia of S. mansoni are positively phototactic and negatively geotactic (Chernin and Dunavan, 1962), and immediately swim to a location likely to be inhabited by these snails. The miracidia of Schistosoma haematobium that parasitise Bulinus globosus snails are negatively phototactic at temperatures above 18°C. At these temperatures the snails inhabit the bottom of ditches or are to be found in areas of shade. If the temperature drops the snails move to surface un-shaded water and the miracidia become positively phototactic (Shiff, 1974).

Once in the correct site the miracidia begin a 'scanning phase'. They swim in long sweeping lines until they encounter chemicals (miraxones) in the water of snail origin, (Chernin

1970). These chemicals cause the miracidia to increase their turning rate, thus keeping the miracidium in the vicinity of the snail and increasing the chance of an encounter between them. These behavioural adaptations bring the miracidium into the ecological location of the correct host but the actual penetration appears to be non-specific and in the laboratory miracidia have been shown to attempt entry into agar blocks (Wright and Southgate, 1981). Entry is not immediate on contact with the snail and there first occurs an exploration of the surface for preferred entry sites, usually in the foot. Then the folded membrane structure at the apical papilla acts as a sucker to hold the miracidium in place (Jourdane and Théron, 1987). Penetration is the result of mechanical boring and the release of histolytic enzymes (Wadji, 1966).

If the miracidium has entered a compatible snail then transformation to the second larval stage, the mother sporocyst, takes place. For most schistosomes this occurs near the entry site but the miracidia of Schistosoma japonicum migrate deeply into the tissues before transformation. This strategy possibly allows for the development of more miracidia since the snails are known to harbour as many as 32 miracidia (Jourdane and Xia, 1987), whereas in B. glabrata the intermediate host of S. mansoni only 8 miracidia may develop and any miracidia exceeding this number are destroyed by the snail's immune system (Kassim and Richards, 1979). The first stage of miracidial transformation entails the shedding of the ciliated epidermis. This is in line with the change of function for the outer tegument from one of motility to one of

nutrient absorption. Schistosomes do not shed their ciliated plates on entry into the snail but do so within the snail tissue. With the loss of the ciliated plates a tegument forms to cover the sporocyst and the apical and sensory papillae are lost. Initially the sporocyst is an oval sac (figure 2c) but as development continues the morphology changes and the mature mother sporocyst resembles a convoluted tube. The mother sporocyst is responsible for the first round of asexual multiplication that takes place in the snail. The germinal cells present in the miracidium multiply and take up more and more space within the sporocyst. As development continues the germinal cells stop dividing and begin to differentiate, with each cell becoming a second stage, daughter, sporocyst. When the daughter sporocysts are mature, the tegument of the mother sporocyst ruptures and they are released. At the time of release each daughter sporocyst contains 50-100 germinal cells (Schutte, 1974). Further development does not occur until the sporocysts have migrated through the snail tissue to the hepatopancreas. The next stage of the life cycle is one of cercariogenesis. Successive cell divisions within the daughter sporocyst produce a morula, i.e. an outer epithelium surrounding the ball of cells. Differentiation into the final larval form, the cercaria, then occurs. The mature cercariae (figure 2d) are divided into two distinct parts, the body and the tail. The body consists of a muscular head organ, various penetration glands and a prominent ventral sucker, the acetabulum. The muscular tail ends in two furcae and enable the parasite to swim. The excretory system consists of flame

cells in both body and tail with excretory ducts running the length of the cercaria and terminating in pores at the furcal tips.

The production of cercariae in most species of schistosome occurs daily and can proceed for several months and often stops only with the death of the snail. The number of cercariae produced depends on the species of schistosome, the degree of compatibility between parasite and snail host and the physical state and age of the snail.

It has been reported that the daughter sporocysts of several species of schistosome are capable of sporocystogenesis producing secondary daughter sporocysts (Kechemir and Théron, 1980; Jourdane, Théron and Combes, 1980; Touassem and Théron, 1986). It is not yet known whether this is true for all species of schistosomes.

On completion of their development the cercariae escape from the daughter sporocyst. They do not leave the snail immediately but remain within the mantle cavity until an external trigger causes them to be shed. The shedding of the cercariae takes place daily with generally one emission per day. The most important factor influencing the timing of this emission appears to be the photoperiod, as artificial inversion of the photoperiod produces an immediate inversion of the rhythm (Glaudel and Etges, 1973). The response to light is the property of the schistosome itself and occurs regardless of snail species (Mouahid and Théron, 1986). The timing of this release once more shows behavioural adaptations of the schistosome that optimise the chances of encountering

their favoured hosts. This is well illustrated in the different strains of S. mansoni found on the island of Guadeloupe. In urban areas where transmission is adequately maintained through man, the schistosome has an 'early' daily shedding pattern. For most areas, the parasite relies on both man and the reservoir host Rattus rattus. Here S. mansoni displays an 'intermediate' shedding pattern. There is also one focus where 80% of transmission occurs via the rat and these schistosomes have a 'late' shedding pattern (Théron, 1985). The schistosome S. margrebowiei is one of the few schistosomes to have two emission peaks, one at dawn and the other at dusk. This release strategy correlates strongly with the behaviour of their vertebrate hosts, the antelope, that visit the water pools at these times. (Pitchford and Dutoit, 1976).

Like the miracidia, the cercariae are adapted to life in fresh water and host invasion. They are motile and swim by means of their muscular tails. Since they are non-feeding they have a relatively short life span. The cercariae of most species of schistosome are positively phototactic and negatively geotactic. On release from the snail they swim to the surface of the water where they remain stationary. In fast flowing water they actively swim to maintain this position and this uses up their glycogen reserves rapidly. Therefore the larvae in such waters are correspondingly shorter lived. This is also true for warmer waters, as the higher temperatures cause the cercariae to move more rapidly (Webbe, 1982). There is no evidence that chemotaxis is involved in host location, and penetration of the mammalian skin appears to be

non-specific. On contact with a vertebrate host the cercaria attaches itself to the skin by means of the acetabulum. Histolytic enzymes are secreted from the penetration glands and the cercaria burrows through the tissue. The tail is shed on penetration and the tailless cercaria is known as a schistosomulum. Within the host epidermal tissue, the transformation from cercaria to schistosomulum continues with the shedding of the glycocalyx. The schistosomulum is now no longer able to survive in fresh water and the transformation continues with the formation of a new outer membrane, the tegument. This is composed of two closely opposed lipid bilayers (McLaren and Hockley, 1977).

The schistosomula travel through the subcutaneous tissue to the peripheral vessels of the blood or lymphatic system and are carried to the lungs. Schistosomula remain in the lungs for several days. They do not actively feed but there is evidence of nutritional uptake across the tegument at this time (Stirewalt, 1974). Growth and maturation do not begin until the schistosomula have left the lungs and have arrived in the liver. In the liver the schistosomula feed on red blood cells and in vitro studies indicate that erythrocytes are essential for the continued development of the schistosomes (Clegg and Smithers, 1972; Foster and Bogitsh, 1986). The absorption of nutrients across the tegument still continues and this is thought to be the major route for the uptake of glucose and amino acids. Schistosomes are dioecious and the male has a long ventral groove known as the gynaecophoric canal. When mature the worms pair and the 'threadlike' female

is held within this canal (figure 2e). After the worms have paired they leave the liver and migrate to the blood vessels of their final destination, either the mesenteric veins or the veins of the vesicle plexus, depending on the species of schistosome.

Once in the blood vessels, the paired worms start producing fertilised eggs and continue to do so throughout their lives which, can be as long as 15 years. Egg production varies with species. A single pair of S. mansoni worms produces approximately 300 eggs per day and a pair of S. japonicum worms, about 3,500 (Webbe, 1982). When the eggs are laid they are non-embryonated and develop in the host's tissues. Mature eggs migrate across the tissue layers in order to gain access to the lumen of the bowel or the bladder. They are then voided with the faeces or the urine.

2 Schistosomiasis: Human Pathology

The initial invasion of the skin by the cercariae can cause dermatitis but this is usually the case when non-human schistosomes have attempted to enter the skin. This is an immune reaction and is specific for each species of schistosome (Colley, Magalhãe-Filho, Coelho, 1972). In permissive hosts there is little or no reaction to this invasion. The post-invasion transformation of a cercaria into a schistosomulum presents a different surface to the vertebrate immune system. Remarkably, this surface itself continues to change as the schistosomulum matures. The outer membrane of the adult worm has been shown to have a high turnover rate and incorporates antigens of host origin

including molecules of the major histocompatibility complex (MHC).

The disease schistosomiasis as it affects the human host can take one of two forms. In a small number of individuals, infection produces a severe reaction known as acute schistosomiasis which quickly proves to be fatal (Warren, 1982). However the majority of infected individuals have long lasting chronic infections with progressively debilitating pathologies. The symptoms are not due to the presence of the adult worms but to their eggs. Of all the eggs laid, only approximately 35% mature and successfully escape their hosts. The remaining die within the tissues of the intestinal or bladder walls and many are swept back in the blood stream to become lodged in the liver and the spleen. These eggs become the target for the host's immune responses and the resulting immunopathology accounts for the disease.

The tissue surrounding the trapped eggs becomes inflamed due to the presence of large numbers of lymphocytes, macrophages and eosinophils (James and Colley, 1975). This inflammatory response causes much tissue damage and, especially in the liver and spleen, blocks local blood flow. New blood vessels do develop, but these are only arterial in nature. Later, the damaged tissue becomes fibrous, due to the deposition of collagen, and is then known as a granuloma. Granulomatous tissue causes loss of elasticity in the bladder or intestinal wall and as the disease progresses the tissue can become calcified. (Warren, 1982). Granulomas form around the individual eggs but after several weeks of infection their

size reduces and the progression of the disease is then due to the on-going production of eggs by the worms. Despite living in the blood vessels the adult worms appear to be unaffected by the host immune response. This is thought to be due to the ability of the parasite to take up and express host antigens in their surface membranes thereby evading the immune response by masquerading as 'self'. The severity of the disease is related to the number of paired worms being harboured but there is evidence to suggest that patients suffering chronic infections become resistant to further parasitic invasion (concomitant immunity). This resistance is not well marked in young children who tend to have high worm burdens.

Research into schistosomiasis during the last ten years has done much to explain the phenomena of granuloma formation and its subsequent reduction, together with acquired resistance and its absence in young children. Much of the information has been obtained by studying the course of infection in mouse and rat models. This has shown that the immune response to schistosomiasis is highly complex and multifactorial involving almost every aspect of the vertebrate immune system.

The infection of mice with S. mansoni produces a disease with many similarities to that found in humans. Early studies using the mouse, revealed that it was the host inflammatory granulomatous reaction to the eggs that led to the disease syndrome (Warren, 1961). Schistosome eggs are laid in an non-embryonated state and the miracidia develop whilst the egg is in the host tissues. On maturation, the miracidium within

the egg starts to produce soluble egg antigens (SEAs) and secretes them through microscopic pores in the eggshell (Stenger, Warren and Johnson, 1967). These antigens include enzymes that assist in egg migration through the tissues. The immune response elicited by these antigens is a delayed type hypersensitivity reaction (DTH), i.e. it is an anamnestic cellular response mediated by T lymphocytes. This response is largely schistosome specific, although there is some cross-reaction with S. haematobium eggs (Warren, Domingo and Cowan, 1967). The cellular granulomas produce lymphokines including macrophage migration inhibitory factor and eosinophil stimulation promoter (Boros, Warren and Pelley, 1973; James and Colley, 1975). Eosinophils have been shown to be capable of egg destruction and the use of anti-eosinophil serum in mice leads to the accumulation of large numbers of eggs. This results in increased mortality (Olds and Mahmoud, 1980). The effector mechanisms involved in granuloma formation are modulated by suppressor T lymphocytes but the reduction in size of the granulomas seen in chronic infections appears to correlate with the appearance of anti-idiotypic suppressive T cells (Abe and Colley, 1984). Despite the lack of a suitable rodent model for S. haematobium the mechanisms for granuloma formation and modulation appear to be similar. Infection with S. japonicum differs in that there appears to be an antibody component in granuloma formation (Warren, Grove and Pelley, 1978) and anti-idiotype antibodies are highly suppressive of this formation (Stavitsky, 1987).

Mice with chronic infections of S. mansoni have also exhibited some degree of resistance to super-infection. When this immunity was studied in more detail it was found to be due to the non-specific shunting or trapping of larvae into areas of egg induced pathology. It was not antibody dependent and did not appear to mirror the resistance seen in humans (Wilson, Coulson and McHugh, 1983).

Rats of the Charles River Fischer strain are non-permissive hosts for parasitism by S. mansoni. Experimental infection initially follows the same route as that seen in mice but only for the first three weeks, there then follows a self-cure which produces a long term resistance to reinfection (Phillips, Reid and Sadun, 1977). Superficially this appears to have little in common with the human disease but examination of the humoral aspects of resistance have shown many similarities with concomitant immunity exhibited by humans. Capron and co-workers have used this model to investigate the humoral response to schistosomiasis (Capron, Dessaint, Capron, Ouma and Butterworth, 1987).

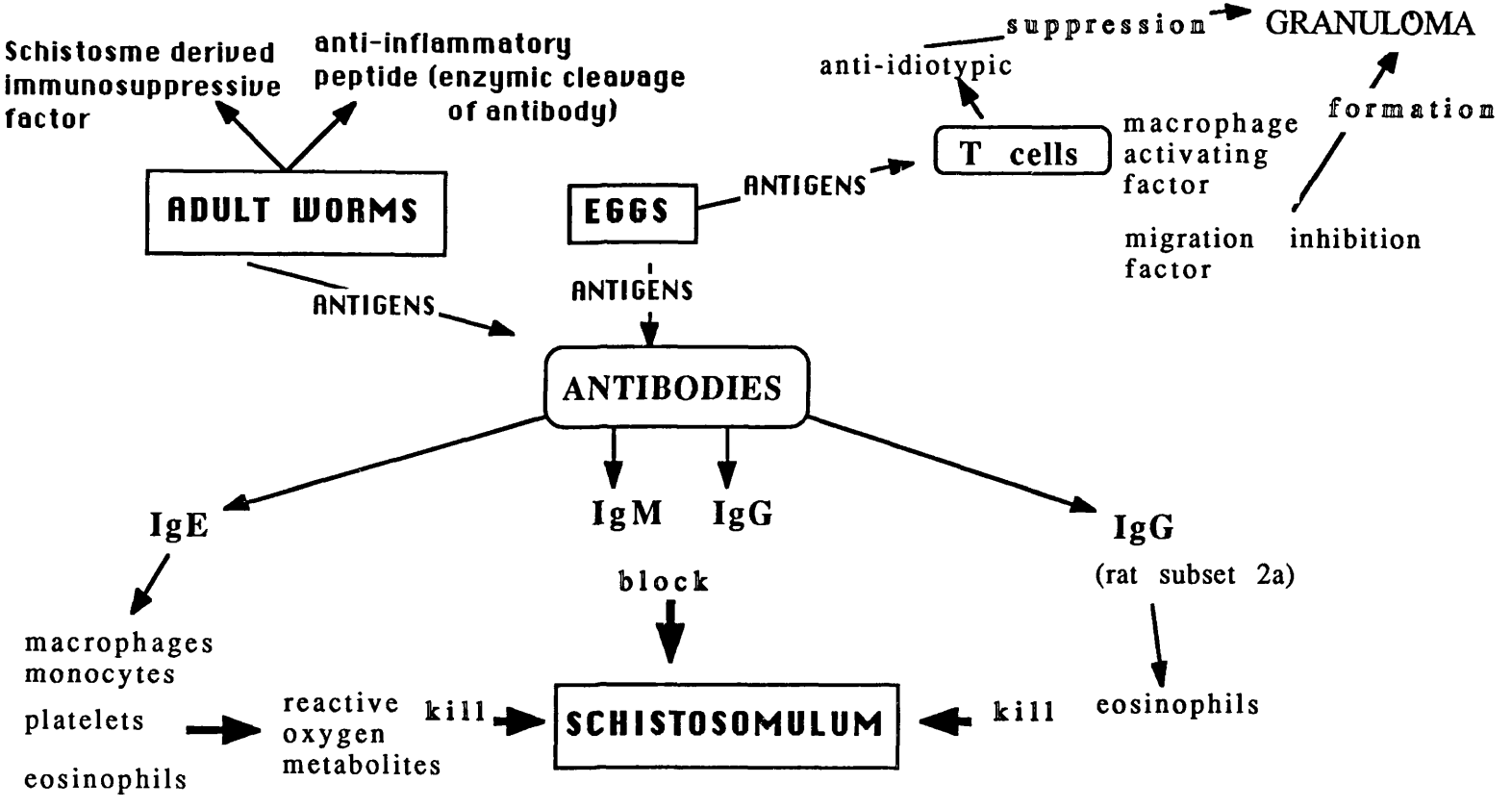
The adult worms secrete/excrete soluble antigens that are highly immunogenic. The antibodies produced to these antigens induce a cytotoxic destruction of the schistosomulum to which the adults are not susceptible. Antibodies produced in response to secreted egg antigens (SEAs) have also been shown to cross-react with epitopes on the schistosomulum surface. Thus both the adult worms and the eggs secrete soluble antigens and are constantly stimulating the host's immune response. Although the schistosomulum soon loses these

epitopes it is thought that this antibody-dependent cell-mediated cytotoxicity (ADCC) reaction is responsible for concomitant immunity. The network of responses is still not fully understood but those reported for S. mansoni infection of rats, mice and humans is outlined in figure 3.

The immunity to infection as manifested by schistosomulum killing has been found to be a T cell dependent antibody response but with non-lymphoid effector cells. These effector cells are monocytes, macrophages, eosinophils and platelets. Of the antibodies produced only those of the IgE isotype were found to induce schistosomulum killing. Patients harbouring chronic infections have been found to have very high levels of IgE in their sera. Further investigations led to the discovery that a specific sub-set of each of these effector cells existed which could be distinguished by a novel Fc receptor in the membrane. This receptor has been designated FcεRII to distinguish it from the FCεRI receptor found on basophils and mast cells. The binding of IgE to these receptors elicits a different response to that hitherto observed in antibody binding to non-lymphoid cells eg. platelets have receptors for both IgG (FcγR) and IgE (FcεR). Binding of IgG causes a release of serotonin which has little effect on schistosomula, whereas the binding of IgE causes the release of reactive oxygen metabolites that are able to kill parasites.

Two other mechanisms have been found to destroy schistosomula. These are the direct activation of macrophages by T cells and the binding of IgG to the surface of eosinophils. The eosinophil reaction in the rat is known to be

FIGURE 3 Immune Responses of vertebrates to infection with *S. mansoni*



mediated by a specific sub-set of IgG antibody, IgG2a, which previously has been shown to be anaphylactic in this animal, (Capron, Nogueira-Queiroz, Papin and Capron, 1984). The reaction has also been observed with IgG antibody from human serum but the subclass is not known (Butterworth, Taylor, Veith, Vadas, Dessein, Sturrock and Wells, 1982).

The stimulation of the immune system by the secreted antigens of adult worms and eggs produces antibodies of all sub-classes. Antibodies of the sub-classes IgG and IgM are especially important. These antibodies have been shown to bind to the schistosomulum surface and block the binding of IgE. This inhibits the ADCC reaction and hence resistance to super-infection. The fact that children have higher levels of the blocking IgM antibodies than do adults is thought to account for their inability to resist compound infections of schistosomes (Capron, Dessaint, Capron, Ouma and Butterworth, 1987).

The study of schistosomiasis has led to a greater understanding of the vertebrate immune system. It also raises the possibility of producing a vaccine that would potentiate IgE production to epitopes on the schistosomulum surface. One potential immunogen is the peptide p28. Antibodies raised to this protein have been shown to cross-react with other schistosome species (Capron, Dessaint, Capron, Ouma and Butterworth, 1987).

3 Schistosome Taxonomy

There are 18 species of schistosome that can infect mammals but the successful outcome of infection is dependent on the compatibility between the parasite and its hosts. This is especially true for the infection of the molluscan hosts since each species of schistosome is restricted by the species of snail in which it can grow and multiply. This species specificity is used as one of the parameters on which to base the classification of schistosomes. The schistosomes found throughout the world are divided into four species groups based on the genus of snail used as intermediate host and the zoographical area that they inhabit. These groups are:-

(i) The S. haematobium group. These schistosomes are found in Africa and some adjacent regions. Three species are known to infect man, S. haematobium, S. intercalatum and to a lesser extent S. mattheei. S. bovis also belongs to this group and is economically important because it infects domestic livestock. Transmission is via snails of the genus Bulinus (though in Spain S. bovis is transmitted by Planorbarius metidjensis). The eggs of these schistosomes bear a terminal spine.

(ii) The S. mansoni group. S. mansoni itself is the only member of the group that infects man. This schistosome is widespread Africa and the Caribbean and is responsible for schistosomiasis in South America. Other members of the group are only found in Africa and parasitise wild animals. The distribution of these schistosomes overlaps with those belonging to the S. haematobium group but they can be distinguished by their eggs which have a lateral spine and by

their intermediate hosts which are snails belonging to the genus Biomphalaria.

(iii) The S. indicum group. These schistosomes occur mainly on the Indian sub-continent and parts of S.E.Asia. They are parasites of the artiodactyls and use snails belonging to the genus Indoplanorbis as vectors. One species, S. incognitum also infects rodents and carnivores and is transmitted by snails of the genera Radix and Lymnaea.

(iv) The S. japonicum group. This group infects a variety of domestic and wild animals, as well as man (Mott, 1982). They are distributed throughout S.E. Asia, China, Japan and the Philippines. These schistosomes differ in certain respects from those in the other groups. Firstly, they show a lack of species specificity, apparent in their wide range of definitive hosts. Also they produce up to ten times as many eggs, better able to withstand desiccation (Garcia, 1976). In the snail up to 4 times as many miracidia can develop (Jourdane and Xia, 1987). In cases of S. japonicum induced schistosomiasis, it has been demonstrated that egg immunopathology is the result of humoral (antibody) responses (Stavitsky, 1987). This differs from infections with S. haematobium and S. mansoni where the eggs elicit a DTH (T lymphocyte) reaction. The intermediate hosts of S. japonicum are amphibious snails of the genus Oncomelania. S. mekongi and S. sinensium are transmitted via snails of the genus Tricula. Schistosome species together with their snail and definitive hosts are given in Table 1.

TABLE 1

DETAILS OF SCHISTOSOMA SPECIES, THEIR SNAIL HOSTS, DISTRIBUTION AND THEIR DEFINITIVE MAMMALIAN HOSTS.

Schistosoma species	Genus of snail host	Continental distribution	Mammalian Orders / important hosts
S. haematobium group			
S. haematobium (Bilharz, 1852) Weinland, 1858	*Bulinus*	Africa and adjacent regions	Primates
S. intercalatum Fisher, 1934	*Bulinus*	Africa	Primates
S. mattheei Veglia and Le roux, 1929	*Bulinus*	Africa	Artiodactyla Primates
S. bovis (Sonsino, 1876) Blanchard, 1895	*Bulinus/ Planorbarius*	Africa and adjacent regions	Artiodactyla
S. curassoni Brumpt, 1931	*Bulinus*	Africa	Artiodactyla
S. margrebowiei Le Roux, 1933	*Bulinus*	Africa	Artiodactyla
S. leiperi	*Bulinus*	Africa	Artiodactyla
S. mansoni group			
S. mansoni Sambon, 1907	*Biomphalaria*	S.America Caribbean Africa Madagascar	Primates Rodentia
S. rodhaini Brumpt, 1931	*Biomphalaria*	Africa	Rodentia Carnivora
S. edwardiense Thurston, 1964	*Biomphalaria*	Africa	Artiodactyla
S. hippopotomi Thurston, 1963	?	Africa	Artiodactyla

Continued.....

.....Continued

S. indicum group

S. indicum	*Indoplanorbis*	India, S.E.Asia	Artiodactyla
Montgomery, 1906		Sri Lanka	
S. spindale	*Indoplanorbis*	India, S.E.Asia	Artiodactyla
Montgomery, 1906		Sri Lanka	
S. nasale	*Indoplanorbis*	India, Sri Lanka	Artiodactyla
Rao, 1933			
S.incognitum	*Lymnaea*	India	Rodentia, Carnivora
Chandler, 1926	*Radix*	S.E.Asia	Artiodactyla

S. japonicum group

S. japonicum	*Oncomelania*	China, Japan	Primates, Rodentia
Katsurada, 1904		Philippines	Carnivora
		Taiwan	Perissodactyla
		Indonesia	Artiodactyla
S. mekongi	*Tricula*	S.E.Asia	Primates
Voge, Bruckner &			Carnivora
Bruce, 1978			
S. sinensium	*Tricula*	S.E.Asia	Rodentia
Pao, 1959			

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4 Invertebrate Immune Response

The elements typified by the vertebrate immune system, lymphocytes, immunoglobulins and molecules of the major histocompatibility complex (MHC), are not present in invertebrates; despite this, invertebrates are capable of mounting an efficient immune response against invasive pathogens.

Unlike the research into vertebrate immunity, no single invertebrate model has been equally explored, although extensive information is known about particular aspects of individual species. The size of many of these organisms limits their usefulness to research, since in many cases only a few microlitres of blood can be collected from any one animal.

Research into the immune systems of invertebrates is in its infancy and has concentrated in many cases on the areas where these organisms are of economic importance. These include pest control, the transmission of disease and aquaculture (the culture of edible crustacea and molluscs). Investigations initially concentrated on discovering an invertebrate system comparable to that seen in vertebrates (Coombe, Ey and Jenkin, 1984). However the invertebrates do not form a single phylogenetic group, as do the vertebrates, there is therefore little reason to expect a unified system. Of those metazoan invertebrates investigated, there appears to be both a humoral and cellular arm to the immune responses. Specialised phagocytic cells are capable of the destruction of invasive pathogens and present in the body fluids are

proteins necessary for immune reactions eg. insect bacteriolysins.

The immune strategies present in one group may reflect aspects of their general biology eg. the prophenol oxidase cascade common to most arthropods and certain crustaceans. Both cellular and non-cellular encapsulation reactions appear to be triggered by this enzyme cascade and the resultant enzyme, prophenol oxidase, is a key enzyme in melanin synthesis and probably originated for purposes of ecdysis and tanning (Gotz, 1986). Likewise the antibacterial proteins, cecropins and attacins, found in lepidopteran insects may be essential during metamorphosis. At this time the gut wall is broken down releasing potentially damaging bacteria into the haemolymph (Boman, 1986).

Self/non-self recognition is a fundamental requirement for all immune systems and probably first arose as a mechanism to distinguish food particles and maintain self integrity. The ability to recognise self is apparent in the free-living protozoa. The amoeba, Arcella polypora, readily fuses with self fragments or fragments from amoebae belonging to the same species but fusion does not take place between different species (Reynolds, 1924). This discrimination is apparent throughout the animal kingdom. In vertebrates the molecules responsible for self recognition (major histocompatibility complex) and non-self recognition (antibodies) belong to the same family, the immunoglobulin superfamily. These proteins are grouped together on the basis of their structure and can be identified at both an amino acid and nucleotide level. Many proteins belong to this family and those whose functions are

known are involved in various aspects of recognition eg. neural cell adhesion molecule (N-CAM) and intercellular adhesion molecule (ICAM) (reviewed by Williams and Barclay, 1988). Members of the immunoglobulin superfamily have also been identified in invertebrates. This has led to speculation that the archetypal gene coding for the immunoglobulin domain is present in the genome of invertebrates and may operate in cell-cell recognition and awareness of non-self (see discussion).

Lectins have been found in the haemolymph of many invertebrates (reviewed by Yeaton, 1981; Renwrantz, 1986), they function as recognition molecules in that they bind to specific carbohydrate moieties. Their presence has been taken to indicate a role analogous to vertebrate antibodies. Investigations into the primary sequence of lectins have shown that, apart from relatively short sequences they differ from antibodies. Such limited amino acid sequence homology may be the result of convergent evolution and reflects physical constraints of the binding parameters. Alternatively the similarities could be the result of the insertion of short DNA segments (minigenes) into different framework sequences (Vasta and Marchalonis, 1984).

a) Repertoire of the snails immune system

(i) Cellular

Phagocytic cells are found in the connective tissue (fixed phagocytes) and in the haemolymph (haemocytes). Haemocytes are motile cells that are capable of leaving the haemolymph and entering the connective tissue. Fixed

phagocytes, as the name implies, remain in the connective tissue where they trap and engulf bacteria (Sminia, van der Knaap and Kroese, 1979).

Examination of the haemolymph of gastropod molluscs by light microscopy reveals different types of blood cell that differ in their morphology. These cells have been variously named by different authors and the terminology is confusing. Basically the population of haemocytes can be divided into three types. Small round cells with a high nucleus to cytoplasm ratio which do not readily attach to glass. Large cells that spread and are motile on glass substrates and cells of intermediate morphology. Whether these morphological differences represent different sub-populations or just one cell type at different stages of differentiation is still open to debate.

Biochemical probes have been used to ascertain whether the members of each of these three morphologically distinct groups are homogeneous. The results so far have shown that all haemocytes from B. glabrata and L. stagnalis snails bear α-D-Glucose and/or α-D-Mannose residues as recognised by Concanavalin A staining. Haemocytes from L. stagnalis snails bind Wheat germ agglutinin but those from B. glabrata do not. Staining with the lectin, ricin, also gives different results in the two snail species. B. glabrata haemocytes all stain with ricin whereas only a proportion of cells from L. stagnalis do so (Dikkeboom, Tijnagel and Van der Knaap, 1988; Yoshino, 1983).

Two monoclonal antibodies (Mabs) have been raised that bind to a sub-population of haemocytes from B. glabrata and L. stagnalis snails. These have been termed BGH1 and LS1 respectively (Yoshino and Granath, 1985; Dikkeboom, Tijnagel and Van der Knaap, 1988). The B. glabrata cells that bind BGH1 were found to have a lower phagocytic capacity and lysosomal enzyme content than non-binding cells and were 'intermediate' in morphology. In these respects they were identical to the LS1 +ve cells found in the haemolymph of L. stagnalis. However there was no cross-reaction and the Mab LS1 did not bind to the surface of B. glabrata haemocytes (Dikkeboom, Tijnagel and Van der Knaap, 1988).

Haemocytes have been investigated for the presence of lysosomal enzymes. The presence of the enzymes acid phosphatase, non-specific esterase and peroxidase has been demonstrated in three snail species; Lymnaea stagnalis, Biomphalaria glabrata and Bulinus truncatus. Haemocytes from L. stagnalis showed higher levels of peroxidase than the haemocytes from either B. glabrata or Bulinus truncatus. Whereas B. truncatus haemocytes appeared to have higher levels of non-specific esterase than either of the other two species. No differences were observed in the phagocytic capacity of the three species (Sminia and Barendsen ,1980).

A study of two different strains of B. glabrata, one resistant and one susceptible, to infection by S. mansoni, confirmed the presence of lysosomal enzymes. Quantitative and qualitative differences were found between these two strains. The susceptible snails were found to have fewer cells per cm^3

with a higher percentage of cells negative for all enzymes (Granath and Yoshino, 1983). These haemocyte populations were monitored during the course of infection with S. mansoni. Susceptible snails showed little alteration in cell profile, this was in contrast to the resistant snails whose cell profile fluctuated dramatically. The decrease in circulating haemocytes seen as a result of the encapsulation reaction appeared to be due to the selective depletion of acid phosphatase positive cells (Granath and Yoshino, 1983a).

Enzyme analysis of haemocytes has also been carried out on histological sections of tissue taken from B. glabrata snails infected with echinostomes. A comparison was made between haemocytes found in the amoebocyte producing organ and those encapsulating the parasites. Acid phosphatase was present in both cell populations but those haemocytes encapsulating the parasite had a higher level of expression of this enzyme (McKerrow, Jeong and Beckstead, 1985). In contrast to the reports cited above, peroxidase activity was not discernible in either cell population. Vertebrate cells of the macrophage/monocyte line also show variable peroxidase levels. Peroxidase can be demonstrated in circulating monocytes but not in tissue macrophages (at the light microscope level). The earlier snail studies used haemocyte monolayers, derived from snail haemolymph, this is taken as an indication of similarity between the invertebrate cells and vertebrate macrophages (McKerrow, Jeong and Beckstead, 1985).

The enzyme peroxidase is involved in the production of reactive forms of oxygen implicated in the killing of invasive

pathogens by vertebrate cells of the macrophage and neutrophil lineages (in Klein, 1982). In the mammalian host the binding of IgE to effector cells is known to cause the 'respiratory burst' that results in the release of these reactive oxygen metabolites and they are thought to be responsible for the killing of schistosomula (Capron, Dessaint, Capron, Ouma and Butterworth, 1987). The metabolites include superoxide anions, hydrogen peroxide and singlet oxygen. Superoxide can be detected by the reduction of (yellow) nitroblue tetrazolium, to (blue/black) formazan. Singlet oxygen can be detected by the emission of chemiluminescence that occurs as these excited atoms return to their ground state.

The respiratory burst can be triggered by perturbation of the cell membrane through phagocytosis or treatment with phorbol myristate acetate. Haemocytes from the snails L. stagnalis, B. glabrata, Helix pomatia and Planorbarius corneus were tested for the presence of reactive oxygen metabolites after stimulation with zymosan. Superoxides were discovered in the haemocytes from all four species. Singlet oxygen was also detected in L. stagnalis and H. pomatia but its presence in B. glabrata and P. corneus was less well defined. No correlation could be found with regards resistance to infection by trematode parasites (Dikkeboom, Van der Knaap, Van den Bovenkamp, Tijnagel and Bayne,1988).

Adult and juvenile L. stagnalis snails differ in their resistance to trematode parasite Trichobilharzia ocellata. The juveniles are susceptible to invasion, whereas the adults are not. A comparison of the haemocytes revealed certain

differences. Juvenile snails had fewer haemocytes with a higher proportion of small round cells, these cells are considered by the authors to represent less differentiated cells since they have a higher mitotic index than the large spreading cells. Haemocytes from juvenile snails were found less able to phagocytose foreign particles when compared with those from adults. (Dikkeboom, Van der Knaap, Meuleman and Sminia, 1984; Dikkeboom, Van der Knaap, Meuleman and Sminia, 1985). These differences were considered sufficient to account for the lack of immunity of these snails. However challenge with the parasite S. mansoni does elicit a successful immune response from juvenile snails (Dikkeboom, Bayne, Van der Knaap, and Tijnagel, 1988).

(ii) Humoral Factors

The haemolymph of snails consists of cells, proteins, glycoproteins etc. in a fluid matrix. The cell free portion of this fluid carries the respiratory pigment which, unlike the respiratory pigments of vertebrates, is not membrane bound but found free in solution. The snails Helix pomatia and Lymnaea stagnalis have haemocyanin as their respiratory pigment whereas snails of the genera Biomphalaria and Bulinus have haemoglobin. These pigments are high molecular weight glycoproteins made up of a large number of subunits (Afonso, Arrieta and Neves, 1976; Hall and Wood, 1976). The role of the carbohydrate content is not known but is thought to be involved in the association of these subunits (Viana de Freitas, Afonso and Neves, 1985). Respiratory pigments account for 97% of the total protein content of the haemolymph

(Figueiredo, Gomez, Heneine, Santos and Hargreaves, 1973; Granath, Spray and Judd, 1987).

The cell free haemolymph of snails contains factors capable of modulating the immune response. This has been demonstrated with different strains of B. glabrata that are either resistant or susceptible to infection by S. mansoni. Work carried out by Loker and Bayne (1982) and Bayne, Boswell, Loker and Yui (1985) shows that haemocytes from susceptible snails are able to encapsulate S. mansoni sporocysts but only if the haemocytes had first been incubated in the plasma from resistant snails. Granath and Yoshino (1984) injected plasma from resistant snails into susceptible snails and found that this conferred resistance on the susceptible snails.

The haemolymph of B. glabrata has also been shown to contain 'miracidial immobilising substances'. The snails used were an albino strain susceptible to infection by most trematodes but the adults are resistant to infection by the echinostome E. lindoense. Experimental infection of juveniles with this echinostome induced the production of haemolymph factors that immobilised the miracidia of not only E. lindoense but also E. paraensei and E. liei. However there was no reciprocal inducement following infection with E. liei. Neither infection produced haemolymph factors reactive against S. mansoni (Lie, Jeong and Heyneman, 1980).

Bactericides have not been found in the haemolymph of snails (Bayne, 1983) but in vitro experiments have shown that bacteria are recognised and agglutinated. It is possible that agglutinins function by marking targets for haemocyte attack.

Agglutinin molecules have been found in the haemolymph of many invertebrate species (reviewed by Yeaton, 1981; Renwrantz, 1986). They are often associated with haemocyte membranes and are thought to be synthesised by these cells (Amirante and Mazzalai, 1978; Vasta, Sullivan, Cheng, Marchalonis and Warr, 1982; Van der Knaap, Boots and Sminia, 1983). In many cases their presence has been detected by their ability to bind to and clump vertebrate erythrocytes, in which case they are known as haemagglutinins. The terms agglutinin, haemagglutinin and lectin are all used to describe protein or glycoprotein molecules with multiple binding sites capable of recognising and binding to specific carbohydrate moieties.

The agglutinins of some invertebrate species show an increase in titre after the animal has been challenged by foreign antigens. Cooper, Stein and Wodjani (1984) demonstrated that the plasma agglutinin from the earthworm Lumbricus terrestris displayed a higher haemagglutination titre after the animal had been injected with foreign particles. This was also shown to be true for the insect agglutinin from Anticarsia gemmatalis investigated by Pendland and Boucias (1985).

Several haemagglutinins have been found in the plasma of B. glabrata snails (Michelson and Dubois, 1977; Stein and Basch, 1979; Jeong, Sussman, Rosen, Lie and Heyneman, 1981; Boswell and Bayne, 1984) but their presence shows little correlation with resistance to infection by S. mansoni. The titre of one such agglutinin, found in both resistant and susceptible snails, did not alter on challenge with S. mansoni

miracidia but increased when the snails were exposed to Echinostoma lindoense larvae (Jeong, Sussman, Rosen, Lie and Heyneman, 1981).

An agglutinin specific for human type A erythrocytes was found in the plasma of PR-B snails, a strain susceptible to infection by S. mansoni. This agglutinin bound to the tegumental surface of the S. mansoni miracidia, sporocysts and cercariae as visualised by direct fluorescence (labelled agglutinin) and indirect fluorescence (labelled anti-agglutinin antibody). In this case the agglutinin is thought to play a role in masking the larvae from haemocyte attack (Stein and Basch, 1979).

As yet the only direct proof of an opsonising role for a haemolymph agglutinin has come from work on the bivalve Mytilus edulis. This agglutinin has been isolated and purified and was shown to enhance phagocytic uptake of yeast cells by the haemocytes of these animals (Renwrantz and Stahmer, 1983).

(b) Manifestations of the snail immune response.

(i) Wound Repair

Snails, when wounded, first seal the lesion by muscular contraction and exude mucus (Bayne, 1983). In many cases this mucus has not been analyzed but an antibacterial factor has been isolated from the mucus of the giant land snail Achatina fulica (Kubota, Watanabe, Otsuka, Tamiya, Tsuchiya and Matsumoto, 1985). It is possible that snails possess antimicrobial peptides similar to the 'magainins' found in the skin of Xenopus toads (Zasloff, 1987). The wound soon becomes plugged with haemocytes and concomitantly there is a sharp

increase in the number of these circulating in the haemolymph. During the course of repair collagen is secreted by fibroblasts and epithelia grows over the haemocyte plug (Sminia, Pietersma, and Scheerboom, 1973). The behaviour of the haemocytes during wound repair is reminiscent of that during the encapsulation reaction. The cells flatten and interdigitate with one another but do not form tight or septate junctions (Sminia, Pietersma, and Scheerboom, 1973; Loker, Bayne, Buckley and Kruse, 1982).

(ii) Phagocytosis

Bacteria and other small pathogens are rapidly cleared from snails. This reaction is so efficient that practically no bacterial pathogens of snails are known to exist. Knowledge of the immune response elicited by bacteria has come from directly inoculating bacteria into snails and in vitro experiments using monolayers of haemocytes. High doses of bacteria (>5 x 10^7) injected into the snail are cleared within 2h (Van der Knaap, Sminia, Kroese and Dikkeboom, 1981). Initially the number of circulating haemocytes drops, returning to normal levels after 24h (reviewed by Bayne, 1983). Radioisotope labelling of the bacteria indicates that they accumulate in regions of the snail rich in haemolymph, particularly the digestive gland (Bayne, 1974). In vitro experiments indicate that plasma agglutinins are important in this response. The plasma from both the land snail Helix pomatia and the water snail Lymnaea stagnalis has been shown to agglutinate various strains of bacteria. One bacterium that is not agglutinated by H. pomatia is the bacterium Aeromonas

formicans and this has proved to be pathogenic for the snail (Bayne, 1982). Bactericides have not been found in snail plasma and the role of the agglutinins appears to be an opsonic one (Bayne, 1982).

Injections of bacteria alter certain aspects of the snail's immune system. These changes become apparent on challenge with further doses of bacteria. The recovery of haemocyte number is faster in snails previously exposed to foreign antigens. The individual haemocytes appear larger with increased cytoplasmic projections and greater phagocytic capacity (Van der Knaap, Boots, Van Asselt and Sminia, 1983). The level of lysosomal enzymes found in the haemolymph has also been shown to increase (Cheng, Chorney and Yoshino, 1977). Unlike the vertebrate immune response, these changes are not specific but reflect a general activation of the entire system. This 'memory' response is relatively long lasting, 64 days in the case of L. stagnalis (Van der Knaap, Boots, Van Asselt and Sminia, 1983).

(iii) Encapsulation

Foreign particles too large to be phagocytosed are encapsulated by haemocytes and destroyed. This is the fate of schistosomes entering a resistant host and is the immune reaction most extensively studied in snails.

An account of the encapsulation reaction is given by Van der Knaap and Meuleman (1986). The haemocytes surround and cover the parasite, forming a loose capsule. These cells then disappear and are replaced by others that flatten and form a compact capsule approximately 15 cells deep. Ultrastructural

examination of the haemocytes in the capsule show these cells to have an electron dense cytoplasm, extensive rough endoplasmic reticulum, with numerous mitochondria and various lysosomal structures.

(c) Genetics of snail/schistosome interactions.

Snail infection by schistosomes is restricted on a species and population level. Each individual species of schistosome is only able to develop within particular species of snail. On a population level it has been found that a snail susceptible to infection by a local strain of parasite may prove to be resistant or incompatible with the same species of schistosome isolated from a different area.

Many factors influence the infection of snails by schistosomes, these include the age of both snails and miracidia, the number of miracidia per snail, the temperature of the water, length of contact time etc. (Webbe, 1962). The inter-relationship between snails and schistosomes is highly complex and snails differ in their capacity to act as hosts. In some cases the biochemical constitution of the snail does not facilitate schistosome invasion or development, the snail and the schistosome are then considered to be incompatible. A snail is said to be resistant if there is an active cellular response to the invading parasite and susceptible if no cellular response is evoked.

The outcome of successful invasion of the snail host by schistosomes is also under genetic control. This genetic basis for resistance has been studied by Richards (1970, 1974, 1985) using different strains of Biomphalaria glabrata snails, that vary in their

resistance to infection by S. mansoni (Richards, 1970, 1975, 1984; Richards and Minchella, 1987). Four patterns of B. glabrata susceptibility have been identified:-

Type I. Resistant at any age.

Type II. Juvenile susceptible, adult resistant.

Type III. Susceptible at any age.

Type IV. Juvenile susceptible and adult variable.

Resistance and susceptibility are under the control of several genes with additional factors regulating their expression. The ability of a miracidium to penetrate a snail host depends on the snail-schistosome combination and is known as the infectivity rate. In rare cases the infectivity rate can reach about 100% but in most cases it is between 30 and 60% (Jourdane and Théron, 1987). Research by Richards (1975) has shown that this also has a genetic component.

(d) Schistosome Evasion of the Snail Immune Response

Successful infection of snails by schistosomes requires either the evasion or perturbation of the snail's defence mechanisms. When a miracidium enters a resistant host its presence elicits an encapsulation reaction. In susceptible snails the haemocytes are activated and accumulate around the sporocyst but do not encapsulate it. This benign association reaches a peak approximately 2h after penetration, the cells then gradually disperse. While associated with the sporocyst, the haemocytes engulf the discarded ciliated plates but do not attack the parasite (Meuleman, Lyaruu, Khan, Holzmann and Sminia, 1978).

It is apparent that the haemocytes of susceptible snails are, in most cases, capable of encapsulation reactions since the same snails are resistant to attack by other schistosomes. The innate cytotoxicity of haemocytes has also been demonstrated by in vitro encapsulation experiments. A strain of B. glabrata susceptible to infection by S. mansoni can be rendered cytotoxic if pre-incubated with plasma from resistant snails (Granath and Yoshino, 1984). The snail Bulinus wrighti has proved to be susceptible to experimental infection by all schistosomes of the S. haematobium group. When this snail is infected with miracidia of S. mansoni (a schistosome usually associated with Biomphalaria spp snails) the larvae are unable to develop but no haemocyte accumulations are seen (Wright and Southgate, 1981). It is possible that the haemocytes of these snails do not have any innate ability to encapsulate schistosomes.

Evasion mechanisms may be designated as either active or passive depending on whether they act directly on the effector arm of the snail's immune response or merely avoid triggering a defence reaction. Observations have been made on miracidia and sporocysts at both an enzymic and ultrastructural level to see whether they directly attack haemocytes. Miracidia release proteolytic enzymes which are released during their transformation to sporocysts but the sporocysts themselves do not (Pino-Heiss, Brown and McKerrow, 1985). The miracidial enzymes may aid their entry into the snail tissue but no cytolytic attack of haemocytes has been observed at the ultrastructural level (Meuleman, Lyaruu, Khan, Holzmann and

Sminia, 1978). Parasites may also depress the immune response rendering the haemocytes incapable of mounting an effective response. Co-infection of B. glabrata snails with echinostome larvae renders normally resistant snails susceptible to successful schistosome invasion (Lie, Heyneman and Jeong, 1979). When these experiments were carried out with different strains of schistosome, there appeared to be no interference of the immune response, with one parasite being encapsulated in close proximity to a developing sporocyst (Lie, Heyneman and Richards, 1976). The mediator of echinostome induced immune suppression has not been identified. Its effects appear to be localised as schistosome sporocysts are afforded greater protection if they lie in close proximity to echinostome larvae (Lie, 1982). Haemocytes remain capable of encapsulation responses, wound repair and still phagocytose ciliated plates (Lie, Jeong and Heyneman, 1981).

Passive mechanisms require the parasites to appear as self to the host and therefore avoid activating the snail's immune system. Studies with lectins and antibodies to human blood group determinants revealed that parasites and haemolymph have carbohydrates groups in common (Yoshino, Cheng and Renwrantz, 1977). Antibodies raised to the cell-free haemolymph of B. glabrata snails have been shown to cross-react with the surface of S. mansoni miracidia and sporocysts (Yoshino and Bayne, 1983). This may be due to molecular mimicry, an adaptive strategy on behalf of the parasite to produce surface antigens that resemble host molecules (Yoshino and Boswell, 1986). Alternatively it could

simply illustrate that invertebrates have a number of epitopes in common (Dissous, Grzych and Capron, 1986; Bayne, Boswell and Yui, 1987). Antibodies raised against the cell-free haemolymph of L. stagnalis did not cross-react with Trichobilharzia ocellata sporocysts, though the miracidia and cercariae were recognised (van der Knaap, Boots, Meuleman and Sminia, 1985).

The sporocyst tegument is the site of nutritional uptake for the schistosome. Sporocysts transformed in vitro (i.e. in the absence of snail components) and subsequently transferred to snail haemolymph rapidly acquire a coating of haemolymph components (Bayne, Loker and Yui, 1986). Coating the sporocyst in this manner could 'mask' the parasite and allow it to avoid detection. A strain of B. glabrata susceptible to infection by S. mansoni was found to possess a haemagglutinin absent in resistant strains. The haemagglutinin was found to bind to the surface of S. mansoni miracidia, sporocysts and cercariae and this specific adsorption is thought to mask the parasites (Stein and Basch, 1979).

The methods mentioned are all possible ways that schistosomes evade detection but as yet no definitive evidence exists for any evasion mechanism.

5. Interactions of Schistosomes and Bulinid Snails.

The majority of the data pertaining to snail-schistosome interactions are based on the experimental evidence from two model systems. These are :- (a) Different strains of the snail Biomphalaria glabrata that vary in their susceptibility to infection by S. mansoni (b) The snail Lymnaea stagnalis and

the duck trematode parasite Trichobilharzia ocellata. These snails are highly susceptible to infection as juveniles but less so as adults. In contrast very little is known about the defence mechanisms of snails belonging to the genus Bulinus, yet these snails are responsible for the transmission of seven species of schistosome including S. haematobium and S. intercalatum.

(a) Snail Taxonomy

Research into this area of snail-schistosome interactions requires a knowledge of bulinid species and their resistance-susceptibility to the parasites. Formerly, snail taxonomy was based solely on shell morphology; however by 1957 this was extended by G. Mandahl-Barth to include observations on the soft parts of the snails. At the Experimental Taxonomy Division of the British Museum (Natural History) additional methods have been introduced to distinguish snail species using biochemical and molecular biological techniques and on their compatibility with schistosomes. This information provides the necessary background required for the study of molluscan defence mechanisms at a cellular level.

Currently the genus Bulinus (family Planorbidiae) consists of thirty species divided into four species groups (Mandahl-Barth, 1958; Brown, 1980), see Table 2. The shells of these snails are sinistral i.e. they coil in a clockwise direction from the apex and are higher than they are wide. Shell heights range in size from 4mm to 25mm with generally four or five whorls (figure 4). The snails are widely distributed, being found throughout Africa, some islands of

the Indian ocean, Iberia, some Mediterranean islands and SW Asia. This wide distribution reflects the ability of members of this genus to exploit widely varying micro-habitats and most are capable of aestivation, enabling the colonisation of the semi-permanent waterbodies natural to these areas. The taxonomy of the genus is complicated due to local populations often appearing as intermediate in morphology between two established species. Such intraspecific polymorphisms are reflected in the analysis of egg proteins and enzymes (Rollinson & Southgate, 1979). The arrangement of species within groups is not based on their schistosome compatibility. Members of each group are susceptible to infection by S. haematobium whereas others are resistant to such infection but susceptible to others.

1) The africanus group

Members of this group are found in Afrotropical regions only, though in a wide variety of habitats. The group contains ten species, eight of whom serve as intermediate hosts for S. haematobium. B. africanus and B. nasutus are also susceptible to infection with S. bovis. B. globosus is the most widely distributed species and is compatible with S. mattheei and some strains of S. haematobium and S. intercalatum. Laboratory studies show that B. globosus can also be infected with some strains of S. bovis (Southgate and Knowles, 1975). The snails in this group are diploid with a chromosome number, n=18.

2) The truncatus/tropicus complex.

This is a large group of closely related species. Some tetraploid species e.g. B. truncatus, B. rohlfsi are

responsible for the transmission of some strains of S. haematobium. This generally occurs in regions where snails belonging to the B. africanus group are absent. The hexaploid and octoploid populations have colonised areas of high altitude and are generally incompatible with S. haematobium (Webbe, 1982). The diploid snail B. tropicus has been implicated in the natural transmission of S. margrebowiei (Southgate, Howard, Rollinson, Brown, Ross and Knowles, 1985). In the laboratory B. natalensis has been found to be susceptible to infection with S. margrebowiei and shows a low compatibility with some strains of S. haematobium (Lo, Burch and Schutte, 1970).

3. The forskalii group.

The snails of this group can be distinguished by their slender, high spiralled shells. B. forskalii is common throughout tropical Africa and is the intermediate host for S. intercalatum in Cameroon and Gabon (Wright, Southgate and Knowles, 1972). It is also known to transmit S. bovis (Southgate and Knowles, 1975). Other members of the group transmit S. haematobium and some are susceptible to S. margrebowiei in the laboratory.

4. The reticulatus group.

This group of snails contains two species, B. reticulatus and B. wrighti. They have a marked capacity to aestivate and are able to colonise water bodies that are only present during the brief rainy season. The snails are small with shells less than 6mm high. It is thought that B. wrighti transmits S. haematobium in some localities in South Yemen and Saudi Arabia

TABLE 2

THE SPECIES OF *BULINUS*, ARRANGED WITHIN GROUPS IN ALPHABETICAL ORDER

1 *B. africanus* group (= *Physopsis*)

B. abyssinicus
B. africanus
B. hightoni
B. globosus
B. jousseaumei
B. nasutus
B. obtusispira
B. obtusus
B. ugandae
B. umbilicatus

3 *B. forskalii* group

B. bavayi
B. beccarii
B. camerunensis
B. canescens
B. cernicus
B. crystallinus
B. forskalii
B. scalaris
B. senegalensis

2 *B. truncatus/tropicus* complex

B. angolensis
B. coulboisi
B. depressus
B. guernei
B. hexaploidus
B. liratus
B. natalensis
B. nyassanus
B. octoploidus
B. permembranaceus
B. rohlfsi
B. succinoides
B. transversalis
B. trigonis
B. tropicus
B. truncatus

4 *B. reticulatus* group

B. reticulatus
B. wrighti

Reproduced from Brown (1980)

FIGURE 4

Representative shells of snails belonging to the genus Bulinus

Top, snails of the truncatus/tropicus complex.

Centre, snail shell of the africanus group.

Bottom left, a member of the forskalii group.

Bottom right, B. wrighti of the reticulatus group

Scale in mm

FIGURE 4

(Wright, 1963; Arfaa, 1976). B. wrighti is the only species that is compatible with all schistosomes of the S. haematobium group (Wright and Southgate, 1981).

b) Hybridisation of schistosomes

In Africa much of the schistosomiasis is caused by schistosomes belonging to the S. haematobium group. These schistosomes are transmitted by snails of the genus Bulinus. In recent years there have been confirmed reports of infections producing eggs of intermediate morphology between two distinct schistosome species. It appears that these hybrid species have arisen through changes in the environment brought about by man.

One example of hybridisation is that between the schistosomes S. haematobium and S. mattheei. In South Africa the distribution of these two schistosomes overlap and in this area both are transmitted by the snail Bulinus globosus. S. mattheei was primarily a parasite of artiodactyls but it is able to infect domestic livestock and shows a low infectivity for man. The emergence of intensive farming techniques increased the number of domestic livestock and the parasite was able to spread, contaminating water bodies formerly colonised only by S. haematobium. It therefore became relatively easy for the same snail or population of snails to harbour and transmit schistosomes of the two different species (Southgate and Rollinson, 1987).

Natural hybrids between these two schistosomes were first suspected by Alves in 1947 and later confirmed by Le Roux (1954) and Pitchford (1959). In 1980 Wright and Ross not only

proved these crosses feasible but also showed that the F1 generations displayed characteristics attributable to hybrid vigour. The hybrids proved more infective for both definitive and intermediate hosts, they matured quicker and they were more fecund than either parent species.

Hybrids are also recognised between the species S. haematobium and S. intercalatum. Both these schistosomes are parasites of man and readily hybridise. In Loum, Cameroun they differ in their intermediate host specificity; transmission of S. haematobium being via B. rohlfsi and S. intercalatum via B. forskalii. In the environs of Loum the only schistosome found in 1969 was S. intercalatum (van Wijk, 1969), however, in a subsequent survey both S. haematobium and hybrids of the two species were found (Southgate, van Wijk and Wright, 1976). It was considered that recent deforestation may have been a contributory factor for this phenomenon. The snail B. rohlfsi is intolerant of shaded conditions and was therefore able to extend its range and cohabit with B. forskalii. The conditions for hybridisation of the schistosomes were therefore optimised.

(c) Molluscicides

Investigations into the immune mechanisms of marine molluscs have shown that certain environmental factors reduce immunocompetence. The presence of heavy metals can either enhance phagocytic uptake by haemocytes or cause the destruction of these cells, depending on type and concentration of contaminant (Cheng, 1987). In the oyster, Crassostrea virginica, an increase in salinity affects

haemocyte locomotion and hence immunity in this mollusc (Fisher and Newell, 1986). Whether the physiological stress induced by non-toxic levels of molluscicides causes a similar reduction in immunocompetence has not been documented. It is important that the current research on plant molluscicides (WHO report, 1987) includes investigations into this area.

6. Hypothesis

Many factors influence the infection of snails by schistosomes and these have been outlined above. The encapsulation reaction observed when a miracidium enters a resistant snail host requires the recognition of the parasite as foreign in order to trigger the haemocyte effector mechanisms. It has been found that the surgical transplantation of sporocysts, an entirely intramolluscan stage, from susceptible snails (B. truncatus) to resistant snails (Planobarius metidjensis) circumvents the non-self recognition mechanisms. The sporocysts are able to develop and produce cercariae in these normally unsuitable snails (Kechemir and Combes, 1982). This indicates that in this host-parasite combination, the molecules recognised by the snail's immune system as foreign reside on the miracidium and not on the sporocyst.

However it has been demonstrated that haemocytes from resistant Biomphalaria glabrata can encapsulate particular sporocysts in vitro (Bayne, Buckley and DeWan, 1980a). Therefore under these conditions haemocytes of this snail are able to recognise the sporocyst surface as being 'foreign'.

As a working hypothesis, I propose that snail-schistosome interactions are governed by factors in the haemolymph which recognise and bind to surface molecules on the larval schistosomes. Once bound, these humoral recognition factor(s) may then stimulate a cellular response mediated by haemocytes which results in the encapsulation of the invading organism(s). In order to test this hypothesis the major surface molecules of the intra-molluscan larval forms, miracidia, sporocyst (transformed in the absence of snail components) and cercariae, were analyzed. The snail haemolymph from resistant and susceptible snails was investigated for the presence of haemagglutinins that might function as recognition factors. A study was also made of the haemocytes of bulinid snails.

1. Snails and Parasites.

The host/parasite combination chosen for investigation was S. margrebowiei and two species of bulinid snails, B. natalensis and B. nasutus. S. margrebowiei has advantages over other schistosomes in that the patency period in laboratory animals is comparatively short (35 days in mice as compared with 55 days for S. haematobium) and there are no substantiated reports of any human infections (Southgate & Knowles, 1977) thereby reducing the biohazard risk in the laboratory.

S. margrebowiei was first described by Le Roux in 1933, it has a wide range of vertebrate hosts but probably the most important of these is the lechwe (Kobus leche kafuensis) and the puku (K. vardoni) (Rollinson & Southgate 1987). Transmission occurs via snails of the B. forskalii group, B. forskalii and B. scalaris (Wright, Southgate and Howard 1979). In Zambia B. tropicus of the truncatus/tropicus complex have also been found to be naturally infected (Southgate, Howard, Rollinson, Brown, Ross and Knowles 1985). Other snails belonging to the truncatus/tropicus complex have proved to be susceptible to infection in the laboratory, whereas snails of the africanus group are resistant to experimental infection (Southgate and Knowles, 1977).

B. natalensis (figure 5a) is a member of the truncatus/tropicus complex and therefore a suitable host for S. margrebowiei. It shows a low compatibility with S.

haematobium in the laboratory but it is not known for this snail to act as an intermediate host under natural conditions (Lo, 1969).

These snails are distributed throughout eastern Africa from Ethiopia to South Africa, where they live in small pools, slowly flowing rivers and lakes (Brown, 1980). The snails used in this study were first collected from the shores of Lake Sibaya, S. Africa in 1963.

In contrast, B. nasutus (figure 5b) is resistant to infection by S. margrebowiei but is the main intermediate host for S. haematobium in Tanzania (Webbe, 1962). B. nasutus can withstand desiccation and are found in seasonal waterbodies and ditches as well as lake margins. The population maintained in the laboratory were collected from borrow pits near Morogoro, Tanzania in 1984.

The snails and parasites were provided by the Experimental Taxonomy Division of the British Museum (Natural History) (BM (NH)).

2. Animal Maintenance

(a) Snails

The snails were maintained at the University College laboratory by the methods used routinely at the BM(NH). Snails were kept in plastic surgical instrument trays, 35 x 25 x 5 cm (Bell and Croyden), covered with a flat sheet of glass to prevent the snails from escaping and to maintain a high level of humidity. Each tray was filled to a depth of 3cm with fish conditioned tap water (FCTW). London tap water has proved to be toxic for snails due to its chlorine content, the water was

FIGURE 5

a) Photograph of Bulinus natalensis

Bar = 1mm

b) Photograph of Bulinus nasutus

Bar = 1.3mm

FIGURE 5

a

b

therefore 'conditioned' before use. This was achieved by using it as aquarium water for a population of fish (Poecilia sp.) for at least one week prior to its use as snail water. The water was filtered through a Boots wine making kit filter before use. Each week the snails were transferred to a clean tray with fresh water. Snails were kept at 27°C in either a bench incubator with natural sunlight or in a constant temperature room on a 12h light and dark cycle. The snails were fed a variety of foods: fish food (Aquarian• for guppies), lettuce, sycamore leaves and algae. The lettuce was rinsed in tap water and FCTW before being given to the snails. Sycamore leaves (Acer pseudoplatanus) were collected in late spring and dried for storage. Prior to use the leaves were soaked for a minimum of 2 days in FCTW, then rinsed to remove dissolved tannins. The supply of algae (Oscillatoria sp) was provided by the staff of the Experimental Taxonomy Division BM(NH). It was grown as thin layers on sterilised mud (collected from Pevensey, Sussex) and once confluent the culture was dried, pieces of mud with algae were placed in the trays for the snails to graze. The snails would, in their natural habitat lay their eggs on the underside of leaves, in the laboratory plastic sheets were placed in the trays to act as substitute leaves and were readily used for egg laying. The sheets with their egg masses were collected at regular intervals and transferred to new trays for hatching. This prevented cannibalism of the eggs and young snails by the adults.

(b) Schistosome life cycle

The parasites were passaged through outbred mice (Tyllers Original Swiss) and Bulinus natalensis snails using similar methods to those outlined by Meuleman, Huyer and Mooij (1984) and Ogbe (1985).

Mice were infected using the partial immersion technique (Watson and Azim, 1949). Each mouse was exposed individually to approximately 200 cercariae counted directly into $100cm^3$ FCTW in plastic pots. The mice were left to paddle in this water for approximately hour then returned to clean cages. Mice were sacrificed 45 days post infection, the livers removed and placed in a beaker with a 2% solution of Milton's reagent (1% sodium hypochlorite; 16·5% sodium chloride) for surface sterilization then rinsed in 0·85% sterile NaCl. All subsequent procedures were carried out aseptically with sterile glassware and reagents. The livers were chopped finely with scissors and forced through a 1mm mesh into a beaker containing 0·85% saline. The liver suspension was allowed to sediment under gravity for 30 min and the supernatant discarded. The sediment was re-suspended in saline and washed several times by centrifuging at 500 rpm for 1min on a Beckman bench centrifuge, discarding the supernatant each time.

In an alternative method aseptic techniques were abandoned and the livers were homogenised in a Waring blender with 100ml 0·85% saline. The liver suspension was then passed through a screen (mesh size 44μm) that retained the eggs whilst allowing most of the liver tissue to pass through. The trapped eggs were washed thoroughly with a jet of saline from

a wash bottle then transferred to a centrifuge tube and washed as described above.

In order for the schistosome eggs to hatch certain external conditions have to be met, including a drop in osmolarity from saline to that of fresh water, exposure to light and an ambient temperature of between 25-30°C (Erasmus, 1972). After washing, the pellet of liver tissue was re-suspended in 40 ml FCTW, previously sterilised by filtration through 0·22μm membrane (Millipore). The liver suspension was placed on a IKA vibrax shaker for approximately 30 min at 200 rpm. Light and heat were provided by a lamp fitted with a 100 watt bulb. The liver tissue was once more allowed to sediment under gravity. This time the supernatant contained the swimming miracidia and was removed to a fresh centrifuge tube. The tube was covered in aluminium foil to exclude the light from all but the top of the tube. The miracidia of S. margrebowiei are positively phototactic and accumulated at the top of the tube where they could be removed with a pasteur pipette.

Snails were infected by placing them individually in small pots with FCTW containing 10 miracidia per pot. The snails were left overnight with the miracidia at 27°C and then transferred to a plastic tray with fresh water and placed in an incubator 27°C. Cercarial shedding occurs approximately 30 days later, and the snails survive for a further 2 months. During this time cercariae are being produced continually. The shedding of S. margrebowiei cercariae follows a constant circadian rhythm with two emission peaks, one at dawn and the

other at dusk. This pattern mirrors the diurnal behaviour of their definitive hosts that visit the water holes to drink at these times (Pitchford and Dutoit, 1976).

In the laboratory the snails were kept in the dark until cercariae were required, when they were transferred to a beaker with fresh FCTW and placed under a lamp fitted with a 60 watt bulb. Shed cercariae could be seen by the unaided eye accumulating at the water surface from where they were collected using a pasteur pipette.

3. Collection of snail haemolymph

The snails were first dried by placing them, shell aperture downwards, onto absorbent paper. This reduced the contamination by ciliates and rotifers present in the snail trays and ensured that the haemolymph was collected undiluted. Cleaning the snails with 70% alcohol proved to be unworkable as this treatment invariably caused the snails to bleed prematurely. Bleeding was carried out by the non-destructive method outlined by Sminia (1981). The snail foot was firmly pushed back into the shell with the aid of a finely drawn pasteur pipette. Standard glass pasteur pipettes (John Poulton Ltd.) were heated and pulled to produce a bore of approximately 0·3mm for a length of 5cm . Pushing the foot in the manner described caused the snail to retract into its shell and exude several drops of haemolymph from the body cavity via the haemal pore. This is a normal defence mechanism of snails, the loss of blood reduces the body volume enabling the snail to retract deeply into its shell. The haemolymph was then collected from within the snail aperture. Using a finely

drawn pipette allowed better control over the small volumes involved (between 5 and 15 μl) reducing haemolymph loss through technique. After bleeding the snails were returned to their trays. Snails emerge and become active within 15 min., however subsequent successful bleeds could not be achieved till after a recovery period of 24 h when renewal of the haemolymph had been achieved.

4. Microscopy

(a) Phase Microscopy

The in vitro transformation of miracidia was monitored using an Olympus IMT-2 inverted microscope. Video recordings were made using a Hitachi HV-735K camera mounted on the microscope and connected to a Panasonic AG-6010 video cassette recorder. Photomicrographs of haemocytes and larval schistosomes were taken using an Olympus 35mm camera attached to the camera port of the microscope.

(b) Scanning Electron Microscopy

The surface morphology and three-dimensional aspects of the haemocytes and the larval schistosomes were examined using the scanning electron microscope. After fixation the specimens were dehydrated by replacing the water content with the solvent acetone. This was achieved by immersing the specimens in a series of acetone/water mixtures :- 25%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100%. The acetone is next replaced by liquid CO_2 in a closed container. Finally the CO_2 was evaporated using a critical point dryer (BM(NH)). Drying is carried out at the critical point of CO_2, when the conditions

of temperature and pressure ensure that the density of the liquid is equal to the density of the vapour. At the critical point there is no surface tension between the liquid and the gaseous phases, the drying is therefore non-destructive.

The dried specimens were subsequently gold coated using a Polaron E5000 coolsputter unit and viewed using an Hitachi S800 scanning electron microscope.

(c) Fluorescence Microscopy

Fluorescence microscopy was used to study the external and internal structure of the snail haemocytes as well as the surface ligands of the larval schistosomes. This technique exploits the property of fluorochrome molecules to absorb radiation in the ultraviolet range and emit it as radiation of longer, visible wavelengths, thereby appearing luminous. Fluorochromes were used either as direct stains (e.g. propidium iodide, ethidium bromide) or coupled to specific reagents (e.g. lectins). The fluorochromes used are given in table 3.

Specimens were stained for fluorescence microscopy then mounted using 'anti-fade' glycerol/PBSA mixture (Chemistry department, City University) as a mounting medium. The coverslips were sealed using clear nail varnish and viewed using an Olympus IM2 microscope fitted with fluorescence optics. Micrographs were taken using an Olympus 35mm camera fitted to the microscope and Kodak Ektachrome 400 film.

TABLE 3

FLUOROCHROMES

Fluorochrome	Excitation wavelength	Emitted wavelength	Colour
Fluorescein	490-495 nm	517 nm	green
Rhodamine	550 nm	580 nm	red
Ethidium bromide	482 nm	616 nm	red
Propidium iodide	493 nm	639 nm	red

5. Gel Electrophoresis.

The protein composition of snail plasma and the surface membranes of larval schistosomes was investigated using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). This method enables the separation of proteins on the basis of size. Protein mixtures were first dissociated by heating to 100°C in sample buffer containing excess SDS with dithiothreitol (DTE) to break the di-sulphide bonds. The SDS detergent surrounds the polypeptides with negative charge thereby negating any intrinsic charge differences and causing the separation to be effected on size only. Sample buffer, pH 6·8, contained 0·063M Tris, 10% glycerol, 2·5% SDS, 1·5% DTE and 0·002% bromophenol blue.

The dissociated proteins were subsequently loaded onto a poly-acrylamide gel matrix in the presence of SDS. Polyacrylamide gel is a synthetic polymer of uniform pore size that is formed by the polymerisation of acrylamide monomer into long chains. These chains are cross-linked to one another by N,N'-methylene bisacrylamide (bisacrylamide). On application of an electric current these proteins migrate through the gel at a rate dictated by their molecular weight.

The smaller the polypeptide the more readily it travels through the gel.

SDS-PAGE was carried out by the method of Laemmli (1970) using a Biorad Mini Protean II Dual Slab Cell apparatus. The minigels were 1mm in thickness and were 9cm x 6cm excluding the stacking gel. A discontinuous buffer system was used where the pH of the sample buffer and the upper gel buffer was lower than that of the resolving gel and the reservoir buffer. This has the effect of concentrating the proteins into stacks within the large pore upper gel prior to their separation in the resolving gel.

The gels were prepared from the following stock solutions:-

Stacking gel: 4·5% Acrylamide-bis-acrylamide, 0·125M Tris, 0·1% SDS pH 6·8

Resolving gel: 10% Acrylamide-bis-acrylamide, 0·375M Tris, 0·1% SDS pH 8·8

The gels were polymerised by the addition of 12·5% N,N,N'N'- tetramethyl ethylene-diamine (TEMED, BDH) and freshly prepared 7·5% ammonium persulphate. Marker proteins of known relative molecular mass (M_r) were included on each gel. These were obtained from Pharmacia in the form of a kit (Pharmacia Electrophoresis Calibration Kit for "low molecular weight proteins") their respective Mr were as follows; Phosphorylase b 94,000; bovine serum albumin 67,000; ovalbumin 43,000; carbonic anhydrase 30,000; trypsin inhibitor 20,000 αlactalbumin 14,400. The marker proteins were dissolved in 0·5mls sample buffer to give a final protein concentration of

1mg ml^{-1}. Electrophoresis was carried out at 25mA constant current.

Gel Staining.

(i) Coomassie staining.

Gels were fixed and stained in a solution of methanol (50%), glacial acetic acid (10%) with Coomassie brilliant blue (0·05%). After the staining was completed (approximately 1h) the gels were destained in order to remove the background stain and allow the protein bands to be clearly seen. This was achieved by placing the gels in a solution of 15% methanol and 10% glacial acetic acid.

The relative molecular mass of the plasma proteins were estimated from a calibration curve constructed from the relative mobilities of the standard marker proteins plotted against their log M_r.

(ii) Silver staining.

Silver staining was carried out following the method of Sammons, Adams and Nishizawa (1981). The gel was placed in dilute destain solution (7·5% methanol and 5% glacial acetic acid) for 30 min prior to staining. Staining was carried out at room temperature in a solution of 1·9 $mgml^{-1}$ $AgNO_3$. The gel was covered to exclude the light and placed on a rotary shaker for 2h. After staining the gel was washed in distilled water and fixed in a 3% solution of NaOH containing 8·75mg $NaBH_4$ and 0·75 mls formaldehyde for 10 min. The colour was developed for 30 min. in Na_2CO_3 solution (7·5 mg ml^{-1}).

6. Schistosomes.

During the course of development in compatible snails, schistosomes undergo rounds of asexual reproduction. This involves several transformations:- the free-living larval form, the miracidium, having penetrated a snail transforms into the first of the generative forms, the mother sporocyst; the mother sporocyst gives rise to the second generative forms, the daughter sporocyst, which in turn produces cercariae. The miracidia, sporocysts and cercariae are morphologically distinct but it is not known whether they differ in their surface properties.

For S. mansoni it has been found that antibodies raised to the glycocalyx of cercariae also bind to the surface of miracidia (Chiang and Caulfield, 1988). Antisera raised to haemolymph proteins of resistant and susceptible B. glabrata bind to both miracidia and sporocysts (Yoshino and Bayne, 1983). These results suggest that there are similarities in the tegumental constitution of larval schistosomes. However, lectin staining of the miracidia and sporocysts shows that at least one fundamental change occurs with transformation. The receptors for eel agglutinin (L-fucopyranose; galactogen) are present on miracidia but are lost on transformation to sporocysts (Yoshino, Cheng and Renwrantz, 1977).

The surface carbohydrates and proteins of S. margrebowiei larvae were investigated using lectins and protein labelling. In order to produce large numbers of sporocysts free from snail components, the miracidia were transformed in vitro.

(a) The in vitro transformation of S. margrebowiei miracidia.

The transformation from miracidium to sporocyst does not require the presence of the snail tissue and can be effected in vitro (DiConza and Basch, 1974; Stibbs, Owczarzak, Bayne and Dewan, 1979; Samuelson Quinn and Caulfield, 1984; Mellink and van den Bovenkamp, 1985). In these published studies the media used for transformation contained a complex selection of amino acids, salts, sugars and serum. Experiments were carried out to observe the effect of different media on miracidia in an attempt to find a simple defined medium capable of transforming S. margrebowiei miracidia and maintaining the sporocysts.

Miracidia were collected, aseptically, from freshly hatched eggs and washed in sterile distilled water to remove any contaminating liver tissue. A concentrated suspension of miracidia (approximately 5000 organisms ml^{-1}) was prepared in distilled water and 10μl added to the wells of a 24 well Nunclon Delta SI tissue culture plate. The suspension was diluted with 1·5 ml of the trial culture medium. The plates were then incubated at room temperature (20°C), 27°C (snail incubator temperature) or 37°C. Miracidial behaviour and transformation were monitored using an Olympus inverted microscope.

(i) Comparison of different culture media and salt solutions.

The culture media tested for their ability to transform miracidia and maintain sporocyst were as follows:

1. Medium F. (table 4) + 10% Foetal calf serum.

Medium F supplemented with 15% foetal calf serum is used for the routine transformation of S. mansoni miracidia (e.g. Bayne, Loker and Yui, 1986; Yoshino and Bayne, 1983; Dikkeboom, Bayne, van der Knaap and Tijnagel, 1988). The complement factors present in the serum were inactivated by heating the serum for 1h at 56°C. Medium F. was also tested without the addition of serum.

2. Chernin's balanced salt solution (CBSS) (table 5).

This salt solution was developed by Chernin (1963) for the culture of snail cells. It was used both with and without the addition of foetal calf serum. Tests were also carried out using this salt solution without the inclusion of the sugars, glucose and trehalose. Alternative energy sources were investigated. These included the addition of 5mM proline, 5mM glutamine. The in vitro cultivation of trypanosomes has shown that these organisms preferentially utilise the amino acid proline as an energy source, rather than glucose (Evans and Brown, 1972). The adult worms and the schistosomulae of S. mansoni have been shown to actively take up and utilise vertebrate haemoglobin (Foster and Bogitsh, 1986). CBSS without sugars but including snail or human haemoglobin was prepared and tested. Snail haemoglobin is not enclosed in erythrocytes, plasma from B. natalensis snails was used as a supplement to the CBSS. Human haemoglobin was also used as a supplement and was prepared by lysing a 4% v/v solution of human A blood with distilled water, spinning to bring down

TABLE 4

Medium F.

Basal Medium Eagle Amino Acids (GIBCO BRL)

Amino acid	mg L^{-1}	Amino acid	mg L^{-1}
L-Arginine•HCl	21•0	L-Cystine	12•0
L-Histidine	8•0	L-Isoleucine	26•0
L-Leucine	26•0	L-Lysine•HCl	36•47
L-Methionine	7•5	L-Phenylalanine	16•5
L-Threonine	24•0	L-Tryptophan	4•0
L-Tyrosine	18•0	L-Valine	23•5

Additional Amino acids and organic acids (GIBCO BRL)

Amino acid	mg L^{-1}	Amino acid	mg L^{-1}
Serine	0•6	Proline	0•29
L-Alanine	0•24	Aspartic acid	0•28
Glutamic acid	0•47	Glycine	0•24
α-Alanine	0•24	Malic acid	4•0
ß-Ketoglutamic acid	3•0	Succinic acid	1•0
Fumaric acid	0•5	Citric acid	1•0
L-Glutamine	292•0		

Basal Medium Eagle Vitamins (GIBCO BRL)

	mg L^{-1}		mg L^{-1}
NaCl	85•0	Biotin	1•0
D-Ca Pantothenate	1•0	Choline Chloride	1•0
Folic Acid	1•0	i-Inositol	2•0
Nicotinamide	1•0	Pyridoxal HCl	1•0
Riboflavin	0•1	Thiamine HCl	1•0

Salts and Sugars (Sigma)

	mg L^{-1}		mg L^{-1}
NaCl	1500•0	Na_2HPO_4	70•0
KCl	150•0	$MgSO_4.7H_2O$	450•0
$CaCl_2.2H_2O$	530•0		
Galactose	4500•0	Glucose	1000•0

+

0•002M HEPES.

the cell debris and removing the supernatant. Snail plasma and human haemoglobin were sterilised by 0·22 μm membrane filter.

TABLE 5

CHERNIN'S BALANCED SALT SOLUTION (CHERNIN, 1963).

	mg L^{-1}		mg L^{-1}
NaCl	2800·0	KCl	150·0
$CaCl_2$	530·0	Na_2HPO_4	70·0
$NaHCO_3$	50·0	$MgSO_4.7H_2O$	450·0
Glucose	1000·0	Trehalose	1000·0

3. Phosphate Buffered Saline

Experimental reagents eg. lectins, were usually prepared in PBSA. The effect of this buffer on miracidia was therefore examined (Table 6).

TABLE 6

PHOSPHATE BUFFERED SALINE (PBSA)(DULBECCO)

	mg L^{-1}		mg L^{-1}
NaCl	8000·0	KCl	200·0
Na_2HPO_4	1150·0	K_2HPO_4	200·0

4. 145mM NaCl

The change in salt concentration from that found within the vertebrate host to that found in fresh water cause the schistosome eggs to hatch. When miracidia enter the snail host there is a return to a higher salt concentration. Salt at the mammalian physiological concentration of 145mM was tested with the miracidia.

(ii) Bulk transformation of miracidia.

Miracidia were collected, free of liver debris, in 50ml centrifuge tubes. NaCl solution was added to the tube to make a final concentration of 145mM. The tubes were left for 10 min to ensure all the miracidia were immobilised and then centrifuged at 1000 rpm for 2 min. The pellet of miracidia was re-suspended in 20ml CBSS without sugars + human haemoglobin and the suspension transferred to Falcon tissue culture flasks (3013E). The flasks were incubated at 37°C.

(b) Surface Morphology

The surface morphology of the larval schistosomes was studied by SEM. Miracidia, sporocysts and cercariae were fixed in a solution of 2·5% glutaraldehyde in PBSA for 4h. After fixation the glutaraldehyde solution was passed through a square of fine nylon mesh (44μ) which retained the parasites. The mesh was folded in such a way as to form a cone enclosing the schistosomes and sealed at the top with a small bulldog clip. The specimens were prepared for SEM as described earlier.

(c) Surface Chemistry

(i) Carbohydrates

Commercially available fluorescently labelled lectins were used to determine the major carbohydrate groups of the larval surface. The lectins were obtained from Sigma Chemicals and those used included ricin$_{120}$, Concanavalin A (Con A), wheat germ agglutinin (WGA), Asparagus pea (Asp Pea) and peanut

agglutinin (PNA). Lectins were used at a concentration of 100μg ml^{-1}.

Schistosome larvae were prepared as follows:-

The miracidia were hatched from freshly collected eggs and were washed thoroughly in distilled water before use.

Sporocysts were transformed in vitro in CBSS supplemented with human haemoglobin. They were harvested at 24 h post transformation. The culture media containing the sloughed miracidial plates was removed and the larvae washed in PBSA.

The cercariae were collected from B. natalensis snails with patent S. margrebowiei infections. The snails, which had been kept in the dark from the onset of shedding, were placed under a 40 watt bulb to trigger cercarial release. After 2h the cercariae were collected from the surface of the water using a pasteur pipette.

Approximately 300 of each of the larval forms were collected with the aid of a dissecting microscope and pasteur pipette and placed in 1·5 ml Eppendorf tubes. The larval pellets were re-suspended in PBSA or distilled water containing either inhibitory or non-inhibitory sugar (100mM). The schistosomes were pre-incubated with the sugar solutions for 15 min prior to the addition of lectin.

All samples were placed on a Vibrax shaker and left gently shaking (100 rpm) for 30 mins at room temperature. The tubes were covered with aluminium foil to prevent photobleaching of the fluorescent dyes. At the end of the staining period the samples were spun on a bench microfuge and the supernatant removed, the pellet was then washed x 3 in

PBSA. The schistosome samples were transferred onto a glass slides and prepared for fluorescence microscopy.

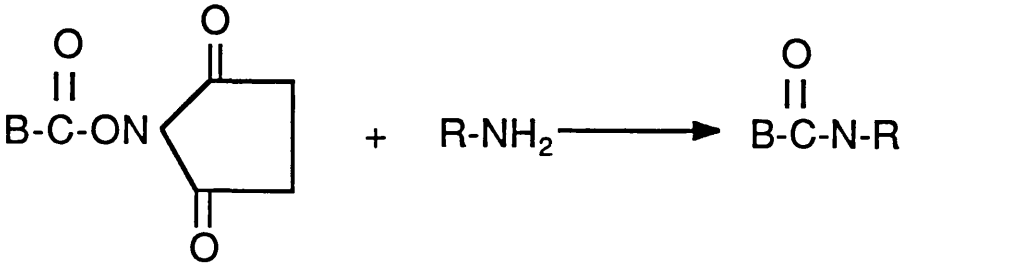
(ii) Surface Proteins

In order to identify the surface proteins of the larval schistosomes they were labelled with the vitamin biotin. The covalent linkage of biotin to surface proteins is maintained throughout subsequent protein separation procedures. The presence of biotin and hence the surface proteins is detected by staining with labelled avidin, a protein that has a high affinity for this vitamin.

Biotinylation of the surface proteins was achieved by using a reactive derivative of the vitamin, sulpho-NHS-biotin which forms covalent bonds with free amino groups. Sulpho-NHS-biotin and labelled avidin products were obtained from GIBCO.

FIGURE 6

Biotinylation: The Sulpho-NHS-Biotin reaction



Approximately 500-1000 larval schistosomes; miracidia, sporocysts and cercariae were placed in an Eppendorf tube and spun in a microcentrifuge (MSE Micro Centaur) for 2 min at high speed. The supernatant was discarded and the pellet taken up with 0·5 ml of sulpho-NHS-biotin at a concentration of 500 μgml-1 in distilled water (PBSA was used in the sporocyst and cercaria preparations). The tubes were rotated on a clinostat for 1-2h at room temperature. Excess biotin was removed by washing the larval schistosomes in PBSA x 3. A sample of cercariae belonging to a different species of schistosome S. nasale was also treated with biotin and compared with S. margrebowiei.

In order to ensure that biotinylation of the schistosome surface had taken place a small sample (approximately 20 schistosomes) was removed and stained with FITC-Avidin. A solution of 1:400 v/v of FITC-avidin was prepared in distilled water (PBSA) and added to the schistosome sample. Samples were incubated for 30 min. at room temperature then washed in PBSA x 3. Control samples of the schistosomes were treated with FITC-avidin without prior treatment with biotin. The schistosomes were viewed using fluorescent microscopy.

Preparation of samples for gel-electrophoresis.

The remainder of the sample was re-suspended in distilled water and the motile forms, cercariae and miracidia were allowed to recover. Only those individuals actively swimming were taken on to the next stage, this was to ensure that only surface proteins were labelled with biotin. These schistosomes were spun into a pellet by once more increasing the

concentration of NaCl to a level that caused inhibition of swimming (approximately 145mM). The pellet was then taken up in 50µl hot sample buffer pH 6·8 and boiled for 10 min. The non-motile sporocysts were examined under the microscope to estimate the percentage of non-ruptured cells. Good samples where 80% of the cells appeared whole were used and samples produced as above. After boiling the samples were spun at high speed for 2 min and the supernatant transferred into Eppendorf tubes and stored at -20°C. The samples were loaded onto acrylamide gels as described above. Between 10-15µl were loaded onto 7.5% gels for the cercariae and onto 10% gels for the sporocysts and miracidia.

Protein Electrophoretic Blotting

The proteins separated by SDS-PAGE were transferred onto nitrocellulose paper using the method of Towbin, Staehell and Gordon (1979). The transferred proteins are bound to the membrane in a way that renders them accessible to probing with biologically active reagents without the protein bands diffusing or losing their resolution. Biotinylated proteins were detected by treatment with peroxidase labelled streptavidin and the addition of the enzyme substrate, hydrogen peroxide with 4-Chloro-1-napthol. The Bio-Rad Trans-Blot system was used.

After electrophoresis the gel was removed from the plates and overlayed with nitrocellulose paper. The gel and paper were then sandwiched between filter papers and support pads and clamped together with plastic support frames. These were then fitted into the electrophoresis tank with the

nitrocellulose sheet towards the positive terminal and the gel towards the negative terminal. The tank was filled with transfer buffer consisting of 2·43g Tris, 11·25g glycine in a 20% methanol water mixture. Electrophoretic transfer was carried out at 100v for 2h, the tank was cooled by placing it in an ice-bucket, this prevented a rise in current during transfer.

After transfer the gel and nitrocellulose paper were separated. The gel was stained with Coomassie blue to assess amount of protein transfer. The nitrocellulose paper was dried between filter papers and stained for total protein with 2% Ponceau red in distilled water. Molecular weight marker bands were measured and marked on the blot in pencil. The presence of protein bands in the other lanes was noted. The blot was returned to the staining tray, placed on a Rotostat shaker at 100 rpm for 30min. in a 5% solution of milk proteins (Rapilait) in PBSA to block non-specific binding. This treatment removes the Ponceau red stain from the blot. The blocking solution was removed and replaced with a 1:400 dilution of horse-radish peroxidase avidin in Rapilait/PBSA for 1h. After washing in 5% Rapilait/PBSA x3 and PBSA x3, the peroxidase substrate was added. 15mg 4-chloro-1-napthol in 5mls methanol + 15 mls tris buffered saline (this saline contained 1·23g tris and 9g NaCl in 1l distilled water), 50μl of 30% hydrogen peroxide added just prior to use. The reaction was quenched with TBS after approximately 10 min. The blots were then dried and photographed.

7. Haemocytes

(a) Haemocyte numbers.

Preliminary studies showed that B. natalensis and B. nasutus, in common with other freshwater snails studied, have relatively few cells per ml of haemolymph. In a review of the literature on gastropod molluscs (Sminia, 1981), put this number at 5×10^4 cm^{-3} as compared with 2×10^7 for the dipteran insects (Gotz, 1986). The low number of cells plus the small volumes of haemolymph available make these haemocytes difficult to study. The haemocytes are fragile and readily lyse, all glassware was therefore cleaned in the free-rinsing detergent Micro and rinsed extensively in distilled water before use. New microscope slides and coverslips were simply cleaned with 70% alcohol and dried with lens tissue.

The terminology used to describe the different types of haemocytes varies with organism and author. In this dissertation the term plasmatocyte will be used to describe large (> 15μm) cells that readily attach and flatten on glass substrates. The small (< 10μm) round cells that do not attach as firmly on glass are termed granulocytes. Occasionally cells were seen that were smaller than plasmatocytes, spread on glass but with no filopodia.

Haemocytes were prepared for examination using a Shandon Elliot Cytocentrifuge, this enabled all of the particulate matter in the haemolymph, including the cells, to be spun onto glass microscope slides or coverslips. The cytocentrifuge comprises of a number of perspex blocks each with an inlet and outlet connected by a conduit (figure 7). A standard glass

microscope slide was attached to the outlet side of the chamber with a filter pad sandwiched between the two. The filter pad has a circular hole that corresponds to the outlet of the chamber. Once in their correct orientation the chambers were loaded into the cytocentrifuge and a known volume of haemolymph was loaded into the chamber inlet together with buffer (PBSA). The chambers were spun and the haemolymph forced out of the chamber and onto the glass slide. The plasma and buffer were absorbed by the filter paper and the cells remained in a discrete circle on the glass slide. Immediately after spinning the slide was removed and the cells covered with 20μl of CBSS. The cells were left in a humid chamber for 10 min to allow the cells to settle. In order to facilitate the attachment of cells to the glass slides bovine serum albumen was included in the buffer at a concentration of 3% w/v, this makes the particulate matter present in the haemolymph 'sticky' and remain on the glass. This method preserves a higher proportion of the smaller granulocyte cells that do not attach to glass as readily as the larger plasmatocytes, these cells are often lost in conventional gravity settling methods of cell preparation.

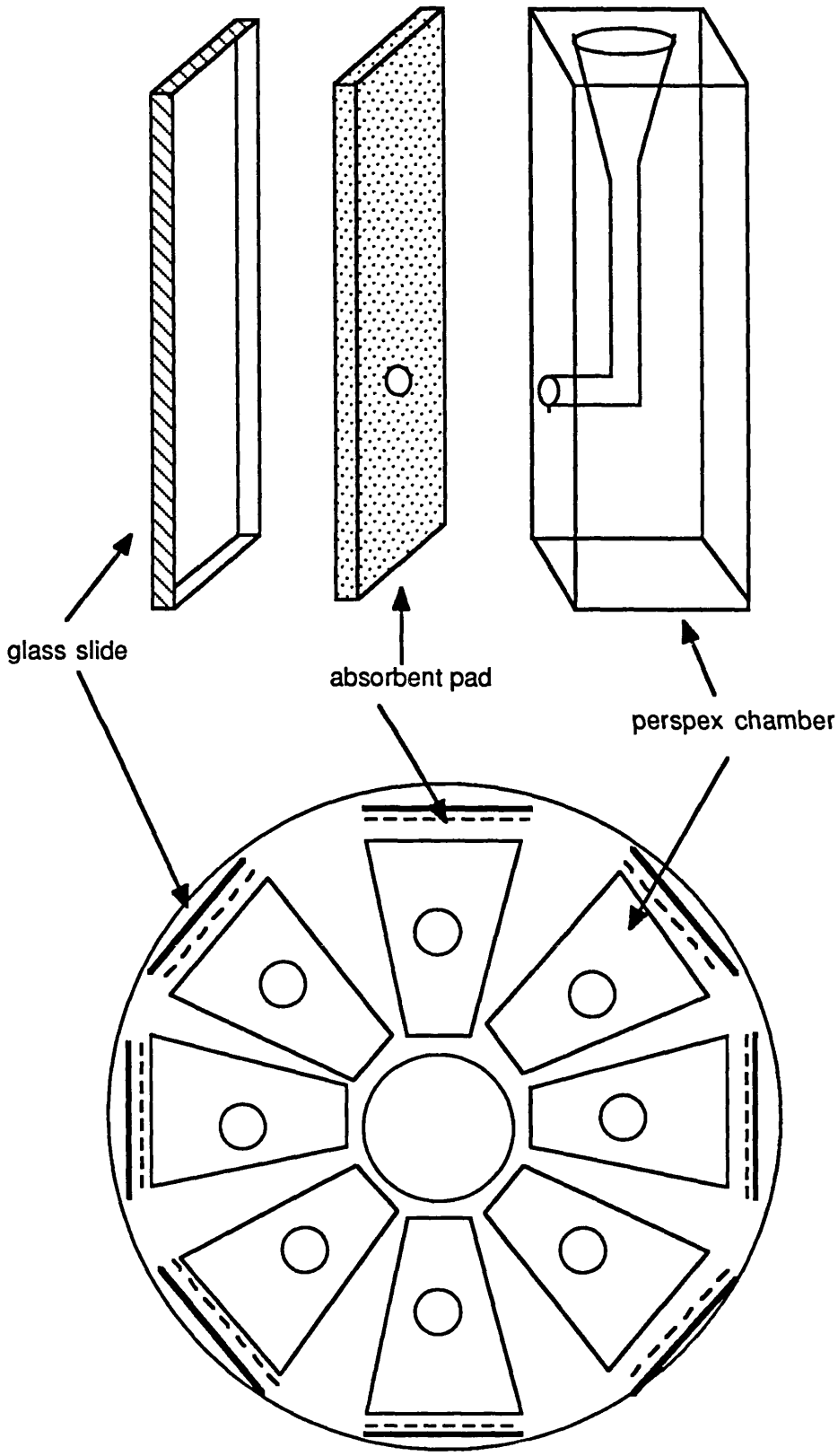
Haemocyte numbers were estimated by counting the total number of cells present on the microscope slide after spinning a known volume of haemolymph in the cytocentrifuge. This method was preferred to that of a haemocytometer as less haemolymph was required for cell number estimation. Prior to counting the CBSS was removed using a finely drawn pasteur pipette, the cells were mounted in glycerol and overlaid with

a coverslip held in position and sealed with clear nail varnish. The total area of the circle containing the cells was counted with the aid of the microscope stage co-ordinates and an eye-piece graticule.

Samples for haemocyte counts were collected as follows. Snails were cleaned and bled as described earlier, the haemolymph was taken up in a pasteur pipette and pooled in an Eppendorf tube kept in an ice-bucket to prevent the cells from settling onto the sides of the tube. Aliquots of 10μl of haemolymph were taken and pipetted into the cytospin chambers, previously filled with 50μl of PBSA with BSA. The chambers were spun at 800 rpm for 3 min.

FIGURE 7

THE CYTOCENTRIFUGE



(i) Comparison of B. natalensis and B. nasutus.

Cell counts were carried out on both adult and juvenile snails. Snails were assumed to be adults if their shell height was greater than 10mm for B. natalensis or greater than 13mm for B. nasutus. Juveniles were taken as being approximately half the adult size. A percentage differential count of plasmatocytes and granulocytes was also made.

(ii) Comparison of cell numbers pre and post exposure to schistosomes.

Haemocyte counts were carried out on individual snails of both species pre and post their exposure to S. margrebowiei miracidia. Adult snails of B. natalensis and B. nasutus were selected and placed individually in plastic pots, each snail was bled and their haemocyte counts recorded. The snails were placed in an incubator at 27°C and left for 24h to recover from this first bleed. The snails were divided into a control group, not exposed to miracidia, and an experimental group, with miracidia: 10 miracidia were introduced into each of the pots with experimental snails. After 2h all the snails were bled and the haemocyte counts taken. A further count was taken 24 h after the introduction of the miracidia.

(b) Haemocyte sub-populations.

The haemocytes were studied to ascertain whether the two major populations distinguished on morphological grounds could be further sub-divided on a functional level or by cell surface differences reflected in differential lectin binding.

(i) Lectin binding of haemocyte surfaces.

Initial experiments with lectins gave highly variable results, this was due to the lectins entering cells rather than binding to the cell surface. A series of experiments was conducted using propidium iodide (PI) to distinguish the living and dead cells. This dye is excluded from live cells and fluoresces red when excited by light of 493nm wavelength. These experiments illustrated that, despite their intact appearance, the haemocytes had often lysed with exposure to air. The best results were obtained from disturbing the cells as little as possible, keeping them covered in CBSS in a humid chamber and reducing the overall staining time to 15 min.

The haemocytes were prepared for lectin staining and fluorescent microscopy as follows:- Cleaned glass coverslips (13mm diameter) were placed in a humid chamber. A drop of CBSS (30μl) was placed on each coverslip and 20μl of haemolymph added to each drop of CBSS. Care was taken to ensure that the haemolymph was introduced below the meniscus of the CBSS drop and the lid of the humid chamber was immediately replaced. The cells were left at room temperature for 10 min to allow them to settle onto the glass, then the haemolymph removed and the cells washed. This was achieved by carefully removing the CBSS and haemolymph with a finely drawn pasteur pipette and immediately covering the cells with fresh CBSS. Fluorescently labelled lectins (Sigma chemicals) were added to each cell preparation at a concentration of 100μg ml^{-1} in CBSS. The humid chamber was covered with aluminium foil to exclude light during the staining time. After 10 min 20μl of PI (Sigma

chemicals) at a concentration of 10μg ml^{-1} was added. The cells were left for a further 5 min before being washed and mounted for fluorescence microscopy.

The lectins used included Concanavalin A (Con-A), Wheat germ agglutinin (WGA), Soya bean agglutinin (SBA), Peanut agglutinin (PNA) and Asparagus pea (Asp Pea). When positive staining did occur further experiments were carried out using sugars inhibitory for the lectin to ensure that the staining was specific.

The percentage of cells staining with WGA was quantified using a flow cytometer. A suspension of haemocytes from 75μl of B. natalensis haemolymph was stained for 15 min with WGA at a concentration of 100μg ml^{-1} in PBSA. No attempt was made to wash these cells as the background fluorescence would not be recorded as an 'event'. Each particle that passes through the laser beam is regarded as an event, the parameters were adjusted so that only particles above 5μm were counted as cells. These are known as 'gated events'. A preliminary run using unlabelled haemocytes was made in order to establish the amount of background fluorescence emitted from these cells. The stained haemocyte suspension was introduced into the sample chamber of a Becton Dickinson FACS analyzer. The suspension was then passed in a stream through a laser beam. As each cell passes through the beam, laser light is scattered. The size and fluorescence of each cell is determined by the pattern of scattering.

(ii) Functional assessment of haemocytes

Haemocyte heterogeneity was also investigated on a functional level. Pooled haemolymph was diluted with CBSS (50μl haemolymph : 100μl CBSS) to which was added either fluorescent beads (Polyscience Inc.), or E. coli. The E. coli were made to fluoresce by staining with the DNA intercalating agent ethidium bromide (50μg ml^{-1}) before use. The haemocytes and particles were kept in suspension by placing them on a Voss Blood Cell Mixer for 5, 10 or 15 min before being loaded onto the cytocentrifuge and spun onto coverslips. The BSA was omitted from the initial buffer, the coverslips were coated in poly-lysine to aid cell attachment. A solution of poly-lysine, 1mg ml^{-1}, was pipetted onto clean coverslips and left for 10 min., the solution was tipped off and excess poly-lysine removed with a jet of distilled water from a wash bottle. After spinning, the cells were left to settle for 10 min prior to staining with fluorescein diacetate. Fluorescein diacetate was used to show the presence of non-specific esterases, this is a lipophilic ester that readily crosses cell membranes where it becomes the substrate for these enzymes. Cleavage of this molecule by the enzymes releases the fluorescent tag, which being no longer lipophilic is trapped within the cell. A stock solution of fluorescein diacetate was prepared by dissolving the solid in acetone at a concentration of 10 mg ml^{-1}, this solution was diluted in CBSS prior to use to produce a solution of 0·5 μg ml^{-1}.

An attempt was made to correlate these in vitro observations with the situation in vivo by injecting E. coli

directly into the snails. Most such attempts failed since the snails' reaction to being injected was to exude haemolymph thus preventing subsequent bleeding. Only one attempt did succeed where a large B. nasutus snail was injected through the foot with 10μl of E. coli in PBSA. The snail was returned to the incubator for 30 min before being bled and the haemocytes examined.

(c) Haemocyte cytoskeleton

Encapsulation and cell spreading requires the participation of the cytoskeletal elements of the cell. The actin and tubulin elements of the cytoskeleton were examined using fluorescently labelled reagents specific for these molecules.

Haemocytes were prepared as described previously, 30μl of haemolymph in 100μl of CBSS containing 1% BSA were spun onto glass coverslips using the cytocentrifuge. After settling the cells were fixed in 3% paraformaldehyde solution. The fixative was prepared by dissolving 10g of paraformaldehyde (Fluka A.G.) in 100ml of distilled water warmed to 80°C, 2-3 drops of 1N NaOH was added to clear the solution. This 10% stock solution was diluted with PBSA to produce a 3% solution of pH 7·2. The haemocytes were fixed in order to preserve the internal components from enzymic degradation, fixation was carried out for 15 min, then the cells were washed x 3 in CBSS with 3% BSA and 100mM lysine, this washing solution was used throughout the subsequent procedures. These additives serve to quench the fixation reaction by reacting with any remaining free aldehyde groups and to prevent any non-specific binding

of the staining reagents. Next the haemocyte membranes were rendered permeable by treating the cells with a 0·2% solution of the non-ionic detergent Triton X 100 for 15 min. After washing (x3) the cellular components other than those of the cytoskeleton were extracted in acetone at 4°C. The coverslips were placed in a 1:1 acetone, water mixture for 3 min then transferred into absolute acetone for 5 min before being returned to the acetone water mixture for a further 3 min. The cells were now prepared for staining.

The first stain used was TRITC-phalloidin diluted 1:9 with CBSS. Phalloidin is an extract from the mushroom Amanita phalloides that specifically binds to actin filaments and stabilises them, when conjugated to a fluorescent label it can be used to stain the actin filaments. The haemocytes were stained with phalloidin for 1h, since this reagent is highly toxic the cells were washed by immersing the coverslips in 500ml of CBSS. The CBSS was further diluted with water before disposal. Next the tubulin components were stained using a rat anti-tubulin monoclonal antibody (MAb) and a fluorescently labelled anti-rat monoclonal antibody as second layer. The anti-tubulin Mab was diluted 1:50 with CBSS and 40μl placed on each of the coverslips. The haemocytes were washed x3 and the presence of the bound antibody was visualised by staining with FITC-αrat antibody for a further 1h. The αrat antibody was used at a dilution of 1:100. Finally the haemocyte preparations were washed and mounted for fluorescence microscopy.

8. Haemagglutinins

The body fluids of many gastropod molluscs have been shown to agglutinate human red blood cells (RBCs). The status of these investigations was reviewed by Pemberton in 1974, at that time 134 species had been investigated, 60% of which were shown to possess haemagglutinins. Four species belonging to the genus Bulinus were tested but only one, B. truncatus, was found to have any agglutinating activity (Pemberton, 1974). Michelson and Dubois (1977) tested 4 populations of snails (B. globosus from Nigeria and S. Africa; B. truncatus from Egypt and Liberia), The S. African B. globosus had a low level anti-A agglutinin, the other snails were negative. No mention was made, in either of these studies, of their resistance/susceptibility to schistosomes. Lectins (agglutinins) have binding sites for specific carbohydrate groups and their presence can be detected by the agglutination of erythrocytes. Mammalian erythrocytes are classically used to test for agglutinating activity as they are a homogeneous set of cells with distinctive carbohydrate groups at their periphery.

(a) Haemagglutination assay.

Erythrocytes in isotonic solution sediment out of solution under gravity and form a tight button of cells in the concave bottom of test wells of haemagglutination plates. Agglutination impedes this sedimentation by the formation of a three-dimensional lattice of cells which results in the solution remaining cloudy. When the agglutinin is present in excess most sites on the cell surface become bound and the

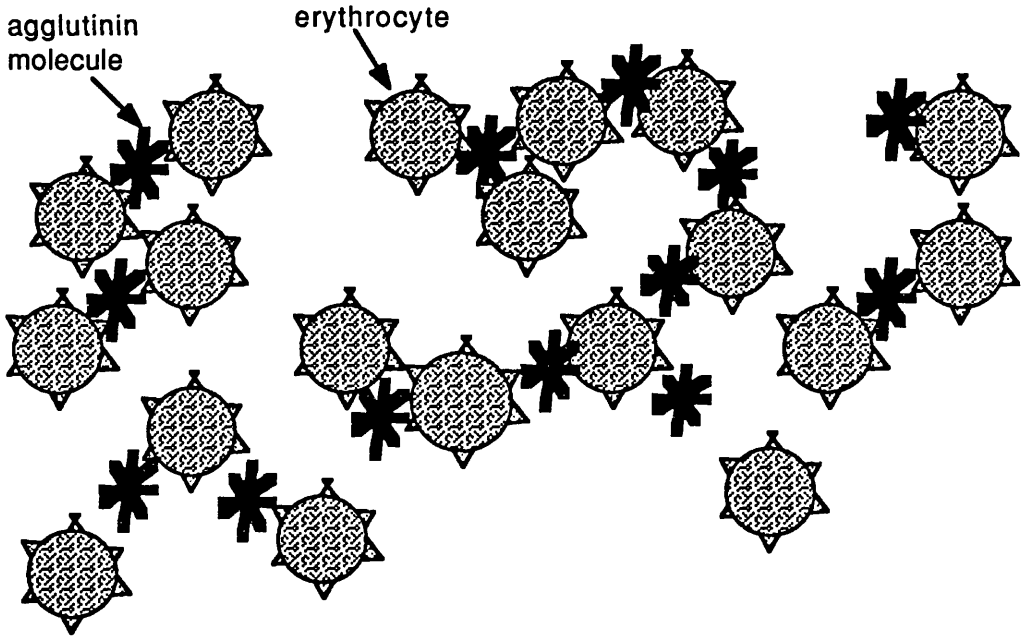
cells form a sheet that descends to the bottom of the well (figure 8). This situation can be distinguished from the negative as the sheet of cells appears angular and often folded. The haemagglutinin titre is taken as the lowest concentration of plasma in a serial dilution that causes agglutination.

The haemolymph was collected as described above, spun to remove the haemocytes and the cell free plasma used in the haemagglutination assay. Plasma samples were titrated in a doubling dilution series using Falcon 3911 assay plates (figure 9). 20μl of un-diluted plasma was pipetted into the first of 12 wells containing 20μl PBSA, the solution mixed and 20μl removed and transferred to the 2nd well. The dilution series was continued for ten wells, the final two wells containing PBSA alone formed the negative controls. 20μl of a 2% v/v solution of RBCs in PBSA were added to each well. Each tray was gently shaken and left for 2h at room temperature in order for the RBCs to sediment under gravity before the results were read.

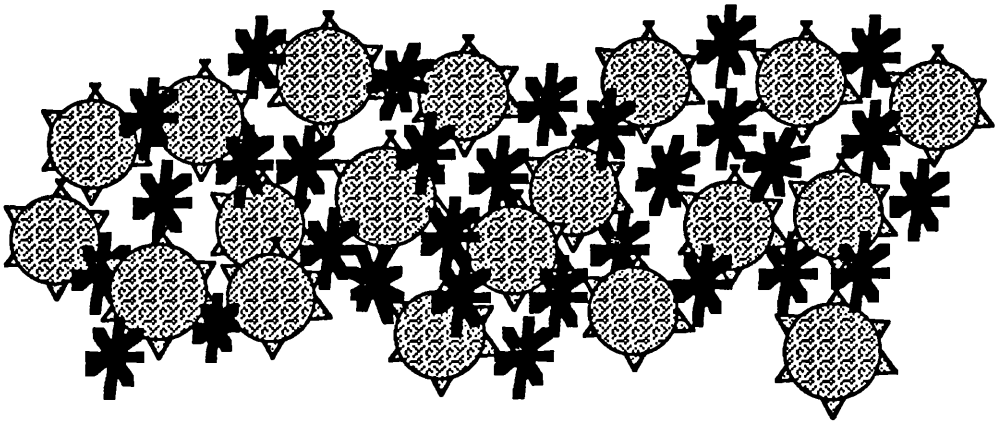
The snails were bled and their plasma tested against the human blood types A1, A2, B & O. These cells differ in the sugars that make up part of the glycophorin molecules. Type A cells have N-acetylgalactosamine residues at the end of the oligosaccharide chains, whereas those of type B these are replaced by galactose. The glycophorin molecules of type O cells have neither of these sugars. The difference between A1 and A_2 is a quantitative one there being fewer of the type A determinants on the A_2 cells.

FIGURE 8

HAEMAGGLUTINATION



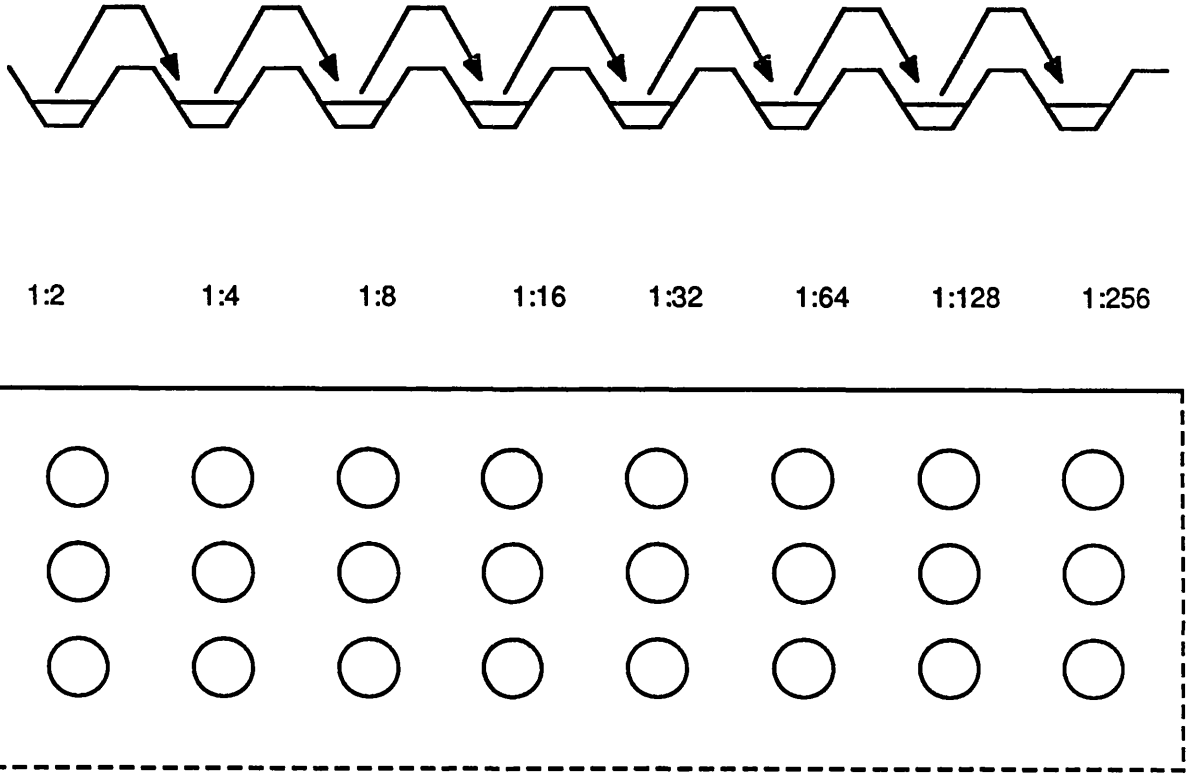
lattice formed with low titre agglutinin



sheet formed with high titre agglutinin

FIGURE 9

Preparation of a two-fold serial dilution series in a microtitre tray



20 µl of PBSA is added to each well.

20 µl of haemolymph is added to the first well, mixed and

20 µl carried over to the second well etc.

(b) Presence of Haemagglutinins in Bulinid Snails

(i) Interspecific variation within the genus Bulinus.

The Experimental Taxonomy Division BM (NH) maintains colonies of snails for experimental purposes. Nine species of bulinid snails were selected and tested for the presence of haemagglutinins using human red blood cells types A and B (Table 7). For each species approximately 10 snails were bled and the haemolymph pooled. These pooled samples were then tested for haemagglutinating activity against the human blood types A and B.

TABLE 7

SNAILS TESTED FOR THE PRESENCE OF HAEMOLYMPH HAEMAGGLUTININS

Snail species	Origin	Date of colonisation	Ploidy (2n)
Africanus			
B. globosus	Malawi	29.08.1985	36
B. jousseaumei	Gambia	28.11.1985	36
B. nasutus	Tanzania	30.07.1984	36
B. africanus	Kenya	16.07.1973	36
B. abyssinnicus	Ethiopia	30.12.1980	36
Truncatus/tropicus			
B. tropicus	Botswana	31.01.1977	36
B. truncatus	Malawi	22.05.1984	72
B. natalensis	S. Africa	06.10.1963	36
Reticulatus			
B. wrighti	S. Arabia	31.04.1980	36

(ii) Intraspecific variation within the species Bulinus nasutus

A comparison of plasma agglutinating activity was made between individual adult and juvenile B.nasutus snails. Adult B. nasutus snails have a shell height of 13mm ± 1mm, snails were considered as juvenile with shell heights were below 6mm.

The haemolymph samples were diluted 1:1024 prior to testing against human erythrocytes belonging to the blood groups A_1, A_2, B and O.

(c) Haemagglutinin induction

The agglutinins found in some invertebrate species have shown an increased titre after the animal has been challenged with foreign antigens (Cooper, Stein and Wodjani, 1984; Jeong, Sussman, Rosen, Lie and Heyneman, 1981).

B. nasutus snails were exposed to S. margrebowiei miracidia to discover whether this would induce enhanced titres of the agglutinin. Five snails were placed individually in plastic pots containing FCTW and 8 S. margrebowiei miracidia. Three pots containing snails but not miracidia were put up as controls. The snails together with the miracidia were placed in an incubator at 27°C for 24h. Haemolymph samples were taken at 2 and 24h post exposure.

(d) Red cell specificity.

The B. nasutus haemolymph under investigation had been shown to agglutinate the human erythrocytes. Sheep, rabbit, rat and mouse red blood cells were readily available in the laboratory and these were also used in 2% v/v solutions in PBSA with the B. nasutus haemolymph. Assays with mouse erythrocytes gave variable results depending on which laboratory strain of mouse was used. It is known that mice together with rats and domestic fowl express their major histocompatibility (MHC) antigens on the surface of their erythrocytes. Mice strains differing in their MHC haplotypes were tested, these included the strains:- CBA (k haplotype),

Balb/c (d haplotype), B10SCN (b haplotype), B10D2 (g1 haplotype) and athymic mice. The athymic mice originated from Balb/c stock and were therefore probably 'd' haplotype. Erythrocytes from the outbred strain Tyllers Original Swiss (T/O) were also tested.

(e) Requirement for divalent cations.

The lectin concanavalin-A requires bound calcium in order to maintain its carbohydrate binding sites. The agglutinins isolated from invertebrates vary in their requirement for divalent cations. The reagents ethylenediaminetetra-acetic acid (EDTA) and ethyleneglycol-bis-(ß-aminoethyl) N,N,N,N,-tetra-acetic acid (EGTA) act as chelating agents for these cations, effectively removing them from solution. Haemagglutination assays were carried out in the presence of EDTA and EGTA at a concentration of 5mM.

(f) Haemagglutination inhibition.

Lectins bind sugars in a specific and reversible manner, a property that can be exploited to isolate them from other constituents in the body fluids. Inhibition tests were carried out in order to discover the binding specificity of the haemagglutinin.

The panel of sugars chosen included different isomeric forms of the same sugar, this reflects the high degree of specificity exhibited by some lectins eg. eel agglutinin binds the L but not the D isomer of fucose. Other lectins only bind sugars when they are presented in a particular configuration. This situation is found in the agglutinin isolated from the haemolymph of the oyster Crassostrea virginica. This

agglutinin binds to sialic residues on the glycoproteins fetuin and mucin but does not bind free sialic acid (Cheng, Marchalonis and Vasta, 1984). Fetuin (type III from foetal calf serum) was used as a potential inhibitor at a concentration of 1mg ml^{-1}.

The sugars (BDH Chemicals), were used at a concentration of 100mM in PBSA. Each sugar solution was used as the diluent in a doubling dilution series with the plasma from B. nasutus snails. Sugars and plasma were allowed to equilibrate for 30 min at room temperature before the addition of 2% v/v solution of human A cells and left for 2h before reading.

Sugars used :- Glucose; Galactose; Mannose; α-D-Fucose ; α-L-Fucose ; Mannose + glucose; Methyl-αD-galactose; Methyl-βD-galactose; Methyl-αD-mannose; (methylation of these sugars prevents the inter-conversion from α to β anomer that occurs when these sugars are in aqueous solution) N-acetyl glucosamine; N-acetyl galactosamine; Sialic acid.

(g) Haemagglutinin absorption by larval schistosomes.

The haemagglutinin present in the haemolymph of B. nasutus snails binds to a variety of vertebrate erythrocytes. This ability can have little practical importance for the snail but it is possible that the haemagglutinin also has the ability to bind to larval schistosomes and thereby be implicated in the snail's immune response.

The following larval schistosomes were used:- S. margrebowiei, miracidia, sporocysts and cercariae. S. mansoni miracidia. S. mansoni eggs were a gift from Mrs Fiona Hackett, Parasitology Division , National Institute for Medical Research.

(i) Preparation of larvae.

The miracidia were hatched from freshly collected eggs as described in Material and Methods section 6. They were washed thoroughly in distilled water before use.

Sporocysts were transformed in vitro in CBSS supplemented with human haemoglobin (Materials and Methods section 6). They were harvested at 2 and 48 h post transformation. Culture media containing the sloughed miracidial plates was removed and the larvae washed in PBSA.

The cercariae were collected from B. natalensis snails with patent S. margrebowiei infections. The snails, which had been kept in the dark from the onset of shedding, were placed under a 40 watt bulb to trigger cercarial release. After 2h the cercariae were collected from the surface of the water using a pasteur pipette.

Approximately 300 of each of the larval forms were collected with the aid of a dissecting microscope and pasteur pipette and placed in 1·5 ml Eppendorf tubes. Miracidial preparations were occasionally contaminated with liver debris. A suspension of liver tissue (about 20% v/v) was included in the experiment. The larvae were used 'live' to avoid the risk of fixatives altering the chemical nature of the tegumental surface. Larval suspensions were pelleted using a bench microfuge (MSE Micro Centaur·) at high speed for 2 min and re-suspended in 0·5 ml of B. nasutus plasma at a dilution of 1:1024 in PBSA. Larvae and plasma were kept in suspension by shaking them on an IKA- VIBRAX-VXR shaker for 2h at 800 rpm. The plasma was separated from the larvae by centrifugation. Supernatants were then tested for agglutinating activity in a doubling dilution agglutination assay using human A_2 erythrocytes as indicator cells. B. nasutus plasma at a dilution of 1:1024 was included in the assay as a control.

The protein profiles of the haemolymph pre- and post absorption were compared. A fresh sample of haemolymph was taken from five adult snails and the plasma incubated, as above, with approximately 10^3 S. margrebowiei miracidia. After incubation the plasma was tested for agglutinating activity and samples were prepared for polyacrylamide gel electrophoresis.

(h) Fast Protein Liquid Chromatography (FPLC)

In order to ascertain what role, if any, the agglutinin plays in the defence of the sail it is necessary to isolate and purify it. As a first step towards this isolation the

plasma was fractionated using FPLC (figure 10). This method of column chromatography is based on the differential movement of molecules eg. peptides, proteins etc through a suitable adsorbent material. This material is packed in a column to form the solid phase and buffer flow provides the mobile liquid phase. The nature of the solid and liquid phases can be adjusted in order to produce a separation of the materials under study. Size, charge, differential binding properties to the solid phase or the differential solubility in the liquid phase will determine the separation process. Most chromatographic techniques have been designed for the separation of small molecules, amino acids, polypeptides etc. The larger proteins fail to adsorb onto the solid phase matrix and are eluted in the void volume. The FPLC has been developed specifically for the fractionation of proteins using conditions that maintain their biological activity.

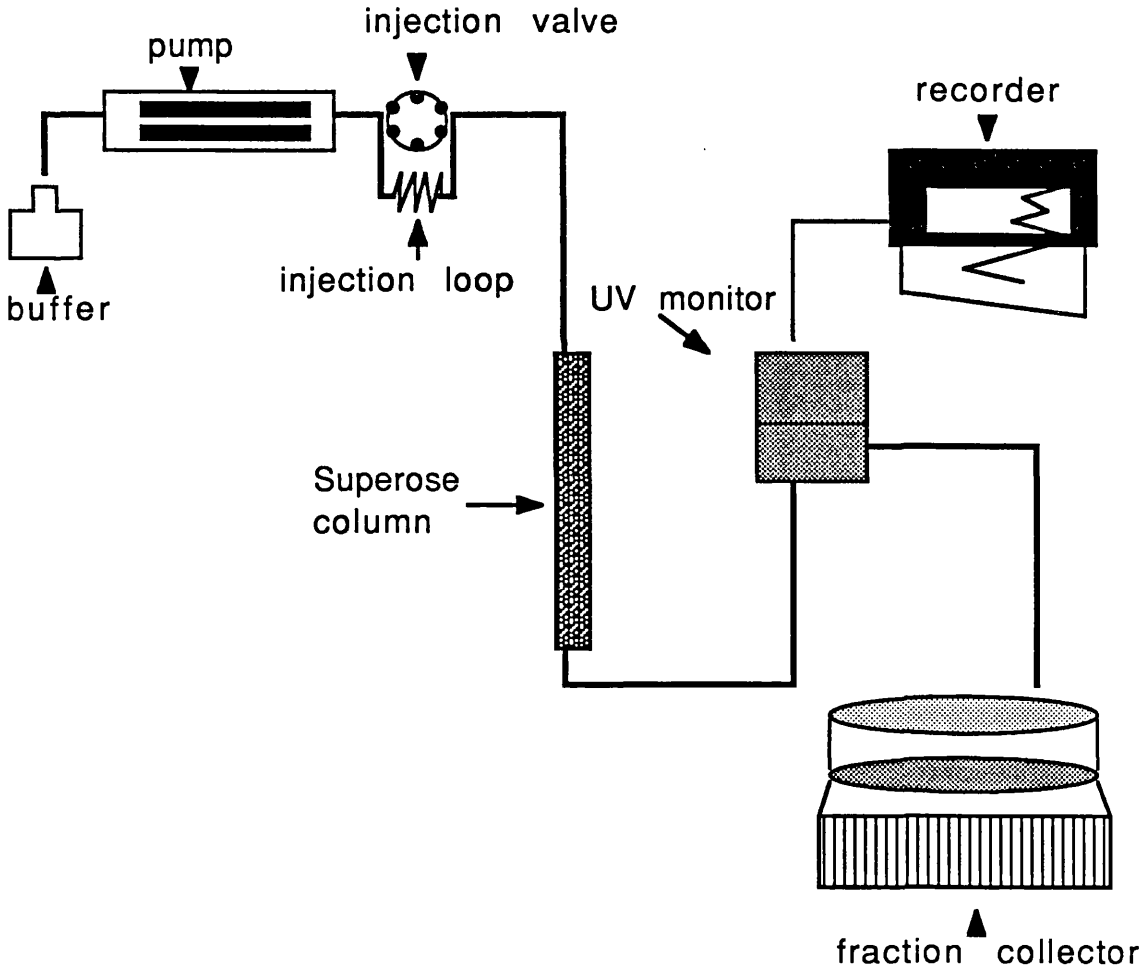
Fractionation was carried out using a Superose 12 column on a Pharmacia GP-250 FPLC. The superose column is composed of a highly cross-linked macroporous agarose gel with uniform particle size (10 ± 2μm) capable of the adsorption and subsequent separation of proteins with a M_r of between 1,000 and 300,000. Phosphate buffer 0·05M, pH 7·3 was used as the eluent, the physiological conditions were therefore maintained and biological activity was not lost in the fractionation procedure.

Samples of between 10 and 50μl plasma were introduced into the sample loop together with approximately 500μl eluent buffer. Phosphate buffer was made up as follows:- Solution A:

0·05M Na_2HPO_4, 0·15M NaCl. Solution B: 0·05M NaH_2PO_4, 0·15M NaCl. Solution B was added to solution B until the pH of the mixture was 7·3. The buffer was de-gassed and filtered through a 0·22μm membrane filter. This treatment removed the air bubbles and dust particles that were liable to block the system. Samples were run at a flow rate of 0·5 mls min^{-1} for 30 min and fractions of 0·5 mls were collected. All fractions were subsequently tested for haemagglutinating activity.

FIGURE 10

Fast Protein Liquid Chromatography



1. SCHISTOSOMES

(a) The in vitro transformation of S. margrebowiei miracidia.

The transformation of a miracidia into a sporocyst occurs in stages. First the cilia stop beating causing miracidia to cease swimming. Next the organelles of the ciliated plates degenerate, the plates becoming detached from the intercellular ridges and the underlying muscle layers. The intercellular ridges then expand and fuse with one another to form the tegument of the presumptive sporocyst. Finally the surface area of the tegument is increased by numerous cytoplasmic projections (figure 11). During the latter stages of transformation the sporocyst changes from an ovoid shape, (essentially the same shape as the miracidium) to pronouncedly tubular.

The study of transformation in vitro revealed that the initial stages could be initiated by an increase in ambient salt concentration. Ciliary beating and swimming was inhibited within 5 min of the miracidia being placed in normal saline solution (145mM), or PBSA. The miracidium/sporocyst have both circular and longitudinal muscle layers under the outer tegument and remains active throughout the transformation period. Inhibition of ciliary motility could be reversed by replacing the saline with FCTW or distilled water. However after 15 min the miracidia were committed to the transformation pathway and replacing the saline with fresh water resulted in the lysis and death of the larvae.

FIGURE 11

The Sloughing of the Miracidial Ciliated Plates and the Formation of the Sporocyst Tegument

A Diagram of the cross-section through miracidial surface

(i) ciliated plate

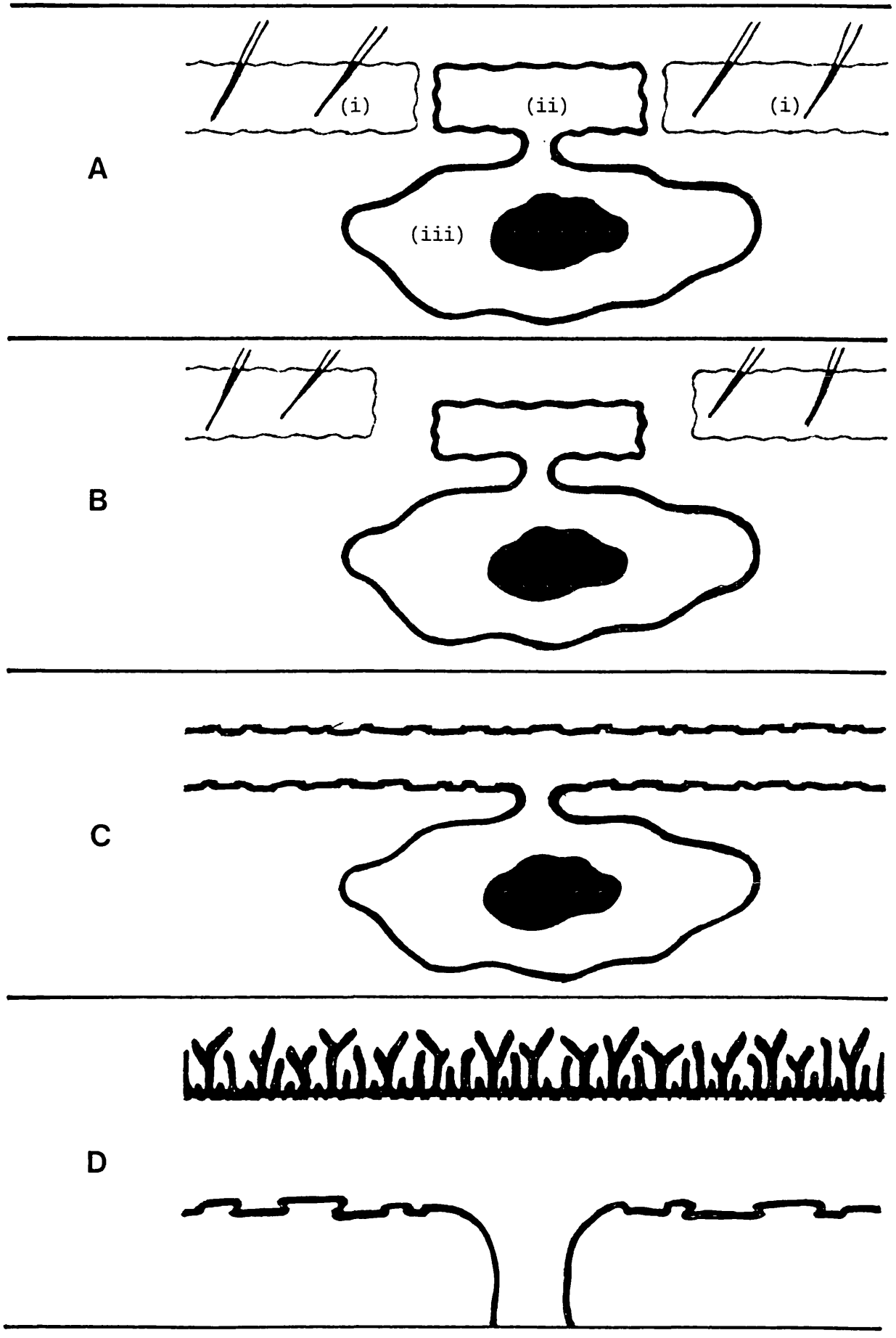
(ii) intercellular ridge

(iii) subepidermal cell

B Ciliated plates being lost from the surface

C Expansion of the intercellular ridge

D Fully formed sporocyst tegument with microvilli


A
(i)
(ii)
(i)
(iii)
B
C
D

Continued incubation in saline solutions resulted in the shedding of the ciliated plates. The time taken for this to occur varied greatly between individual miracidia. On average, at room temperature (approximately 20°C), plate shedding began approximately 2 h post media replacement and was completed after 6 h. Continued maintenance and growth of the sporocysts could not be supported by saline solutions.

Medium F proved to be less efficient than saline solutions at instigating transformation. Miracidia incubated in Medium F immediately after hatching took, on average, 8h to begin plate shedding and after 10h some miracidia (approximately 2%) were still swimming. However after the ciliated plates were shed, this medium, when supplemented with foetal calf serum, supported the continued growth of the sporocysts. By day 4 the sporocysts had become tubular.

Incubation of miracidia in CBSS with 10% foetal calf serum was comparable to Medium F but with a shorter initiating time. Miracidia stopped swimming after 20 min incubation and plate shedding occurred after approximately 3h. During studies to discover alternative nutrients to foetal calf serum it was observed that transformed sporocysts could be maintained for longer periods of time when this salt solution lacked the sugars glucose and trehalose. CBSS without sugars was therefore used as the basic medium for testing the effects of proline, glutamine and haemoglobin. Proline and glutamine proved to be unsuitable for sporocyst development. B. natalensis plasma and human haemoglobin supported sporocyst growth and development. There was little difference between

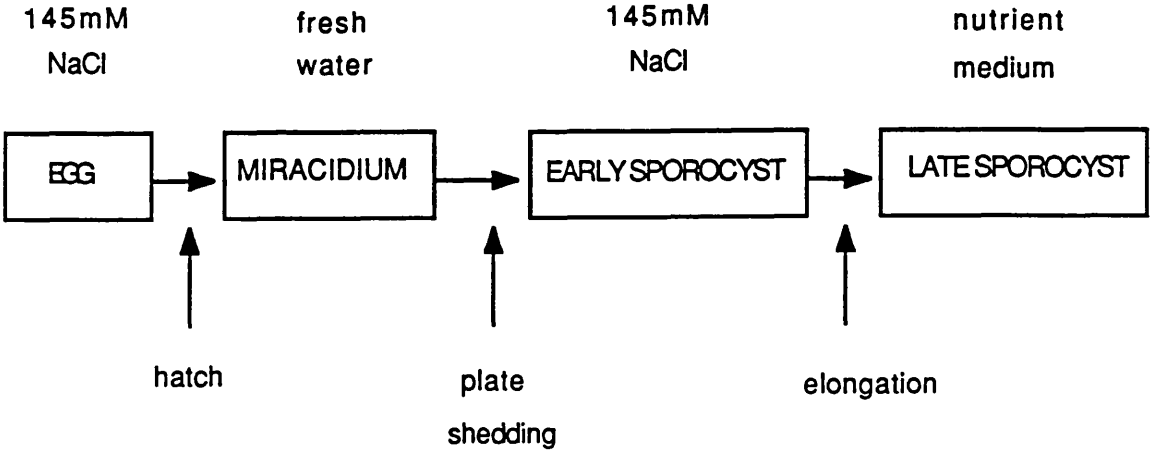
these two media and growth of the sporocysts into the tubular forms was acheived in 3 days at 27°C.

Temperature was also found to effect the rate of transformation. Under optimal conditions (incubation of miracidia in 145mM NaCl for 10 min, replacing salt solution with CBSS containing human haemoglobin and incubating at 37°C) transformation of miracidia into tubular sporocysts was achieved in 48h and were the conditions used for bulk transformation.

The sporocysts were maintained for 2 weeks with changes of media every 48h but no further overt developmental changes were seen. Photomicrographs showing the stages of S. margrebowiei transformation are given in figure 13.

FIGURE 12

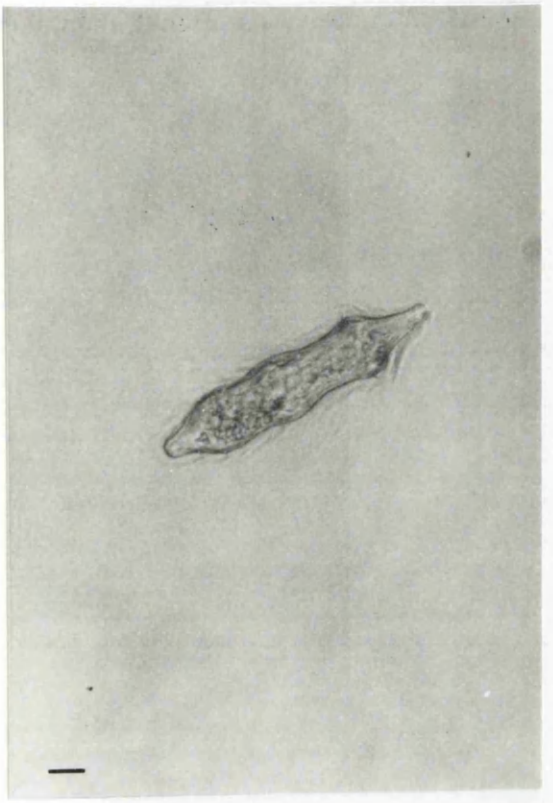
EVENTS IN THE TRANSFORMATION OF MIRACIDIA TO SPOROCYSTS



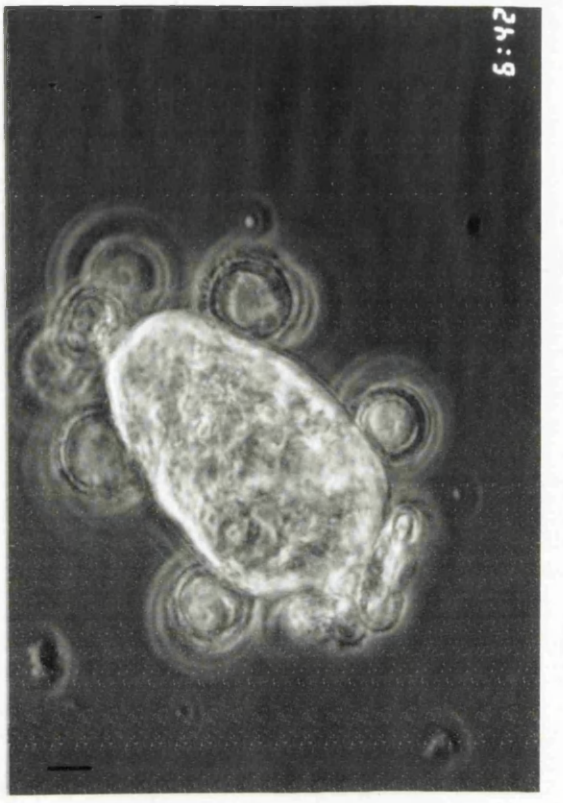
The time required for each stage varies with temperature, salt concentration and nutrients supplied.

FIGURE 13

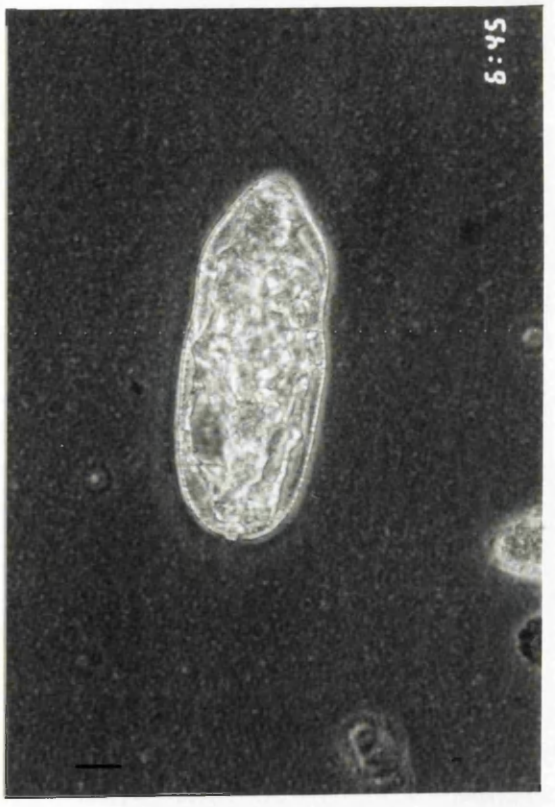
a



b



c



d

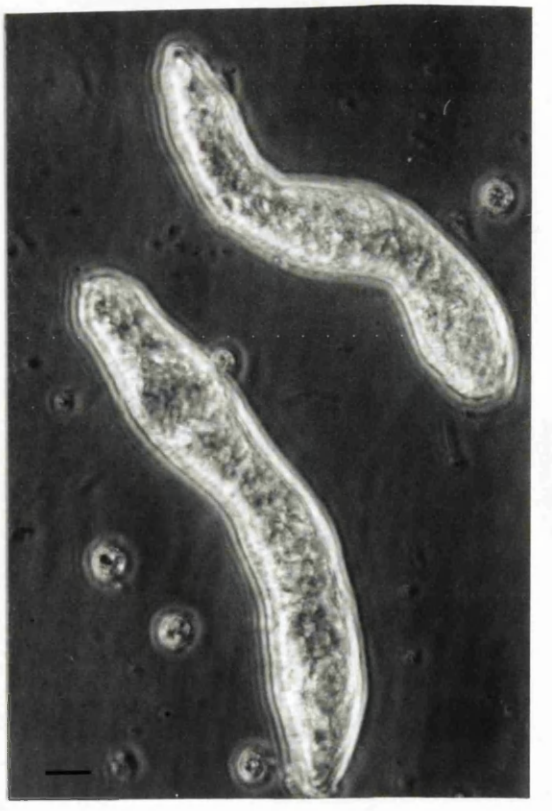


FIGURE 14

Scanning Electron Micrograph of S. margrebowiei miracidium

Bar = 3.3 μm

FIGURE 15

Scanning Electron Micrograph of S. margrebowiei miracidium showing apical papilla

Bar = 0.3 μm

FIGURE 14

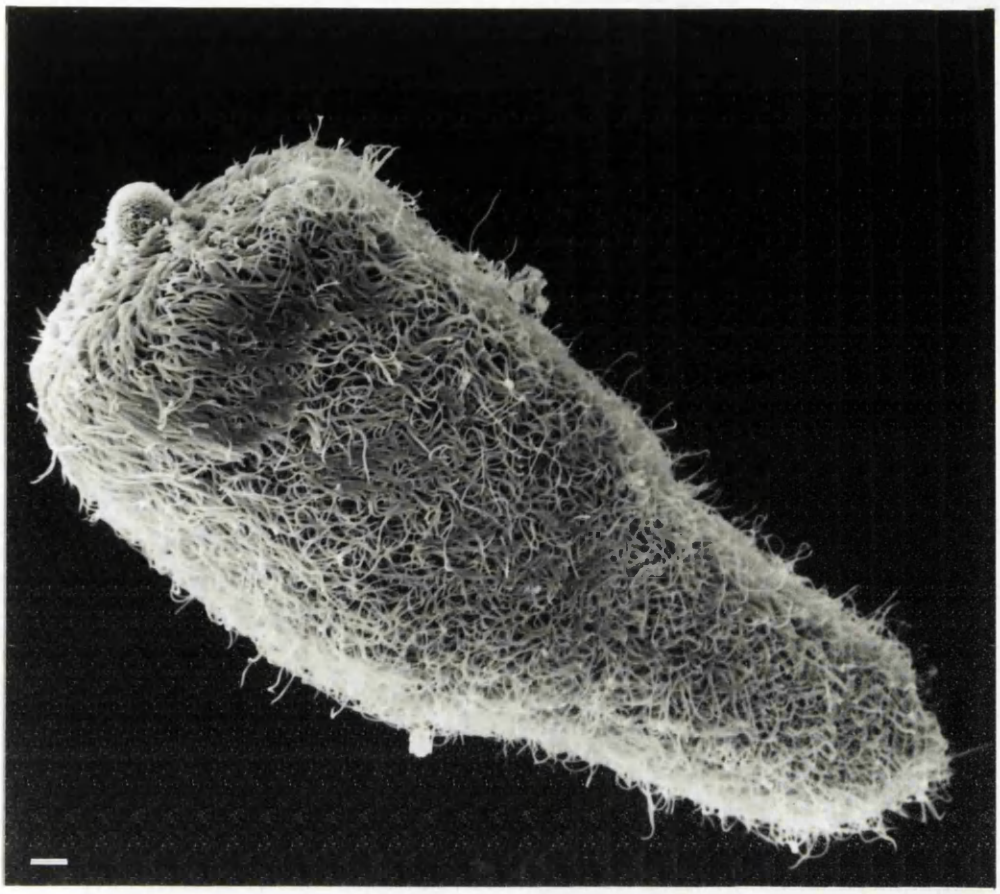


FIGURE 15

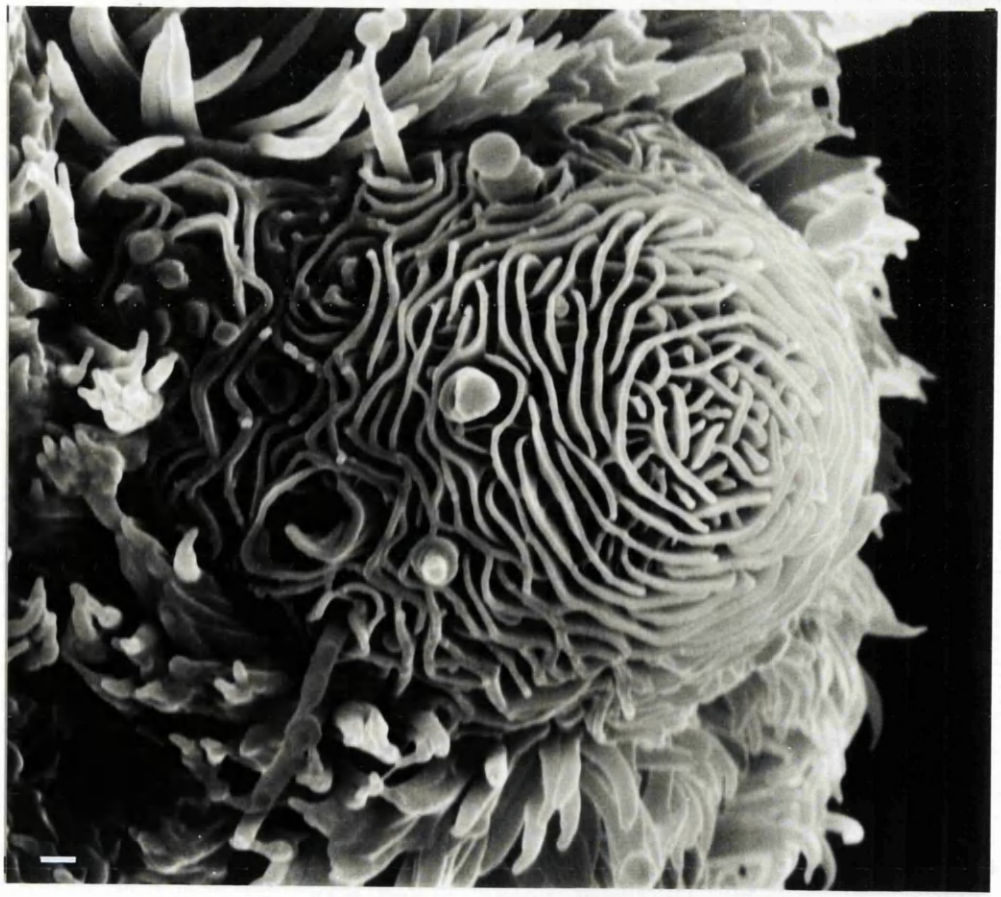


FIGURE 16

Scanning Electron Micrograph of S. margrebowiei newly transformed sporocyst with attached ciliated plates

FIGURE 17

Scanning Electron Micrograph of S. margrebowiei newly transformed sporocyst

Bar = 7.0 μm

FIGURE 16

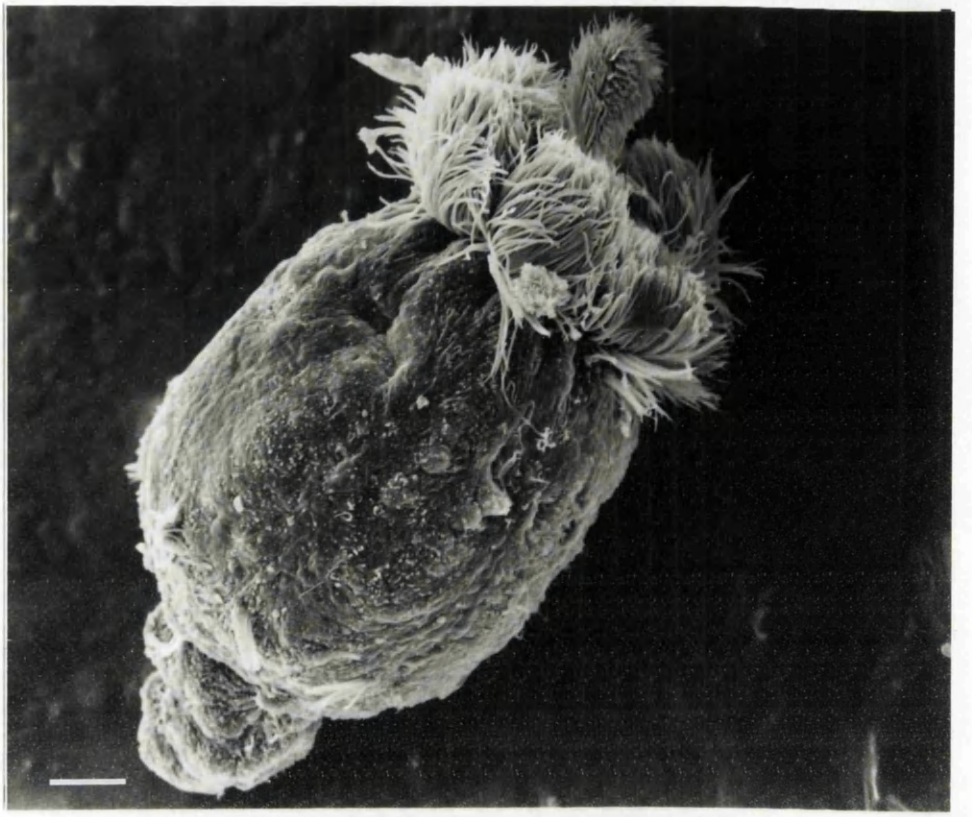


FIGURE 17

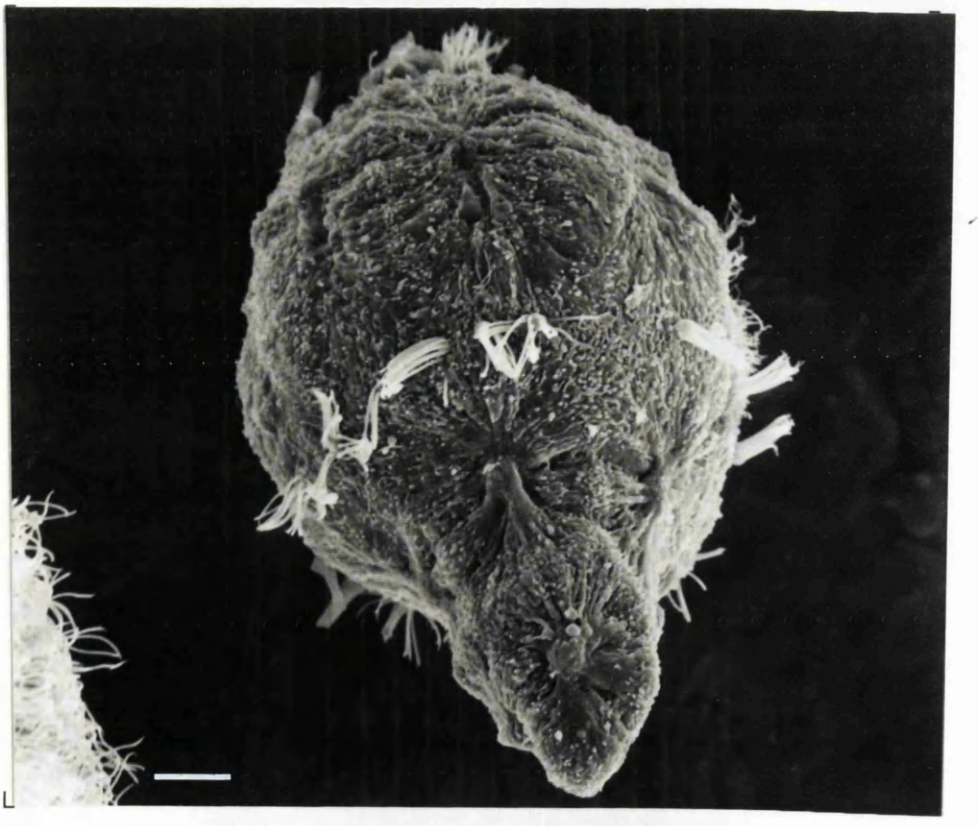


FIGURE 18

Scanning Electron Micrograph of S. margrebowiei newly transformed sporocyst showing sensory receptor

Bar = 2.0 μm

FIGURE 19

Scanning Electron Micrograph of S. margrebowiei cercaria showing sensory receptor

Bar = 0.5 μm

FIGURE 18

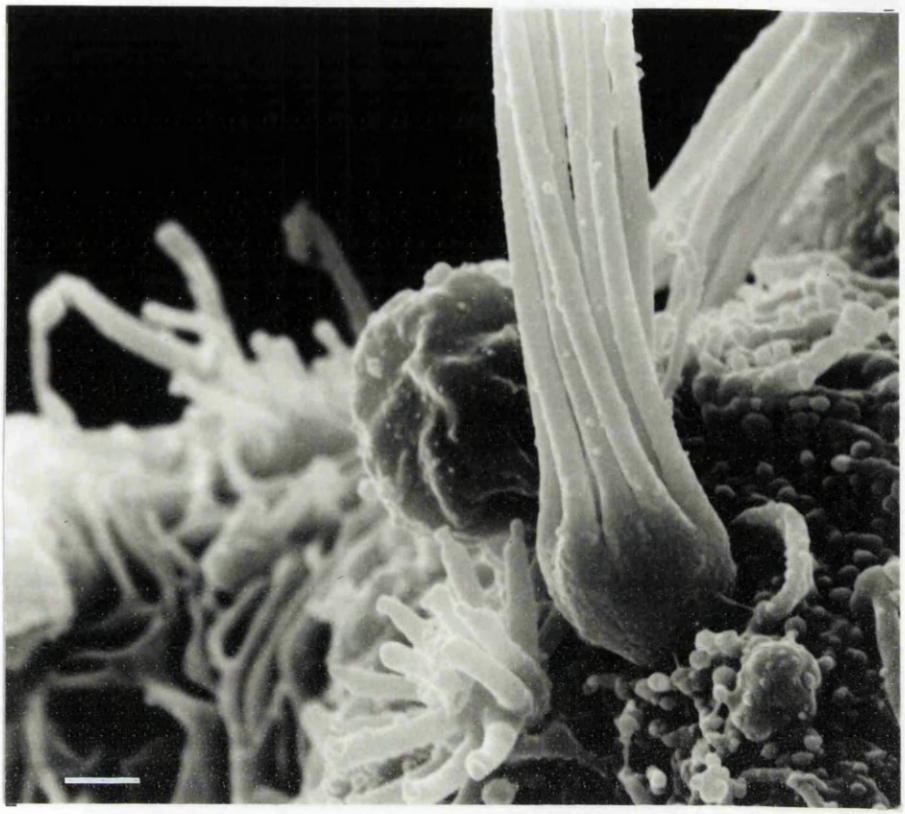


FIGURE 19

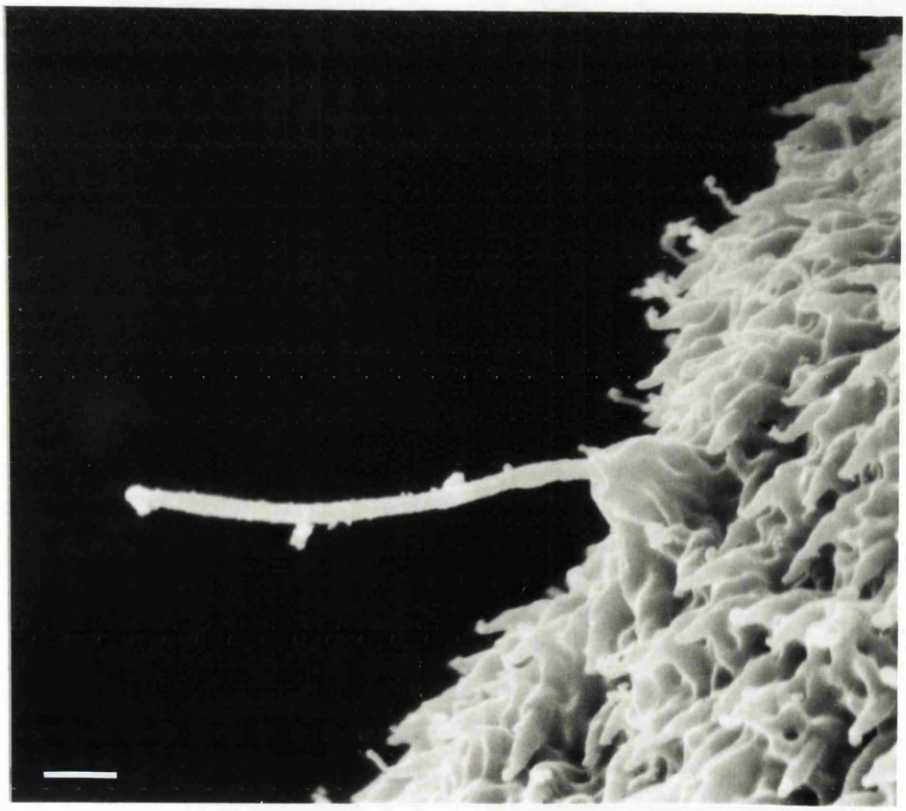


FIGURE 20

Scanning Electron Micrograph of S. margrebowiei cercaria

Bar = 2.2 μm

FIGURE 21

Scanning Electron Micrograph of S. margrebowiei cercaria to show surface spines

Bar = 0.22 μm

FIGURE 20

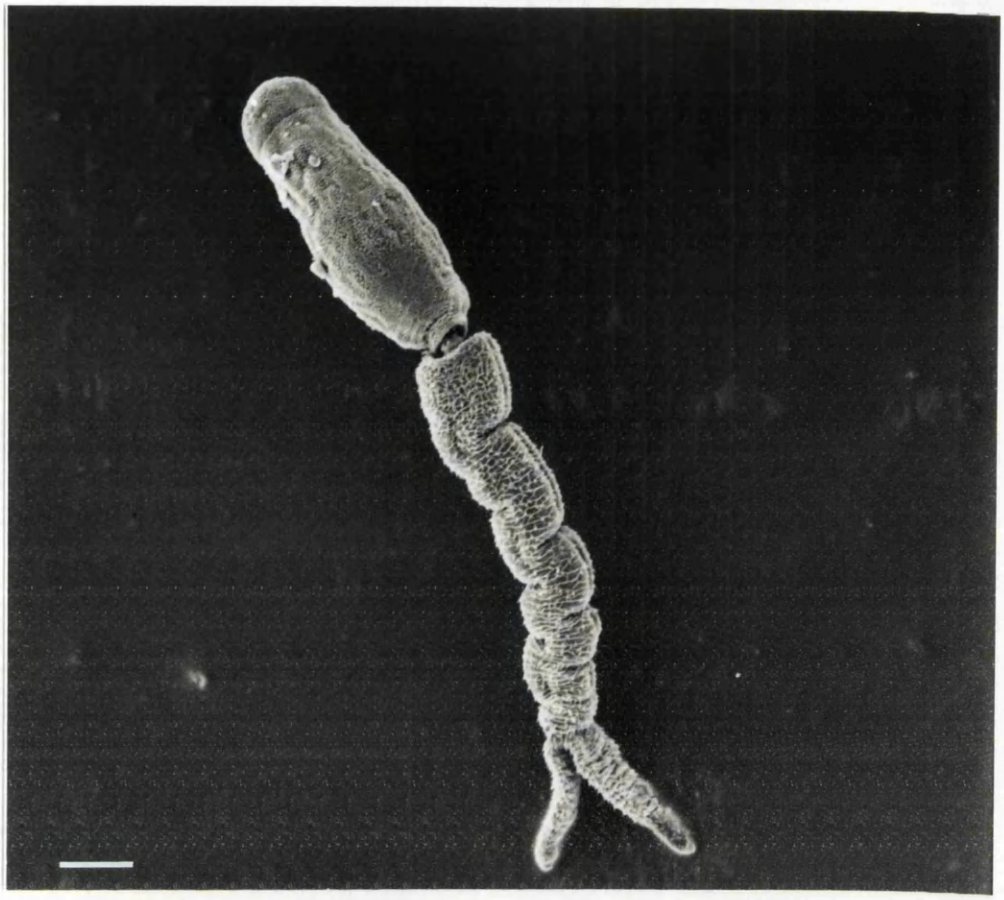


FIGURE 21

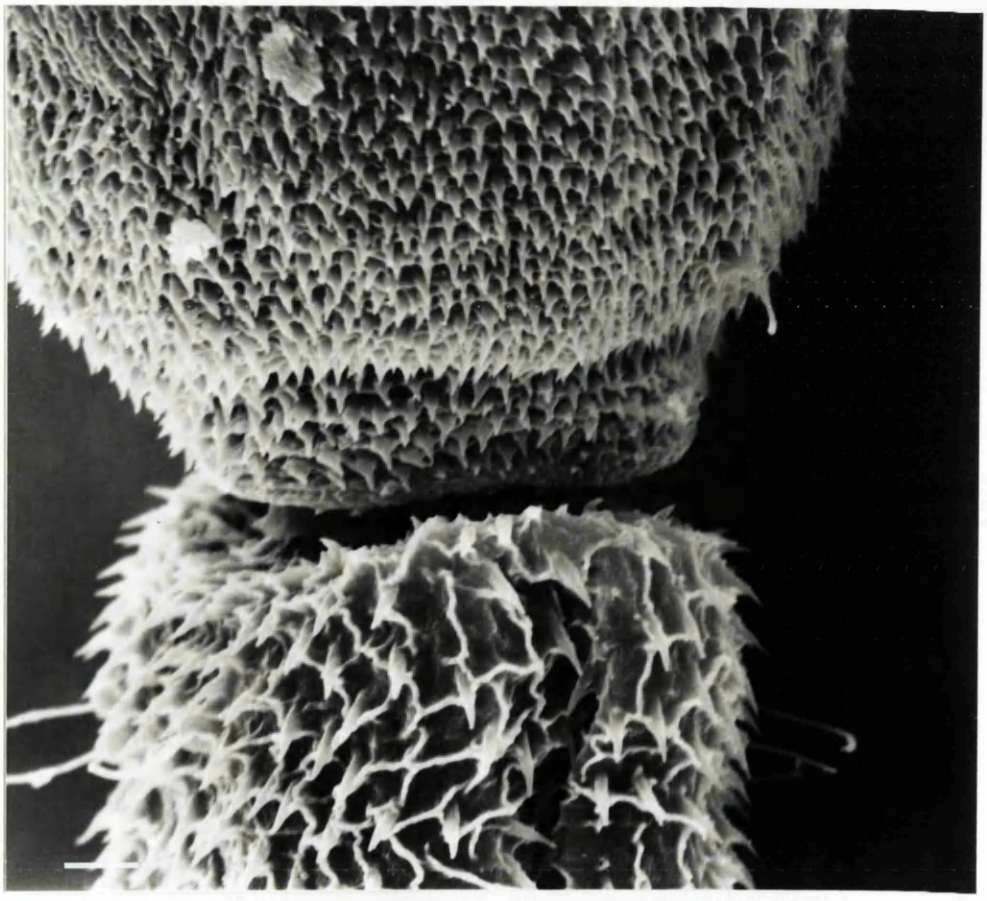


FIGURE 22

Scanning Electron Micrograph of S. margrebowiei cercaria showing the receptors on the apex of head

FIGURE 23

Scanning Electron Micrograph of S. margrebowiei cercaria showing excretory pore

Bar = 0.7 μm

FIGURE 22

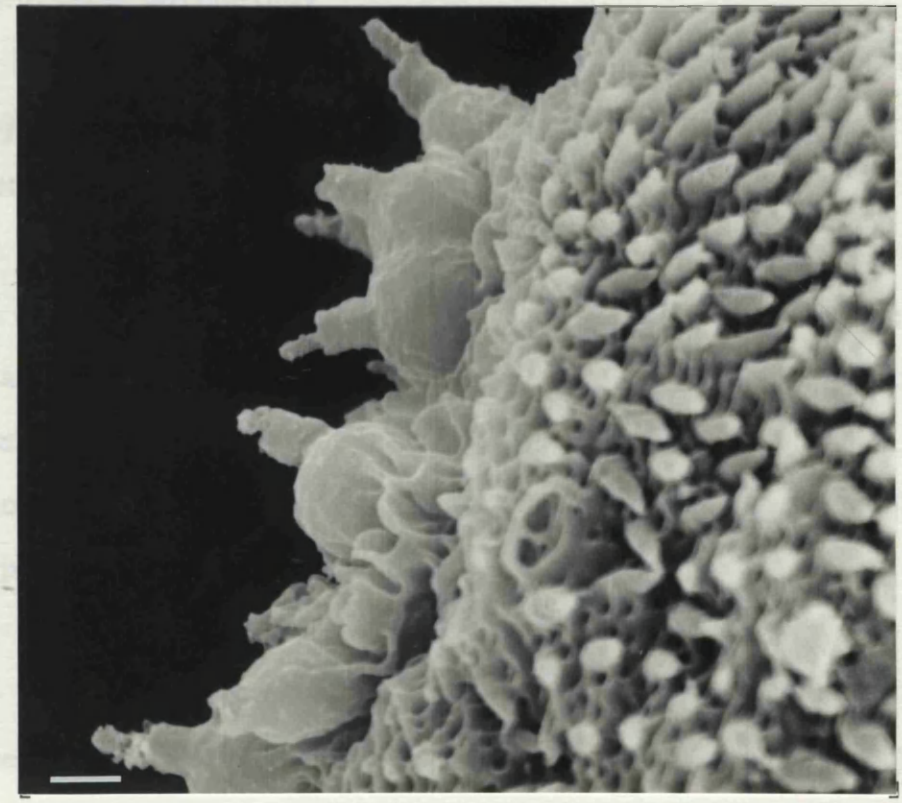


FIGURE 23

(b) Surface Morphology

Scanning electron micrographs of the larval schistosomes are given in figures 14-23

(i) Miracidia

S. margrebowiei miracidia are approximately 80 x 50μm in size and have a dense covering of cilia (figure 14). The cilia appear to be shorter at the anterior end of the miracidium and are absent from the apical papilla. Ciliated and non-ciliated sensory organelles can be seen within the membrane folds of the papilla (figure 15).

(ii) Sporocysts

The newly transformed mother sporocysts are similar in shape and size to the miracidia (figures 16 and 17). The intercellular ridges are not apparent on the surface of the sporocyst but the sensory cilia can be seen. These cilia are present on the miracidium but are often obscured by the motile cilia, they appear to be arranged in groups of 9 joined at a bulbous base (figure 18). Specimens of the later, tubular, sporocysts proved to be very fragile and were lost during preparation.

(iii) Cercariae

The total length of the cercariae in these fixed specimens, is about 260μm (figure 20). Both body and tail are covered in spines. These appear to shorter (0·6μm) and more densely packed on the body than they are on the tail (1·8μm in length) (figure 21). Groups of sensory receptors are visible at the apex of the body (figure 22) and single receptors are present over the entire length of the cercariae. Many but not

all of these receptors bear a single cilium (figure. 19). The presence of cilia varied with specimen and it is possible that all the sensory receptors are ciliated and some are lost during the preparation. Each of the furcae terminates in a hollow tube, the excretory pore (figure 23).

(c) Surface Chemistry.

(i) Carbohydrates.

The results of the lectin labelling experiments are given in table 8 and figures 24-31. The surface of each of the intramolluscan forms displayed a different, stage-specific carbohydrate group.

TABLE 8

THE LARVAL STAGES OF *S. MARGREBOWIEI* AND THEIR LECTIN MARKERS.

Larval Stage	Lectin	Sugar Specificity
Miracidia (cilia)	$Ricin_{120}$	ß-D-galactose
Miracidia (basement membrane)	PNA	ß-D-gal (1-3)-D-galNAc
Sporocysts	Con A	α-D-mannose (α-D-glucose)
Sporocysts	WGA	N-acetyl-glucosamine
Cercariae	Asp Pea	α-L-fucose

Lectin binding was inhibited by the presence of sugars specific for that lectin. However WGA binding was not inhibited by N-acetyl glucosamine, this is due to the inability of this lectin to bind this sugar when it is presented in its monomeric form. Control samples should have been prepared with either fetuin or mucin, both known to bind this lectin.

FIGURE 24

S. margrebowiei whole miracidia stained with FITC-Ricin$_{120}$. Phase contrast (a) and (b) fluorescence micrographs.

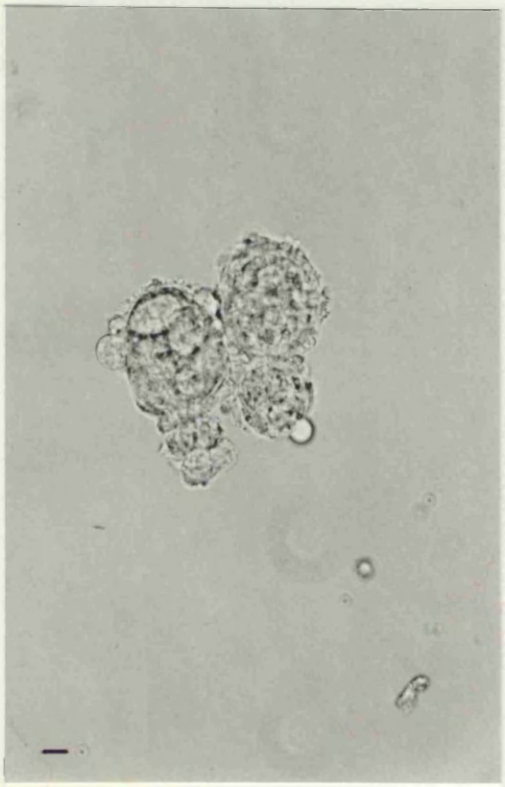
FIGURE 25

S. margrebowiei miracidial plate stained with FITC-PNA. Phase contrast (a) and fluorescence (b) micrographs.

Bar = 10 μm

FIGURE 24

a



b

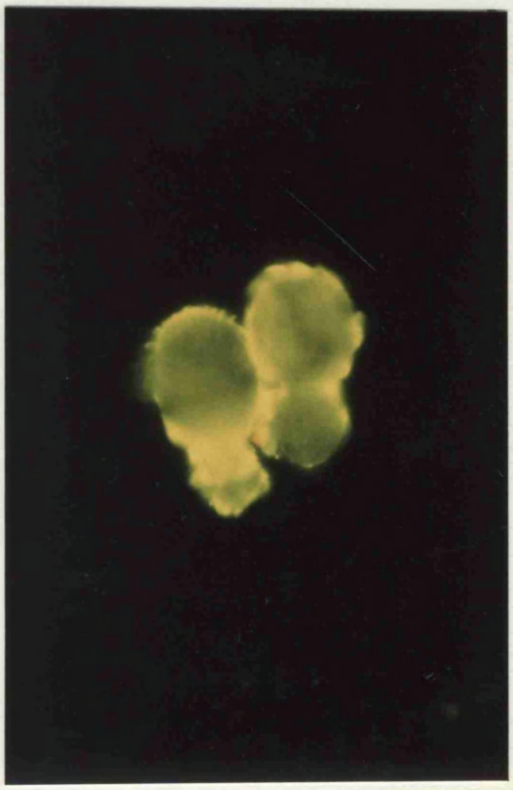
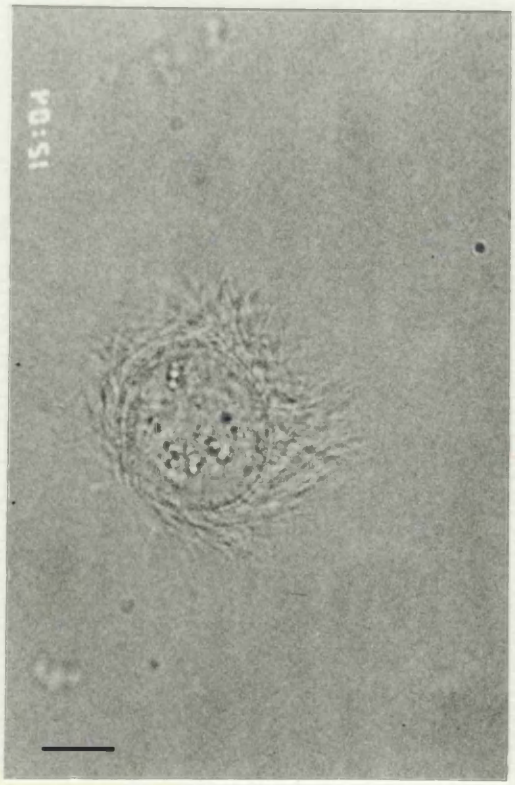


FIGURE 25

a



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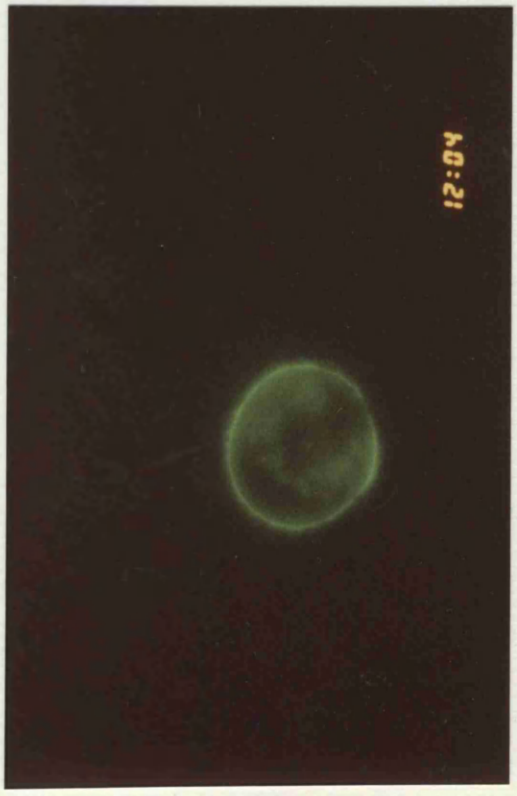


FIGURE 26

S. margrebowiei miracidium at an early stage of transformation stained with FITC-$Ricin_{120}$. Fluorescence micrograph.

FIGURE 27

S. margrebowiei early sporocyst with attached ciliated plates, stained with FITC-PNA. Phase contrast (a) and fluorescence (b) micrographs.

Bar = 10 μm

FIGURE 26

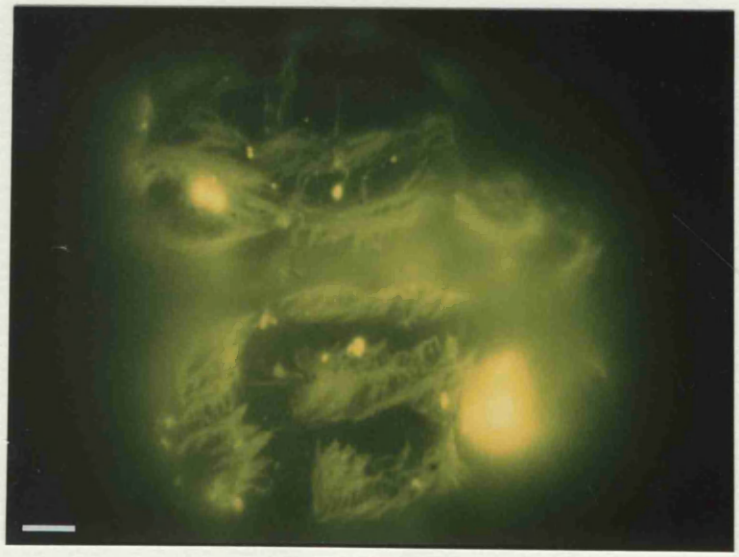
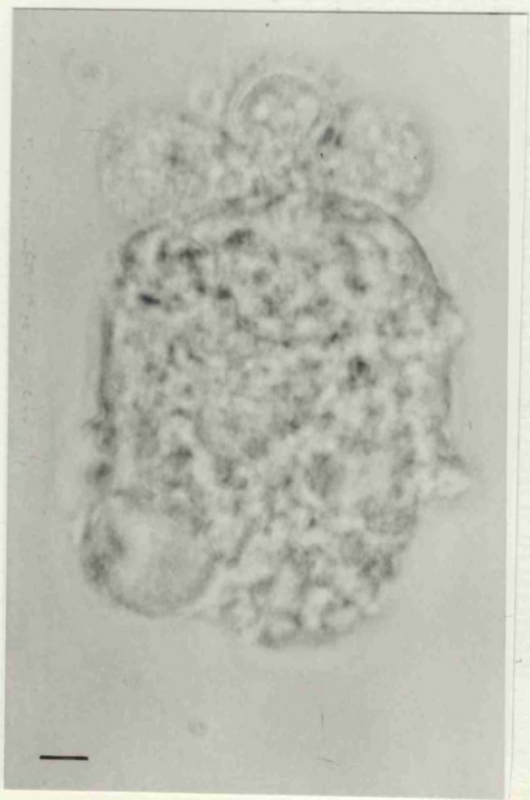


FIGURE 27

a



b

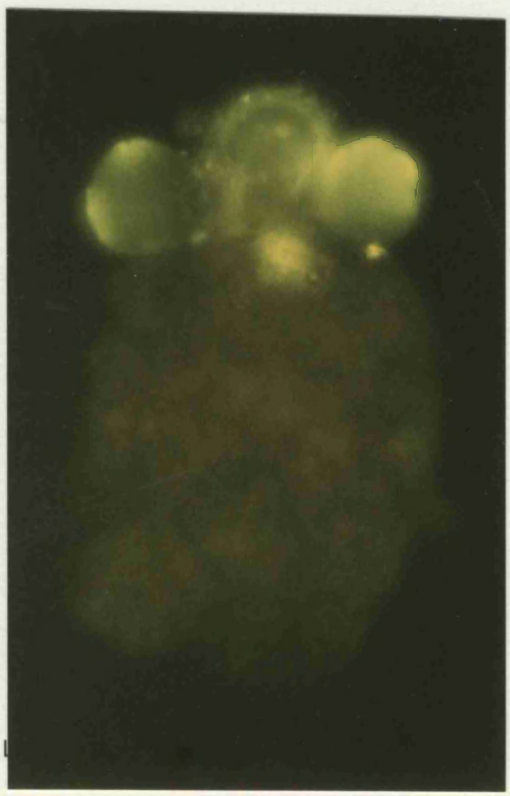


FIGURE 28

S. margrebowiei early sporocyst stained with FITC-WGA showing location of the intercellular ridges. Phase contrast (a) and fluorescence (b) micrographs.

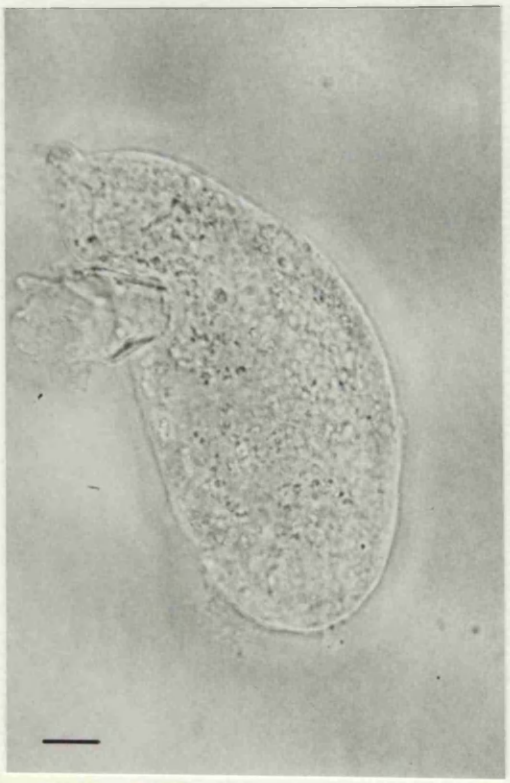
FIGURE 29

Fluorescence micrograph of S. margrebowiei sporocyst after the expansion of intercellular ridge stained with FITC-Con-A.

Bar = 10 μm

FIGURE 28

a



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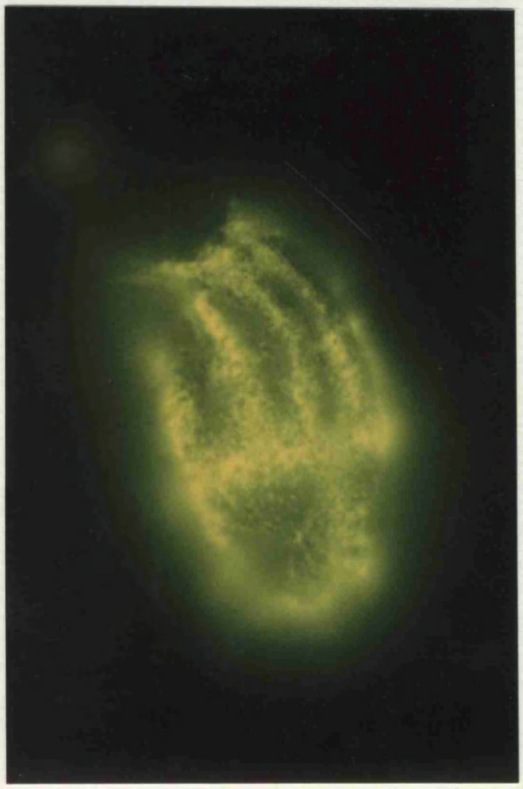


FIGURE 29

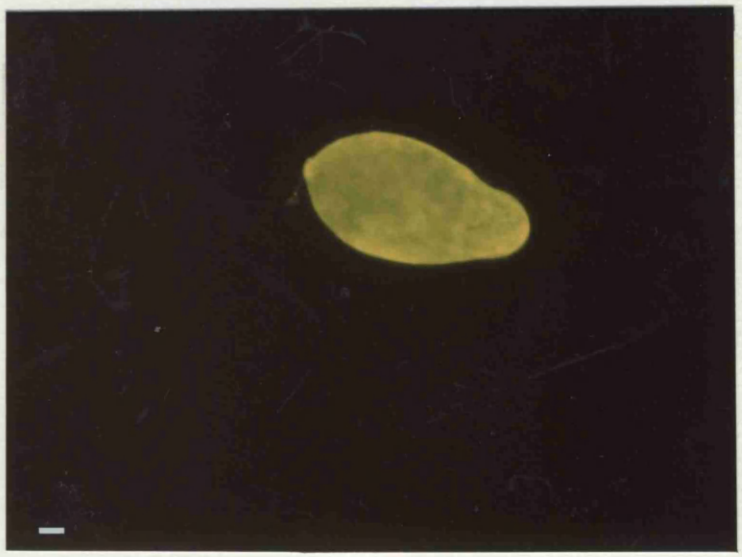


FIGURE 30

S. margrebowiei Sporocysts, approximately 48h after transformation stained with FITC-Ricin$_{120}$. The surface of the sporocysts are unstained whereas as sloughed ciliated plate is stained. Phase contrast (a) and fluorescence (b) micrographs.

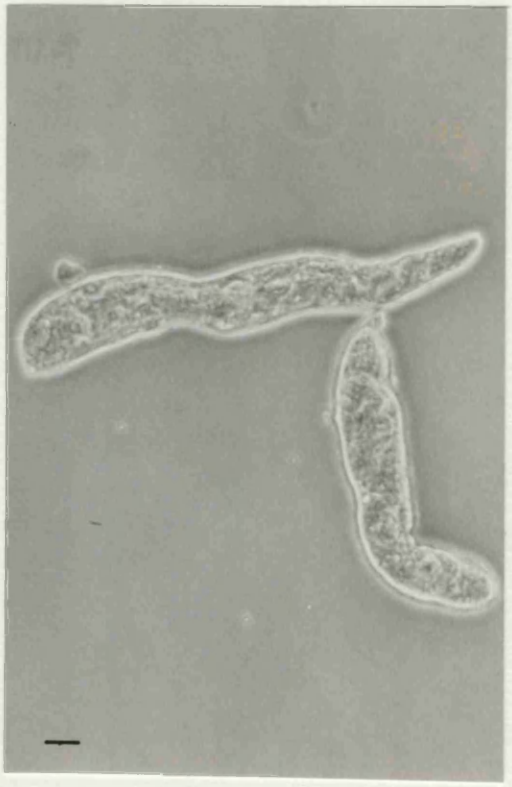
FIGURE 31

Fluorescence micrographs of S. margrebowiei sporocysts stained with (a) FITC-WGA and (b) FITC-CON-A.

Bar = 10 μm

FIGURE 30

a



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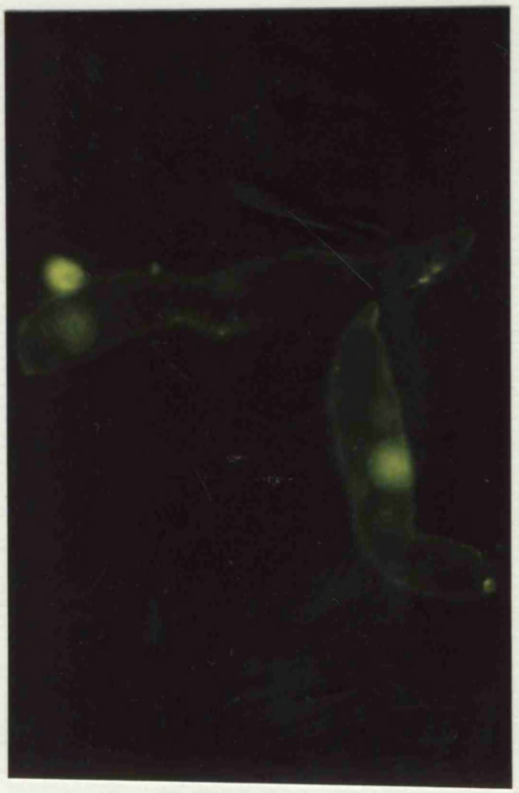
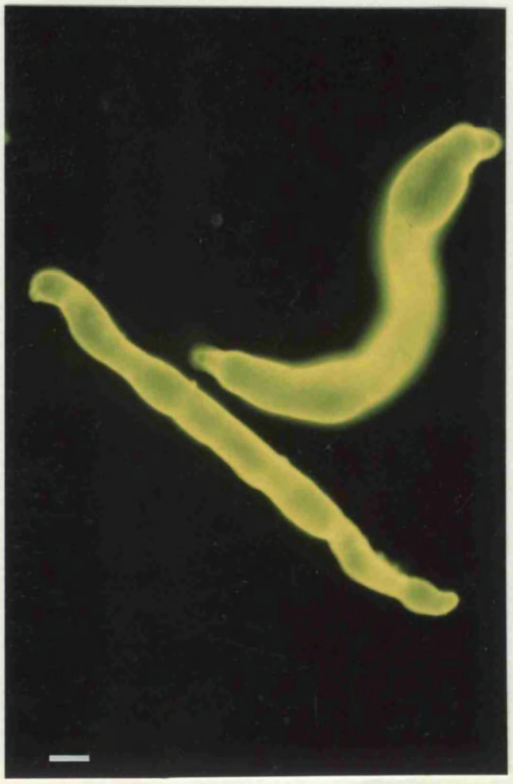


FIGURE 31

a



b

FIGURE 32

Lectin staining of S. margrebowiei cercariae.

(a) Phase contrast micrograph of WGA staining.

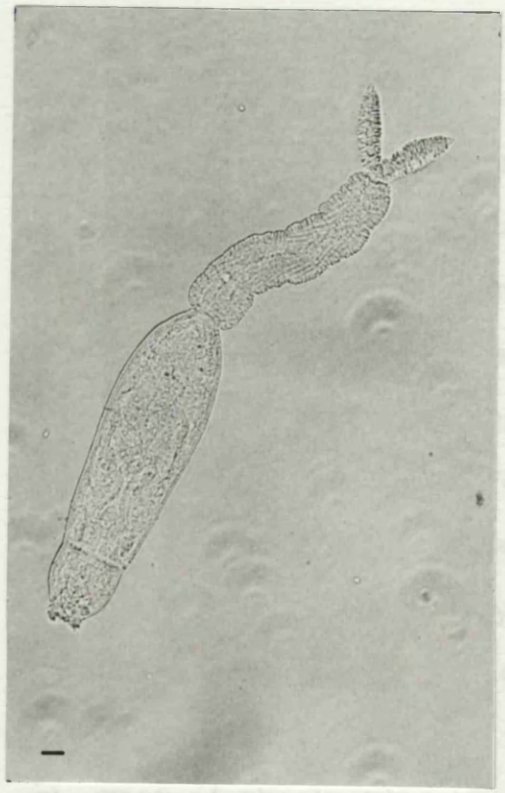
(b) Fluorescence micrograph of the same cercaria.

(c) Fluorescence micrograph of cercaria stained with Asp-pea.

Bar = 10 μm

FIGURE 32

a



b

c

Staining of the overall surface of the S. margrebowiei larvae with these lectins was stage specific. The lectin $Ricin_{120}$ stained the cilia of the miracidia (figures 24, 26 & 30). PNA also bound to this stage but to the basement membrane of the ciliated plates and not the cilia (figure 25). Other lectins did not bind to the miracidia. Lectin staining of miracidia during the course of transformation showed that on early sporocysts the intercellular ridges stained with WGA (figure 28) and Con-A. These lectins came to stain the entire surface of the sporocyst as transformation progressed (figures 29 & 31) whereas the binding sites for ricin were lost (figure 30). The cercaria was the only stage of the life cycle whose surface bore L-fucose residues. This was ascertained by the positive staining achieved with the lectin Asp pea (figure 32 c). Most of the lectins tested stained an area on the apex of the body of the cercariae. This is the location of the apical gland and the site of enzyme secretion for the cercariae. The lectins are probably staining these enzymes and not the tegument surface. The excretory pores situated at the end of each furcae gave positive staining with WGA, not seen with other lectins (figure 32 a & b).

(ii) Proteins

The surface proteins of the larval schistosomes were labelled with sulpho-NHS-biotin, successful biotinylation was visualised by the fluorescent staining of the schistosomes using FITC-Avidin. This fluorescence was compared with that

obtained when FITC-Avidin was used to stain schistosomes that had not been treated with biotin. This is shown for the cercariae in figure 33. Sporocysts were also readily biotinylated (figure 34) but when miracidia were biotinylated the biotin was restricted to underlying membrane of the ciliated plates (figure 35a). The cilia themselves appeared to resist biotinylation, this may be due to steric hindrance created by the presence of carbohydrate groups on the ciliary membrane. Biotinylation was carried out on miracidia post incubation with B. nasutus plasma (see Haemagglutinin section). Subsequent staining with FITC-avidin gave a strong positive result (figure 35b).

The biotinylated proteins of the larval schistosomes were compared after their separation using polyacrylamide gel electrophoresis. The electrophoretically separated proteins were transferred onto nitrocellulose paper. Figure 36 shows the initial protein pattern for the cercarial proteins as seen with Ponceau red staining. Here two species of schistosome have been compared S. margrebowiei and S. nasale. The relative position of the molecular weight markers can also be seen. After the position of these bands was noted the Ponceau red was removed and the blot treated with horseradish peroxidase labelled avidin to identify the biotin labelled surface proteins (figure 37). The cercarial surface proteins of these two species differed, the major protein band of S. margrebowiei corresponding to an apparent M_r of 137,000 was not present on the cercariae of S. nasale.

Figure 38 shows a comparison of the biotinylated proteins of the sporocysts and miracidia of S. margrebowiei. Miracidia after incubation with B. nasutus plasma were also included.

The results indicated that the surface proteins were different for each larval stage of schistosome. The major surface protein of the sporocyst was calculated to have an M_r of 70,000. Two major bands were present on the miracidia corresponding to 46,000 and 74,000 whereas those of the cercariae had a higher M_r at 137,000 and 99,000.

Biotinylation of the miracidia post absorption of B. nasutus plasma factors were analysed alongside native miracidia. Subsequent detection of the biotinylated proteins revealed the presence of a protein at approximately M_r 61,000 absent from the other samples.

FIGURE 33

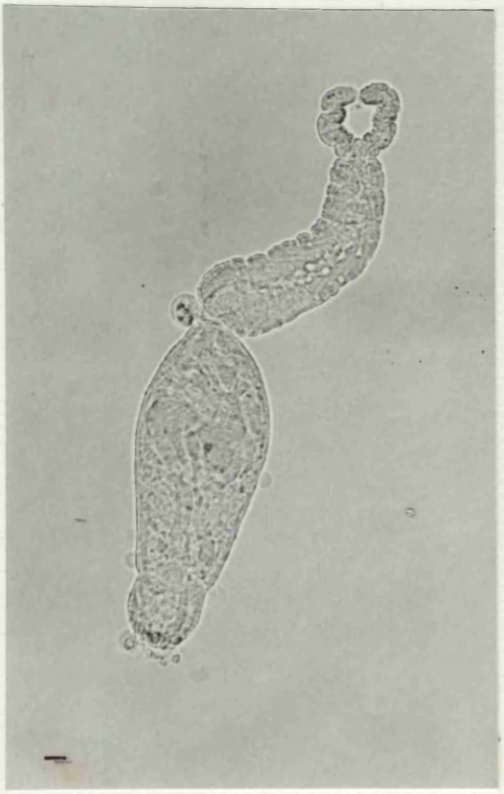
Phase (a) and fluorescence (b) micrographs of S. margrebowiei cercaria after treatment with biotin and subsequently stained with FITC-avidin.

Phase (c) and fluorescence (d) micrographs of control samples stained with FITC-avidin but not treated with biotin.

Bar = 10 μm

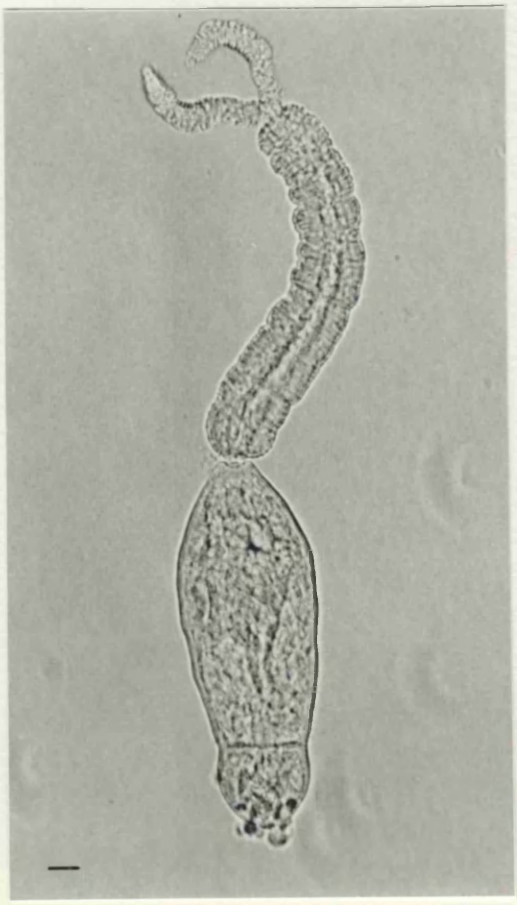
FIGURE 33

a



b

c



d

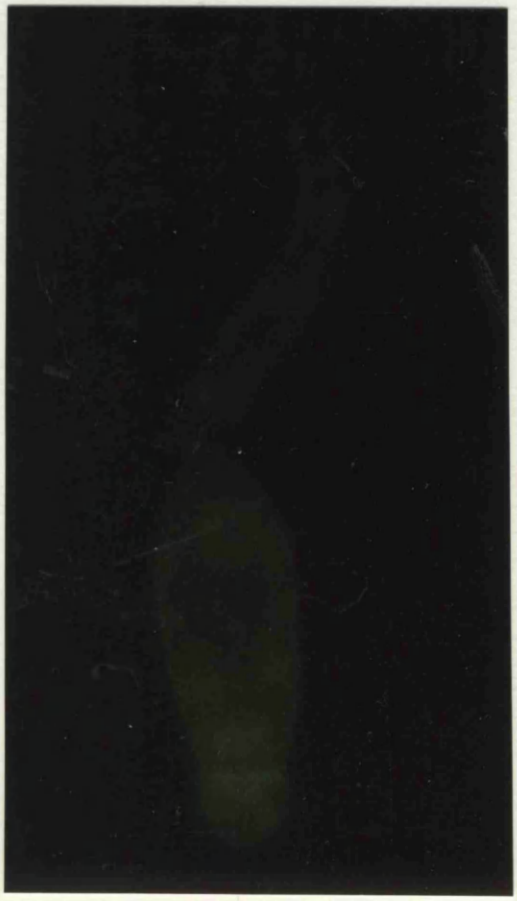


FIGURE 34

Fluorescence micrographs of S. margrebowiei sporocyst after treatment with biotin and subsequently stained with FITC-avidin.

FIGURE 35

(a) Fluorescence micrograph of S. margrebowiei miracidia post biotinylation stained with FITC-avidin

(b) Fluorescence micrograph of miracidia incubated in B. nasutus haemolymph prior to biotinylation.

Bar = 10 μm

FIGURE 34

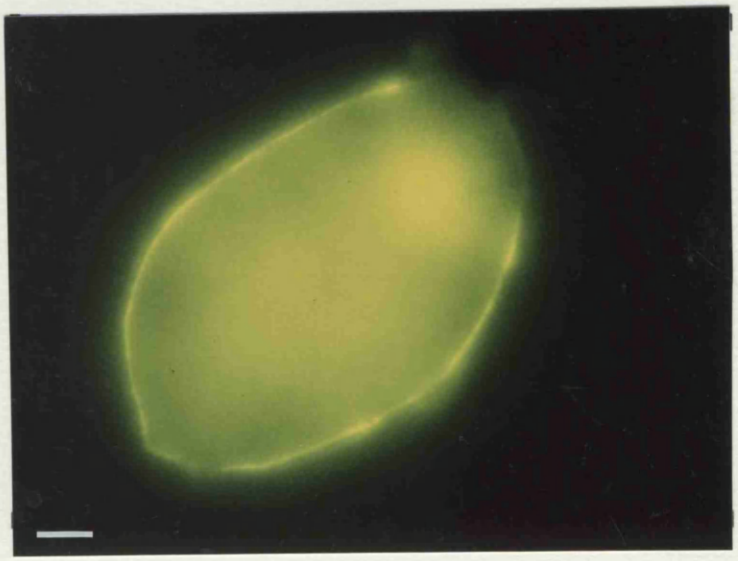
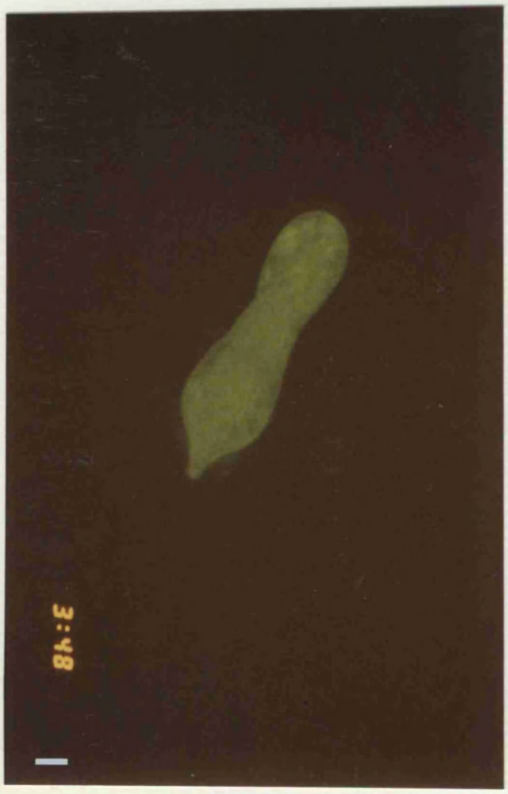


FIGURE 35

a



b

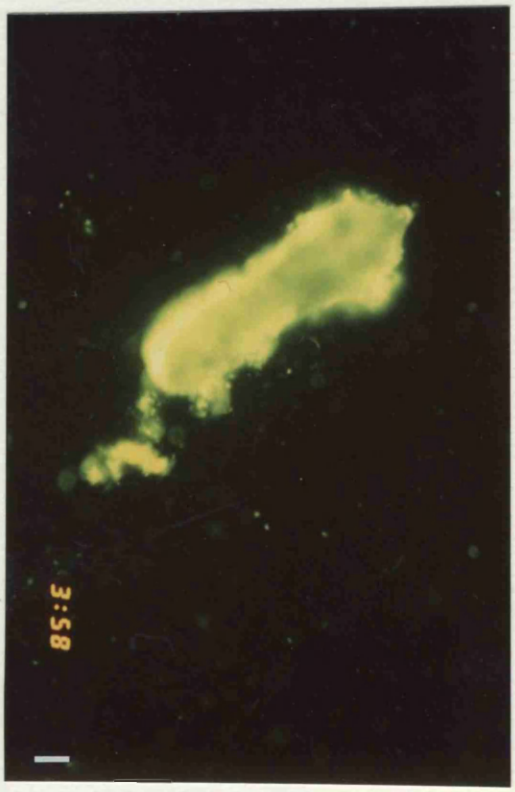


FIGURE 36

Cercarial proteins of S. margrebowiei and S. nasale separated using SDS-PAGE transferred onto nitrocellulose paper. Proteins stained with Ponceau red. Lanes 1 & 2 S. margrebowiei; lane 3 molecular weight markers; lanes 4 & 5 S. nasale.

FIGURE 37

The same blot with Ponceau red removed and subsequently stained with horseradish peroxidase labelled avidin to identify surface proteins.

FIGURE 36

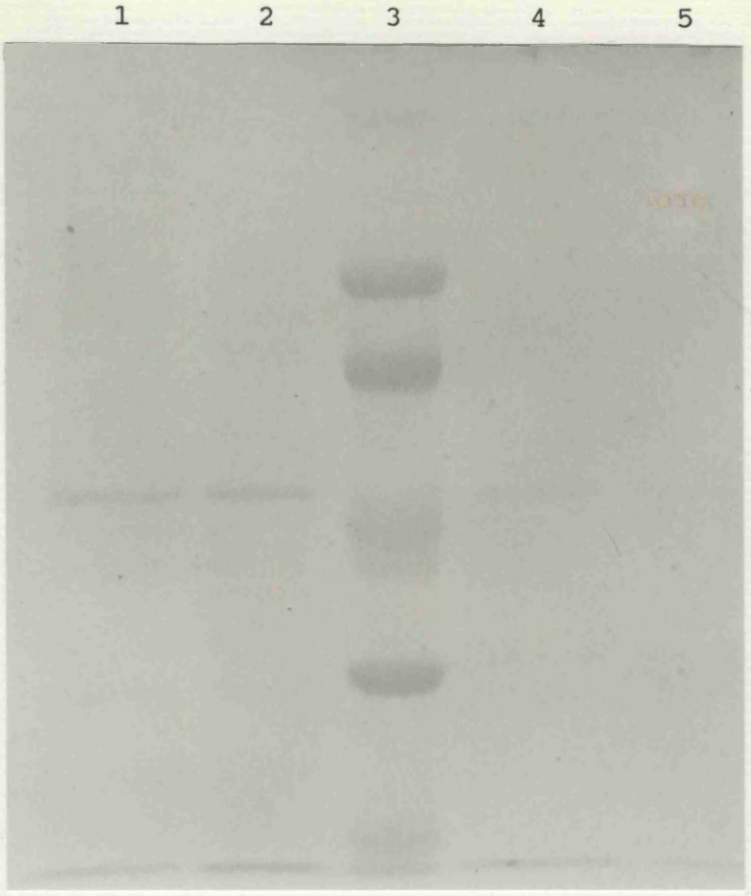


FIGURE 37

FIGURE 38

Surface proteins of S. margrebowiei sporocysts and miracidia after treatment with biotin. Proteins separated using SDS-PAGE and visualised using peroxidase labelled avidin. Computer enhanced photograph to show additional protein band (arrow) in lanes where the miracidia were incubated in snail haemolymph.

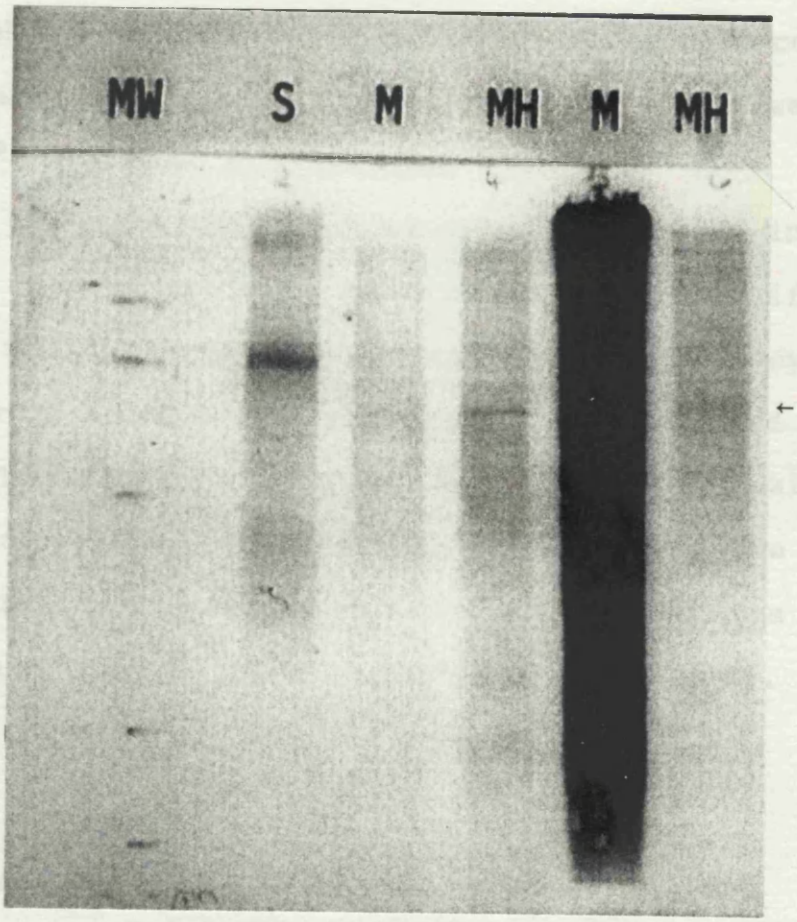
MW = molecular weigh markers (position of bands marked in pencil)

S = sporocysts

M = miracidia

MH = miracidia after incubation in B. nasutus haemolymph.

FIGURE 38



2. HAEMOCYTES

(a) Haemocyte numbers.

(i) Comparison of B. natalensis and B. nasutus.

The haemocyte numbers assessed from pooled haemolymph samples showed that these snails have relatively few cells per ml of haemolymph. B. nasutus appears to have far fewer cells (62×10^3 ml^{-1}) than B. natalensis (188×10^3 ml^{-1}). Yet the ratio of plasmatocytes to granulocytes was similar in both species, 42% : 58%. There appeared to be little difference between the numbers and the ratio of plasmatocytes to granulocytes in the juvenile snails when compared to the adults. This may reflect the fact that the smaller snails have already achieved immune maturity by the time they have reached a size that can be successfully bled. The results of the haemocyte counts are given in table 9 and figure 39.

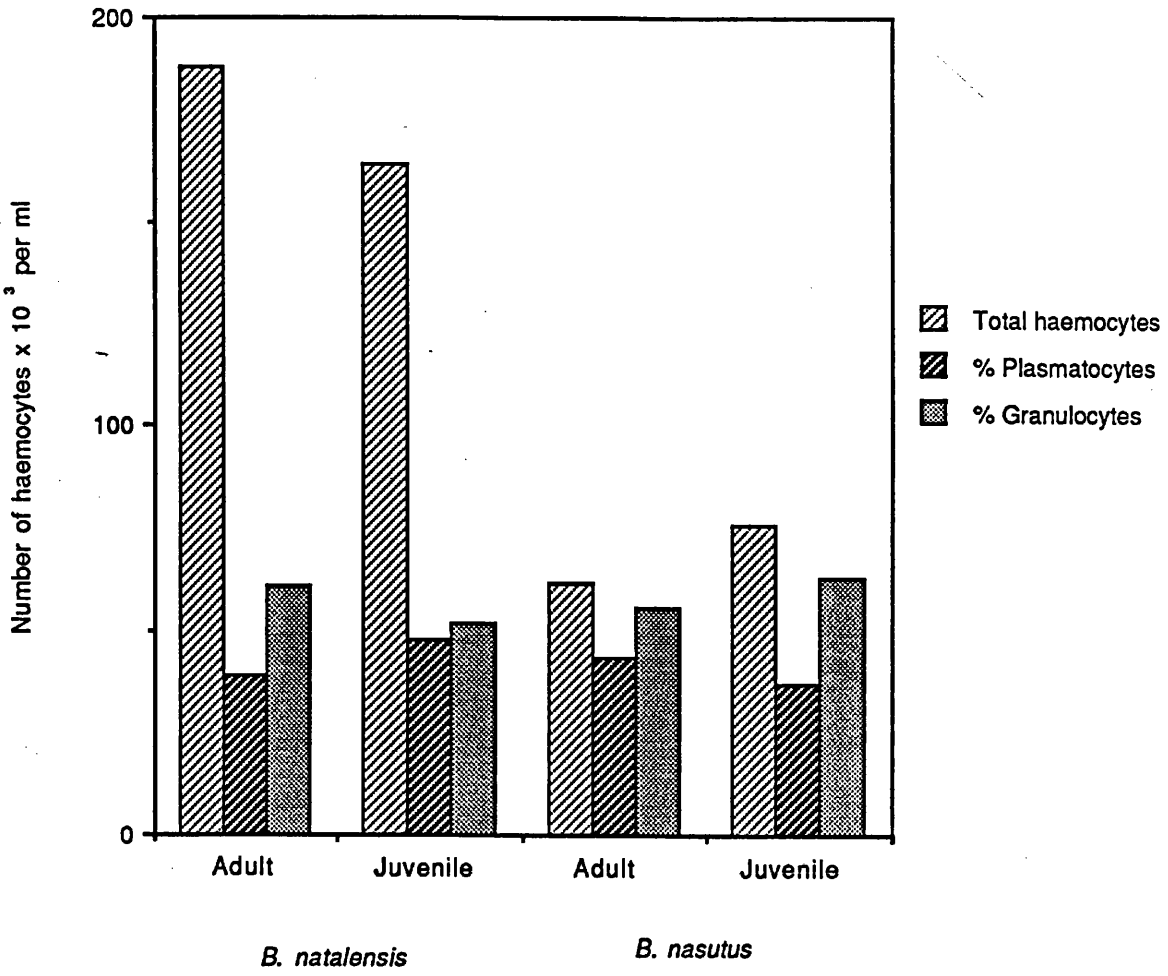
TABLE 9

COMPARISON OF HAEMOCYTE NUMBERS BETWEEN B. NATALENSIS AND B. NASUTUS ADULT AND JUVENILE SNAILS.

	sample size (n)	no. cells ml^{-1}	% granulocytes	% plasmatocytes
B. natalensis				
Adult	15	188 ± 20	61 ± 14	39 ± 14
Juvenile	5	164 ± 15	52 ± 10	48 ± 10
B. nasutus				
Adult	10	62 ± 9	56 ± 9	44 ± 9
Juvenile	3	76 ± 3	63 ± 12	37 ± 12

Figure 39

Haemocyte counts of Adult and Juvenile bulinid snails



(ii) Pre- and Post- exposure to schistosomes.

The haemocyte counts taken before (pre E) and post (PE) exposure to S. margrebowiei miracidia were taken on individual snails over a period of 2 days. The results of these counts were inconclusive as regards the effect of miracidial invasion on the snails. What was apparent, however, was the high degree of variation in cell numbers found between the individual snails. These variations could be due to number of different reasons;

a) the snails represent an outbred population and the differences observed could represent genetic differences.

b) the snails were not kept in sterile conditions and therefore their immune status prior to the start of the experiment was not known.

c) temperature is known to affect haemocyte numbers in limpets (Davies and Partridge, 1972), no attempt was made to keep the temperature constant and snails were transferred from the incubator at 27°C to the bench approximately 20°C.

d) it was not possible to ensure that 10μl of haemolymph was withdrawn with each bleed and at times haemocyte numbers were estimated from 5μl of haemolymph which was a possible source of error.

Although it was possible to bleed the snails 3 times in 48 h, this was obviously detrimental as all the snails died within one week of the experiment. Results are given in figures 40 and 41.

An evaluation of the effect of miracidial invasion on snail haemocyte numbers requires far larger numbers of snails.

FIGURE 40

NUMBERS OF HAEMOCYTES IN THE HAEMOLYMPH OF *B. natalensis* BEFORE AND AFTER EXPOSURE TO *S. margrebowiei* MIRACIDIA

A - D Experimental

E - H Controls

 Total number of haemocytes

 Plasmatocytes

 Granulocytes

X Axis; 2 & 24 = time in hours after exposure
p = haemolymph samples pre-exposure

Y Axis; number of haemocytes per ml x 10^{-3}

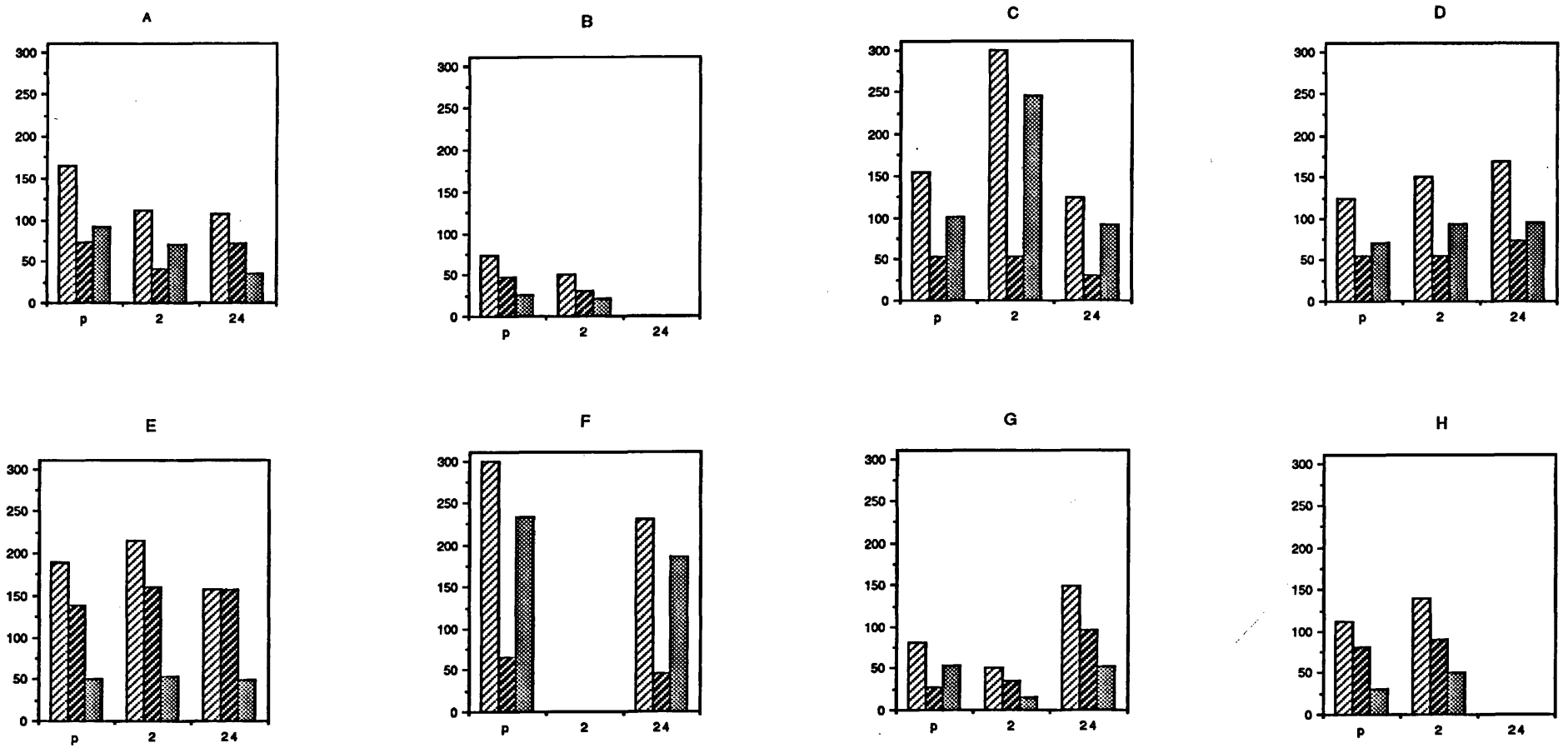

A
B
C
D
E
F
G
H
300
250
200
150
100
50
0
p
2
24

FIGURE 41

NUMBERS OF HAEMOCYTES IN THE HAEMOLYMPH OF *B. nasutus* BEFORE AND AFTER EXPOSURE TO *S. margrebowiei* MIRACIDIA

A - D Experimental

E - H Controls

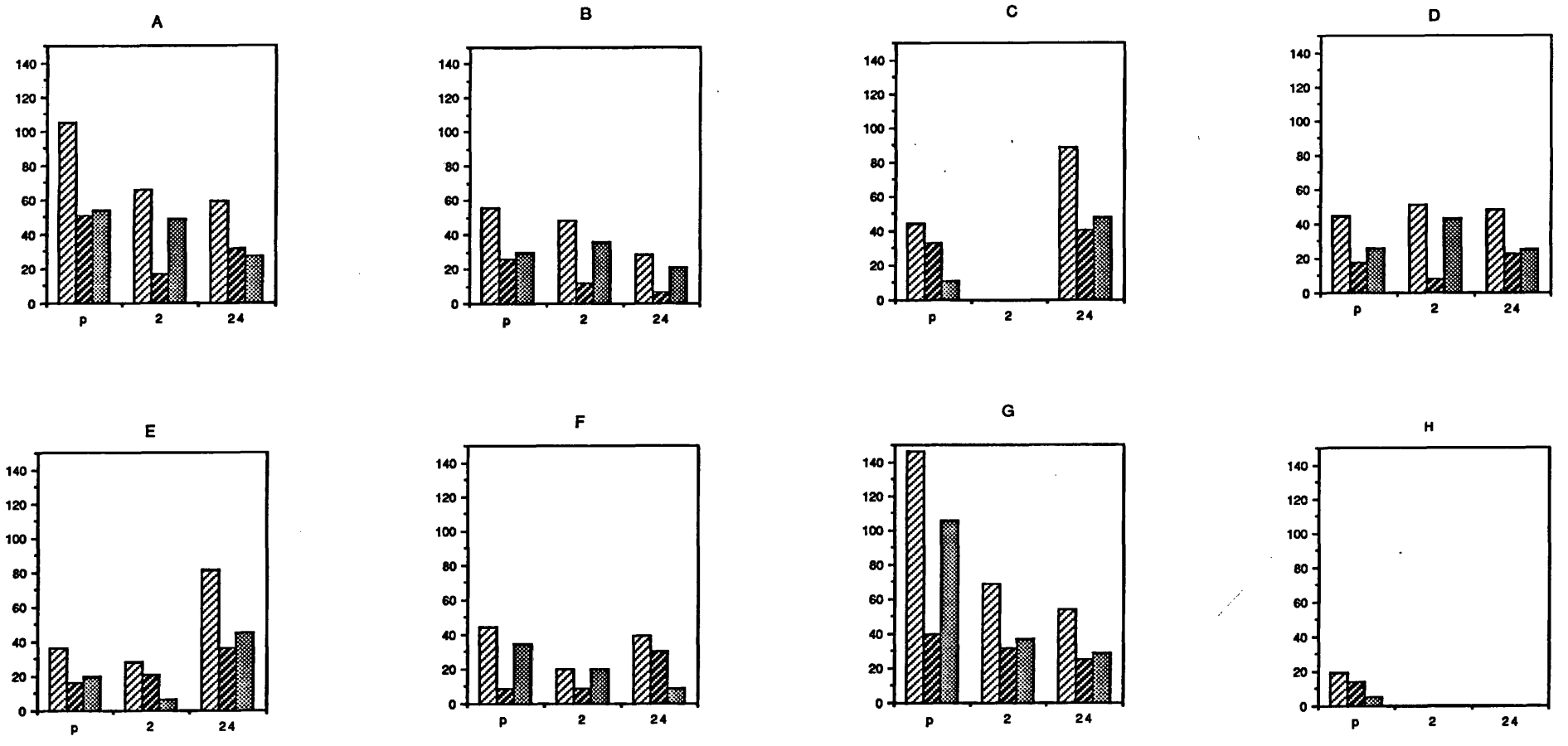
 Total number of haemocytes

 Plasmatocytes

 Granulocytes

X Axis; 2 & 24 = time in hours after exposure
p = haemolymph samples pre-exposure

Y Axis; number of haemocytes per ml x 10^{-3}


A
B
C
D
E
F
G
H
140
120
100
80
60
40
20
0
p
2
24

Haemocyte counts could then be carried out on pooled haemolymph samples from groups of snails. Individual snails need only to be bled once during the course of the experiment. After 48h snails should be sacrificed and histological sections made to ascertain whether miracidial invasion had occurred and the state of the sporocysts within the snail. This was not done due to the lack of snails available for experimentation.

(b) Haemocyte morphology and structure.

(i) Phase contrast microscopy.

The circulating haemocytes of B. natalensis and B. nasutus were indistinguishable both at a morphological and functional level. When freshly extracted haemolymph was pipetted onto glass coverslips the haemocytes settled onto the surface of the glass. The plasmatocytes, having made contact with the glass substrate, spread and extended cytoplasmic projections, filopodia. In contrast the granulocytes settled onto the glass but remained as small round cells (figure 42).

(ii) Scanning electron microscopy.

Electron micrographs of B. natalensis haemocytes are shown in figures 43-45. When viewed at this magnification the granulocytes were seen to possess filopodia and appeared to vary in morphology. Many cells were lost during the preparation for scanning electron microscopy and it is not known how indicative of a normal cell population are the remaining haemocytes.

FIGURE 42

Phase contrast micrographs of bulinid haemocytes

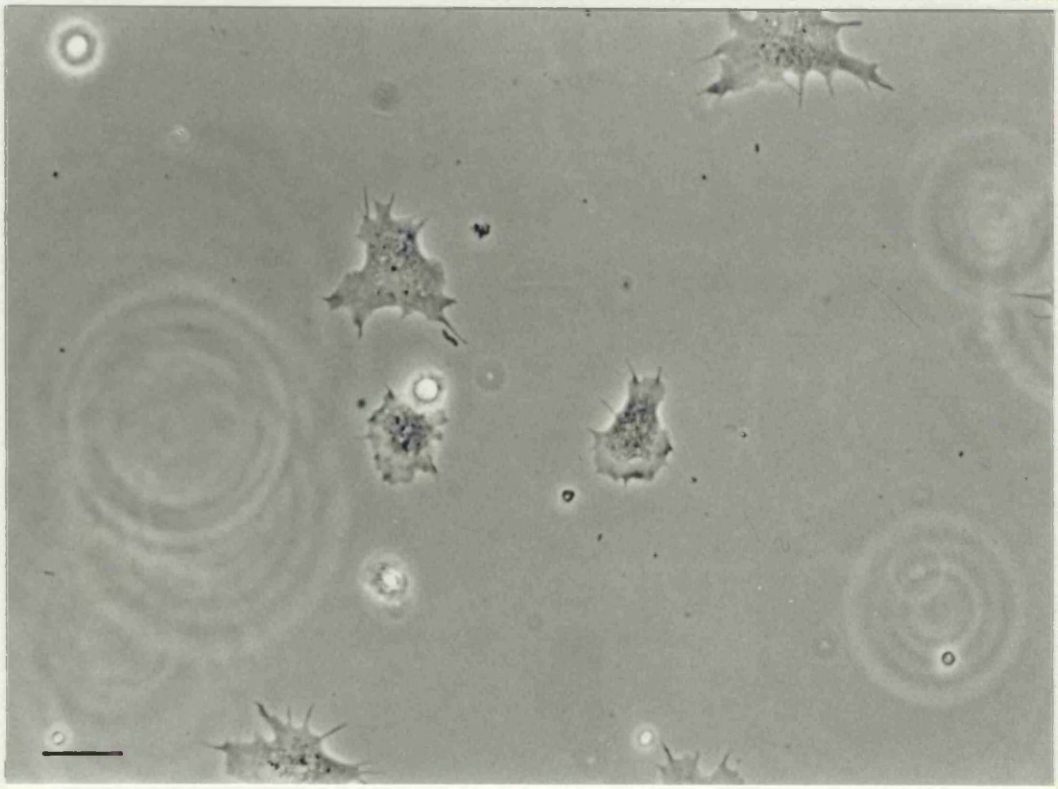
a) After 5 min settling

b) After 15 min settling

Bar = 10 μm

FIGURE 42

a



b

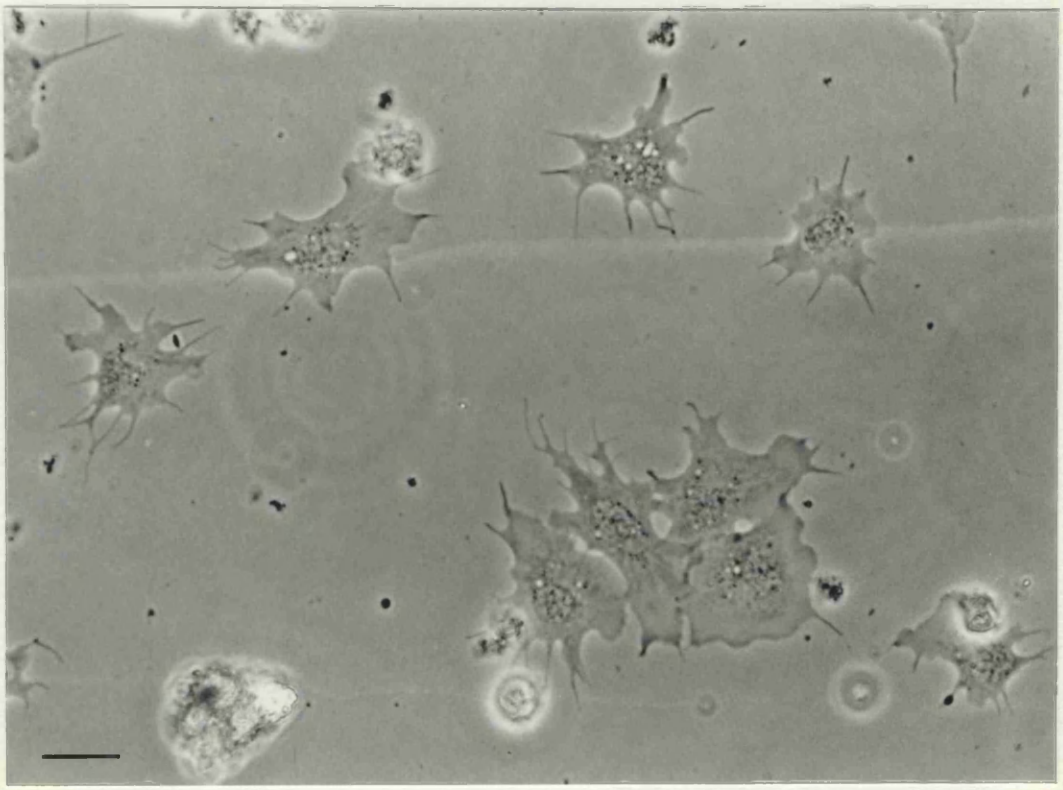


FIGURE 43

Scanning electron micrograph of B. natalensis plasmatocyte

Bar = 2.0 μm

FIGURE 44

Scanning electron micrograph of plasmatocyte and granulocyte

Bar = 4.0 μm

FIGURE 43

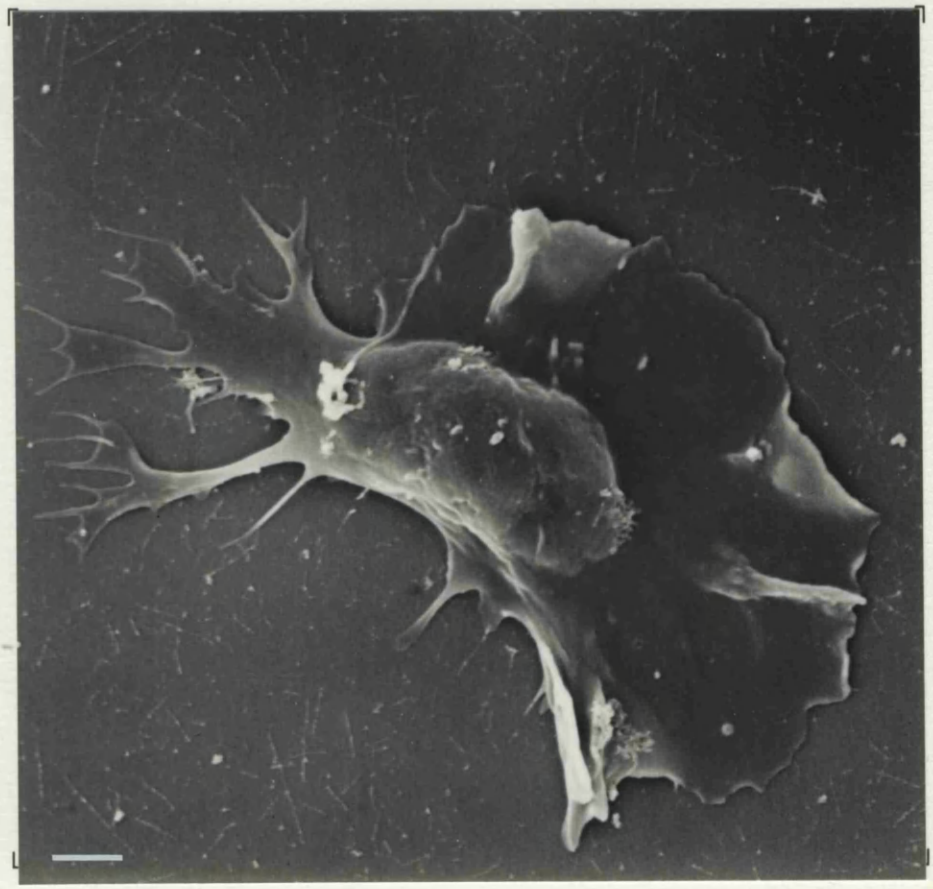


FIGURE 44

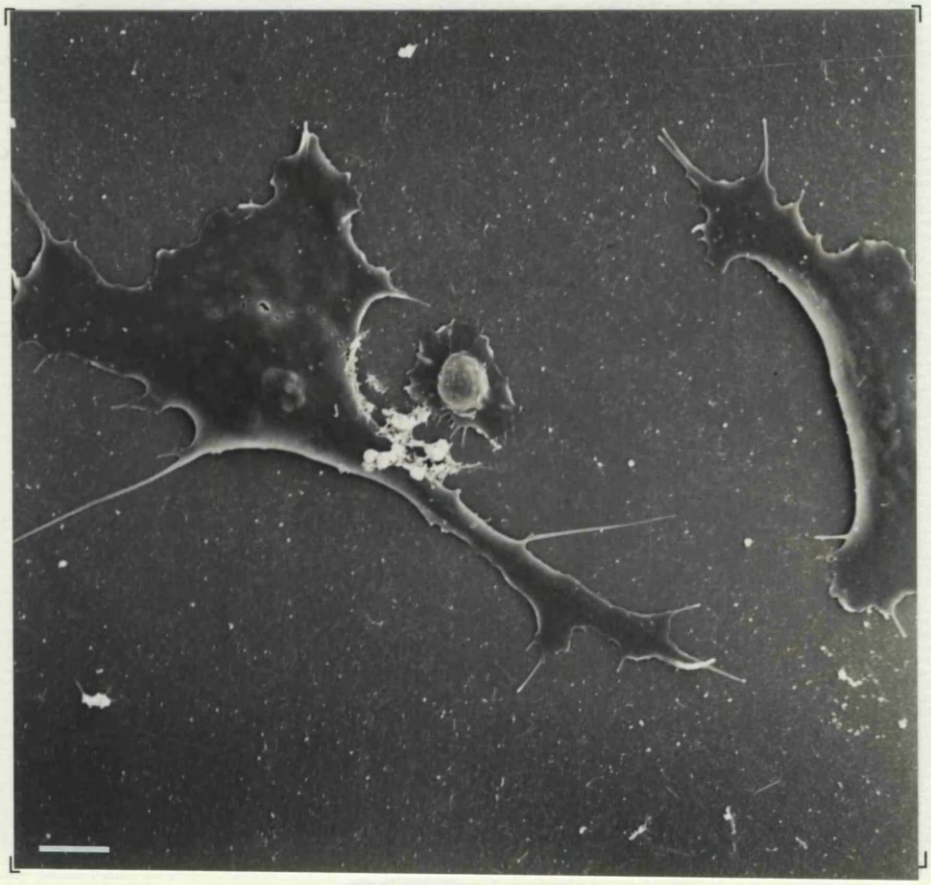
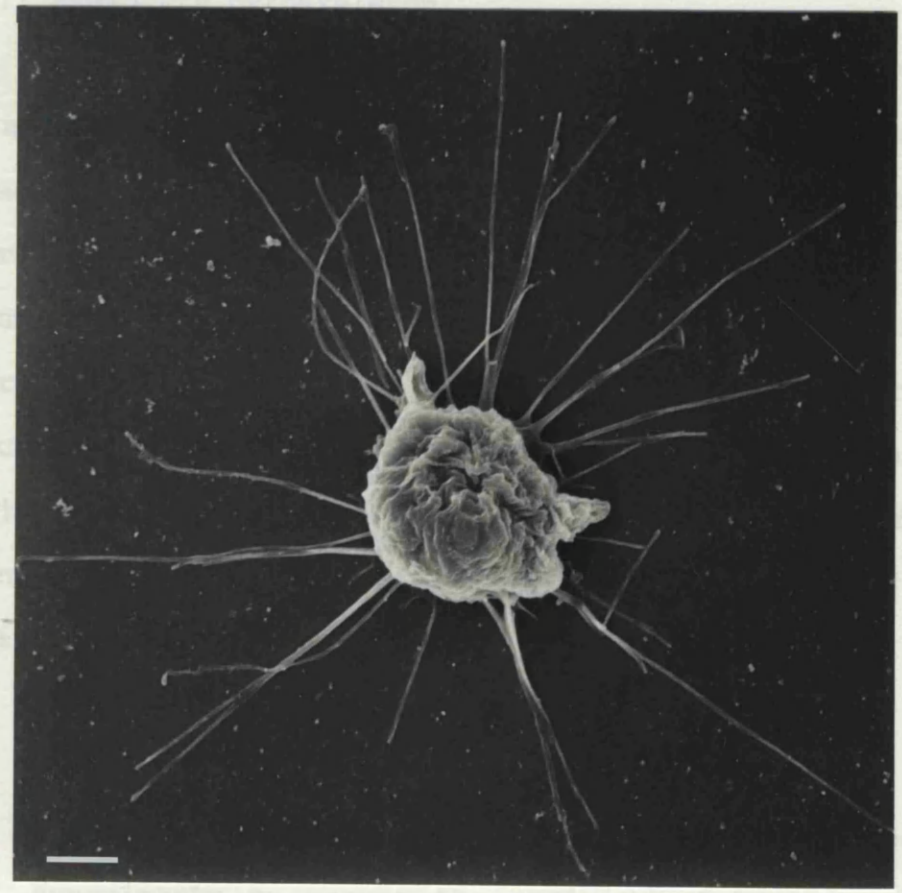


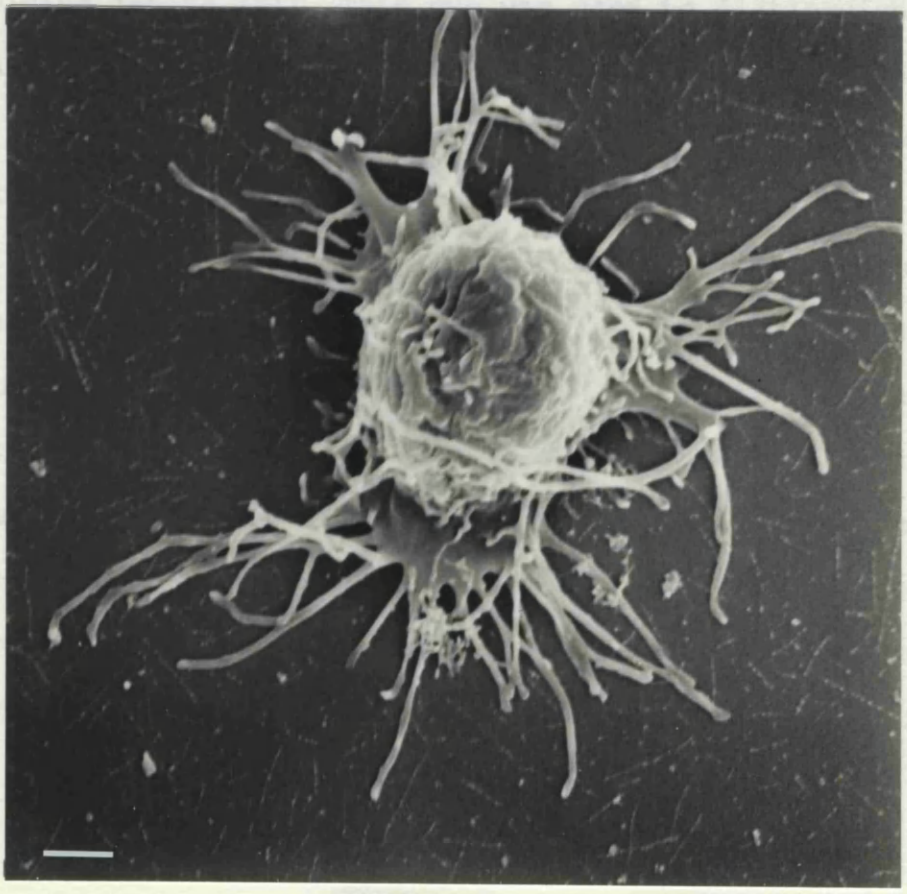
FIGURE 45

Scanning electron micrographs of B. natalensis granulocytes

Bar = 2.2 μm

FIGURE 45





(iii) Haemocyte cytoskeleton.

Extracting the haemocytes and staining for the presence of actin and tubulin revealed the cytoskeletal elements of these cells. Both cell types possess a network of microfilaments and microtubules. The filopodia of the plasmatocytes appeared to be supported by microtubules. The microtubules and microfilaments of the granulocytes were in a circular distribution surrounding the nucleus. The cytoplasmic projections apparent in electron micrographs of the granulocytes were not visible in these preparations (figures 46 & 47).

(c) Haemocyte sub-populations

(i) Binding of lectins to the surface membrane of haemocytes.

Experiments to assess the staining of haemocytes with fluorescent lectins were carried out in conjunction with propidium iodide (PI). This dye was used to distinguish live from dead cells; the staining pattern of those cells that did not exclude PI (dead cells) was ignored. An example of PI staining is given in figure 48.

The lectins Asp pea, SBA and PNA did not bind to the haemocytes of either B. natalensis or B. nasutus. In contrast Con A stained all haemocytes (figure 49). Staining was easily distinguished from the controls that had been incubated with mannose. Selective staining was achieved with WGA (figure 50). This lectin bound to a subpopulation of cells that represented approximately 10% of the total number in both snail species. Surprisingly there was no correlation with morphology since

FIGURE 46

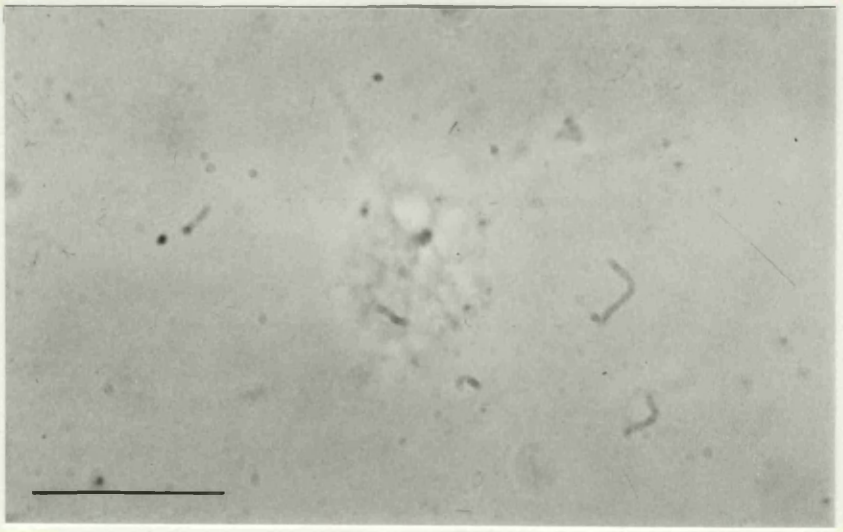
B. natalensis plasmatocyte (a) phase contrast micrograph of extracted cell; (b) fluorescence micrograph, microtubules stained with FITC-anti-tubulin; (c) fluorescence micrograph,

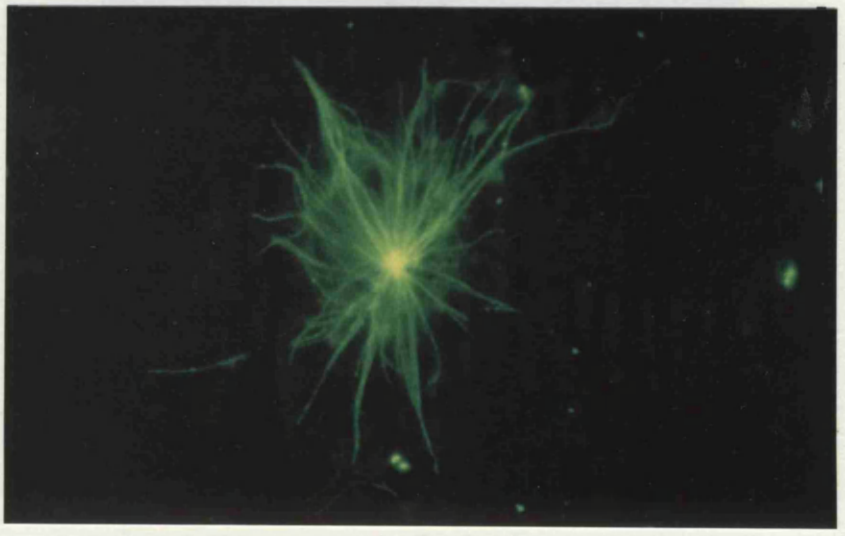
FIGURE 47

B. natalensis granulocyte (a) phase contrast micrograph of extracted cell; (b) fluorescence micrograph, microtubules stained with FITC-anti-tubulin; (c) fluorescence micrograph, microfilaments stained with TRITC-phalloidin.

Bar = 10 μm

FIGURE 46





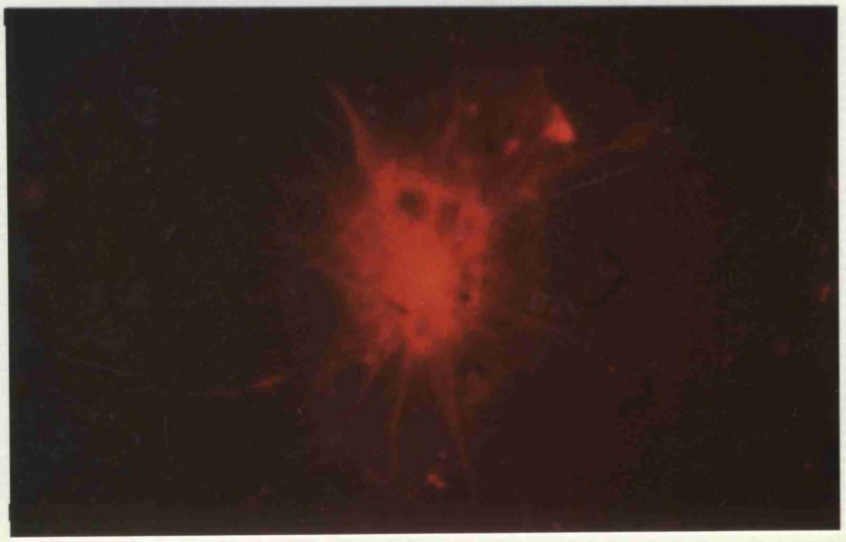
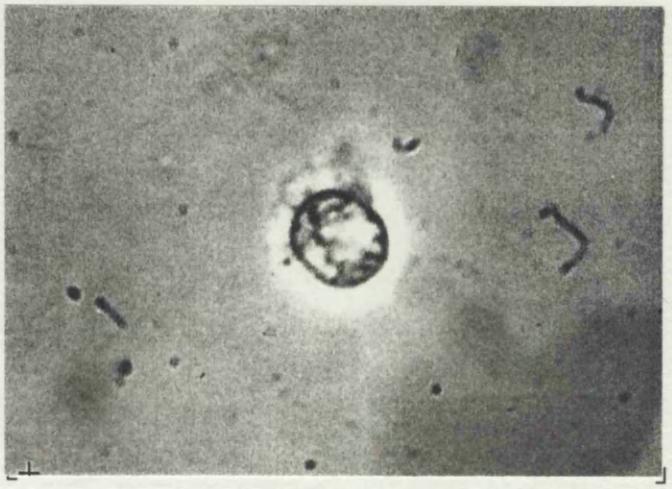
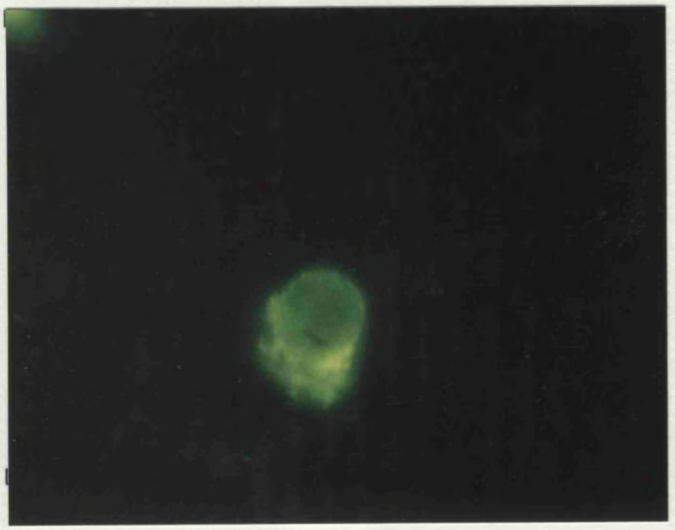


FIGURE 47

a



b



c

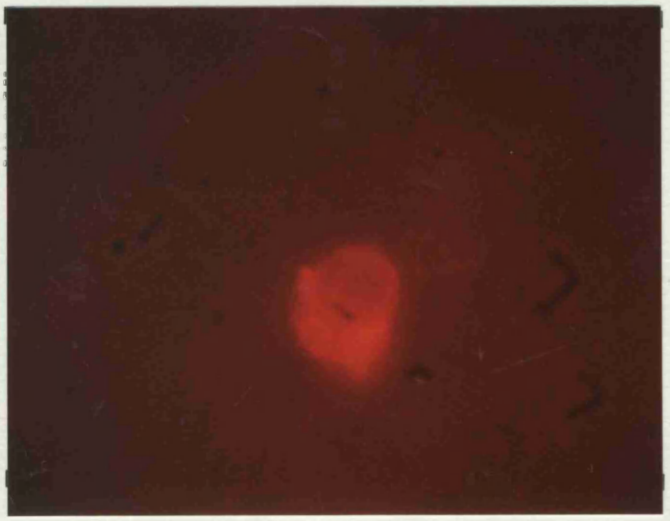


FIGURE 48

Fluorescence micrograph to show Con-A labelling of bulinid haemocytes.

FIGURE 49

Fluorescence micrograph to show haemocyte of 'intermediate morphology' (a) stained with FITC-Con-A; (b) stained with propidium iodide

Bar = 10 μm

FIGURE 48

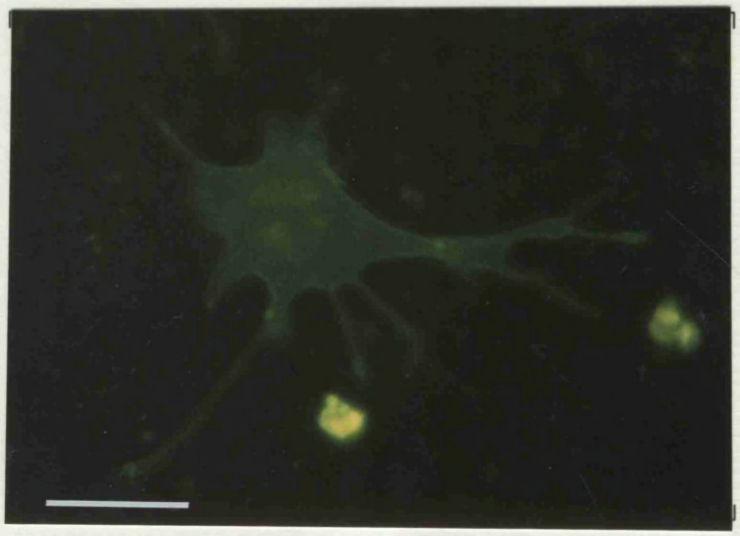


FIGURE 49

a

b

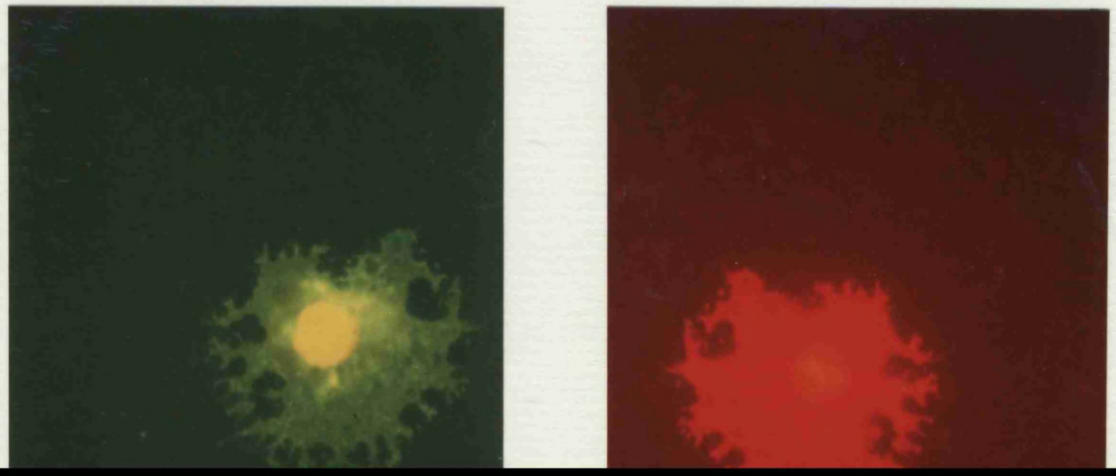
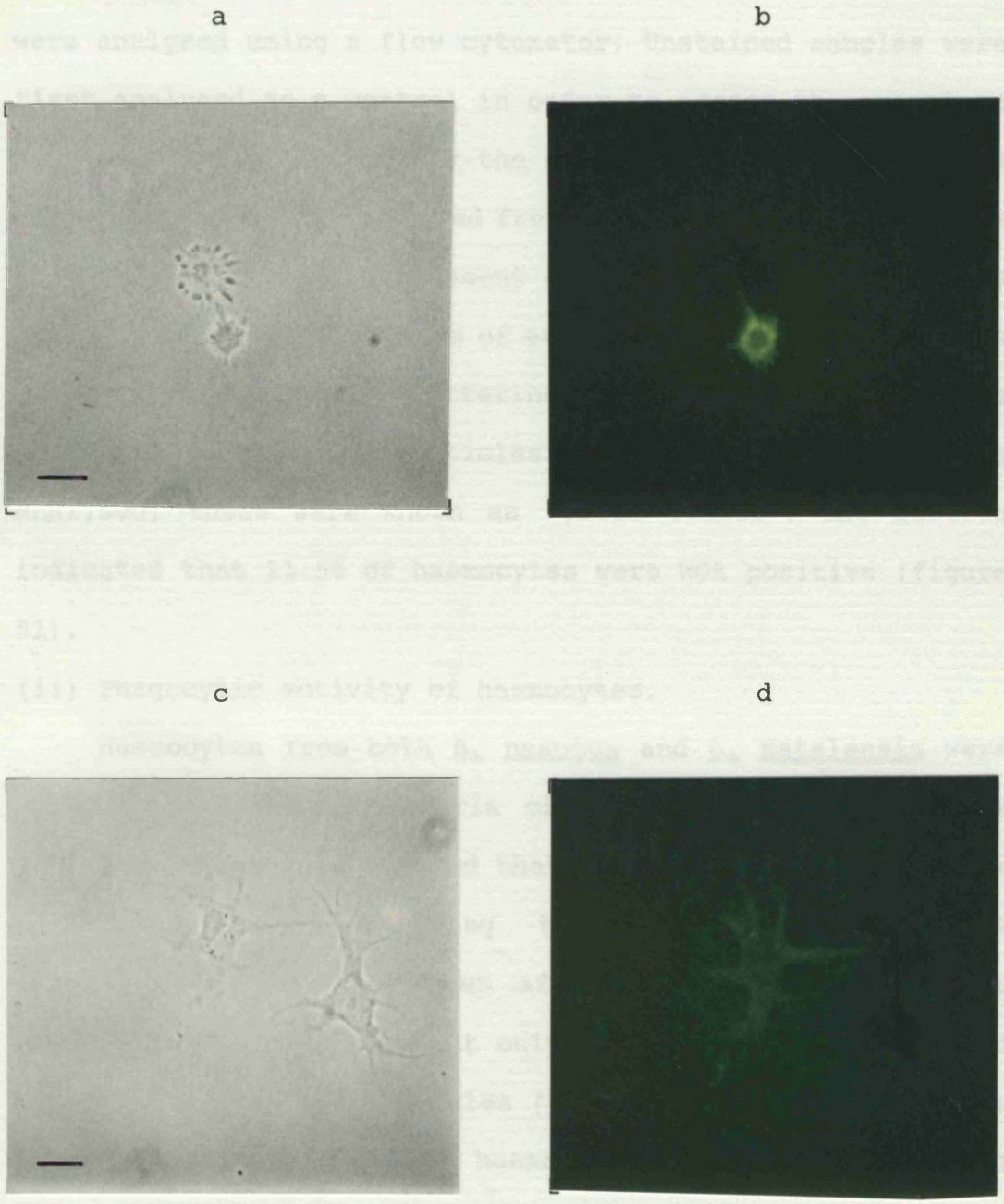


FIGURE 50

Phase (a & c) and fluorescence (b & d) micrographs of B. natalensis haemocytes stained with FITC-WGA

Bar = 10 μm

FIGURE 50



members of both plasmatocyte and granulocyte classes were stained with this lectin.

Haemocytes from B. natalensis snails, stained with WGA were analysed using a flow cytometer. Unstained samples were first analysed as a control in order to assess the amount of autofluorescence emitted by the cells. The FITC-WGA positive haemocytes were distinguished from the FITC-WGA negative cells as those emitting a fluorescent signal above this level. The analyser records the passage of every particle passing through the laser beam by light scattering. The gating parameters were adjusted so that only particles above 5μm in diameter were analysed, these were known as 'gated events'. The results indicated that 11·5% of haemocytes were WGA positive (figure 51).

(ii) Phagocytic activity of haemocytes.

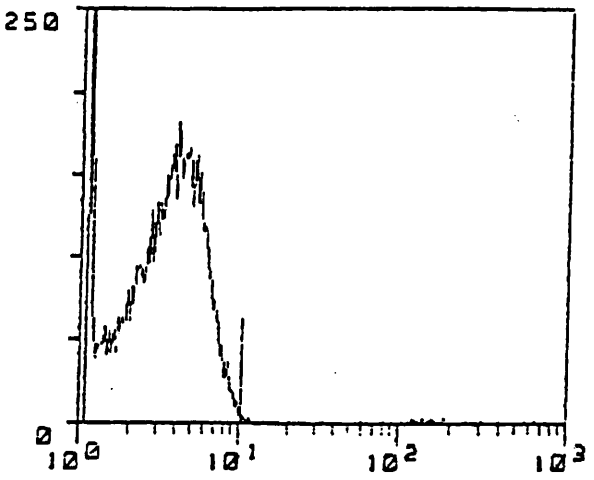
Haemocytes from both B. nasutus and B. natalensis were able to phagocytose bacteria or latex beads to a similar degree in vitro. It appeared that the granulocytes were more efficient at phagocytosing these particles than the plasmatocytes. Samples taken after 5 min incubation with beads/bacteria revealed that only the granulocytes had bound and phagocytosed the particles (figure 52). Incubation times of 10 and 15 min revealed haemocytes of both morphological types associated with the particles (figure 53) but there were always plasmatocytes with no associated particles. In some cases, haemocytes with extremely long filopodia could be seen. These cytoplasmic strands were found to be associated with the latex particles (figure 54), similar cells were not observed

FIGURE 51

FLOW CYTOMETRY OF HAEMOCYTES

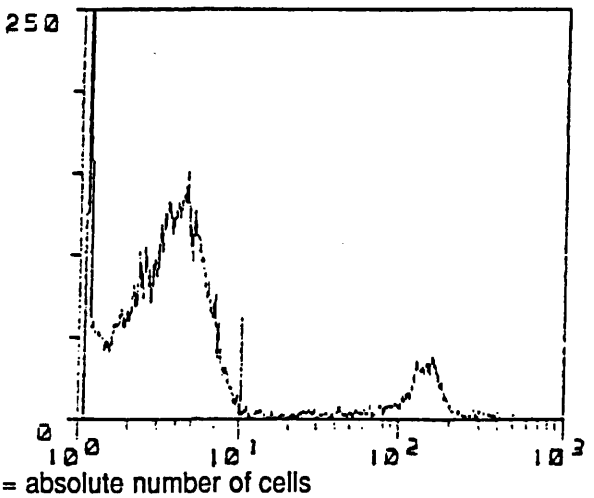
CONTROL

Intensity of fluoresence	Events (total = 8849)	% Gated
10^0 - 10^1	8778	99.2
10^1 - 10^3	71	0.8



EXPERIMENTAL

Intensity of fluoresence	Events (total = 8764)	% Gated
10^0 - 10^1	7755	88.5
10^1 - 10^3	1009	11.5



ORDINATE = absolute number of cells

ABSCISSA = intensity of fluorescence (arbitary scale)

FIGURE 52

In vitro phagocytosis: B. natalensis haemocytes stained with fluorescein diacetate. Haemocytes were incubated with E. coli (stained with ethidium bromide) for 5min prior to preparation onto glass coverslips. Phase (a) and fluorescence (b) micrographs show granulocyte and plasmatocyte. The granulocyte is stained with both fluorescein diacetate and ethidium bromide indicating uptake of bacteria by this cell.

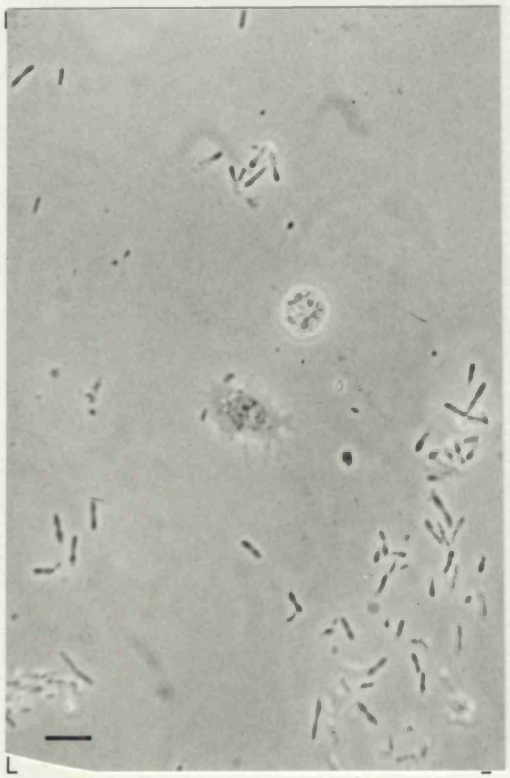
FIGURE 53

B. natalensis haemocytes after 15 min incubation with E. coli. Phase (a) and fluorescence (b) micrographs of plasmatocytes, both stained with fluorescein diacetate showing uptake of bacteria by these cells

Bar = 10 μm

FIGURE 52

a



b

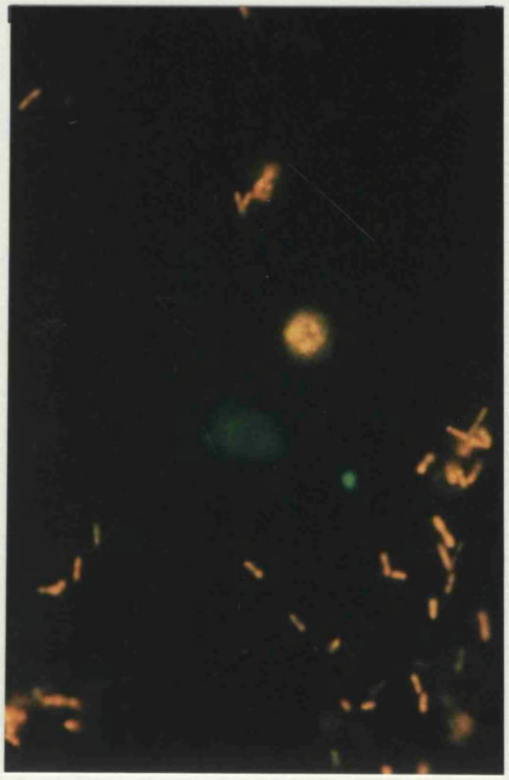
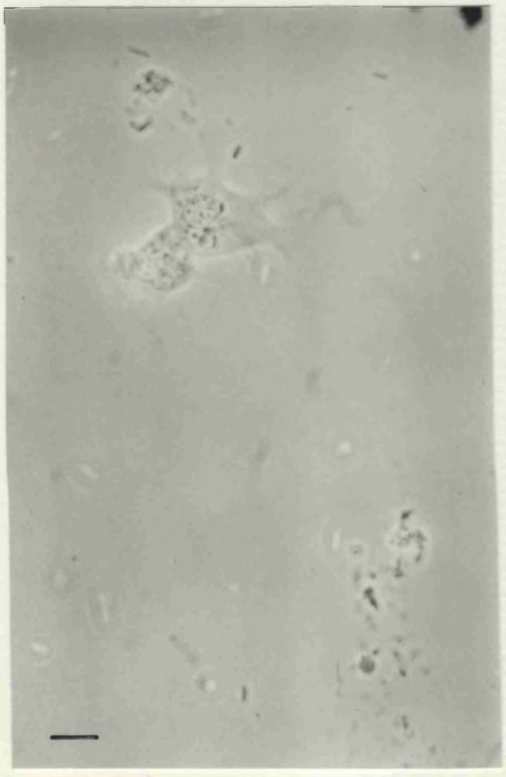


FIGURE 53

a



b

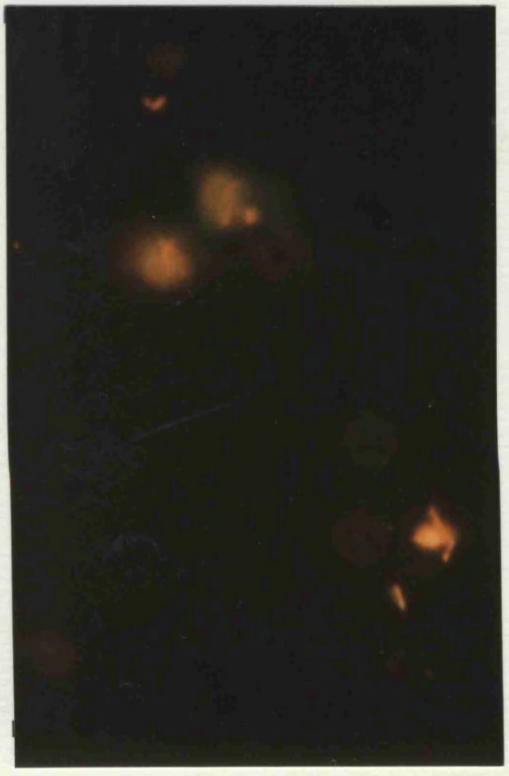


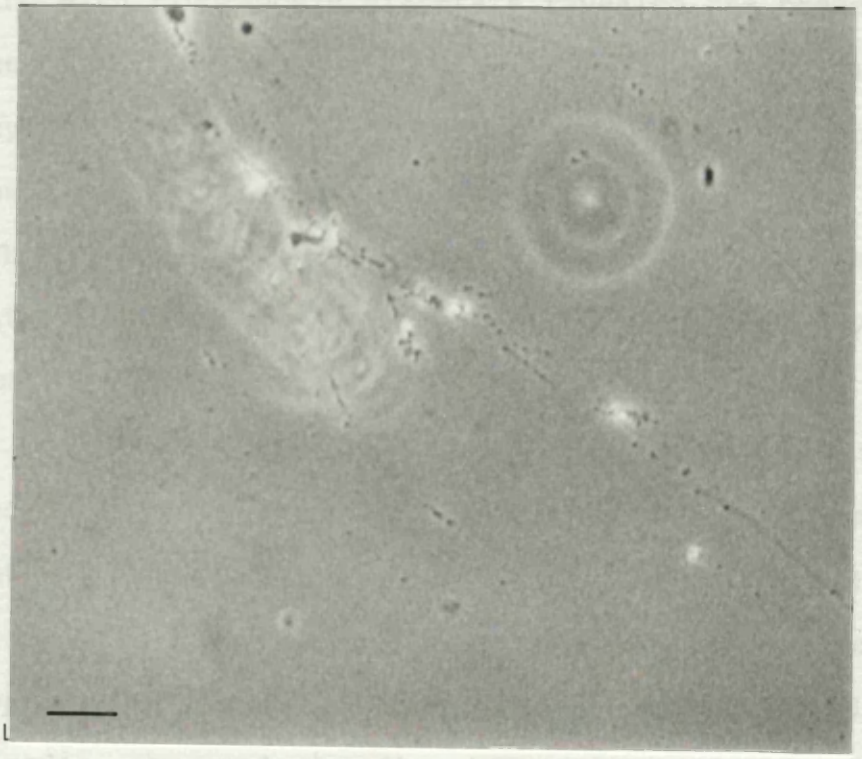
FIGURE 54

In vitro phagocytosis: Phase (a) and fluorescence (b) micrograph of B. natalensis haemocyte after 10 min incubation with fluorescein labelled latex particles, stained with fluorescein diacetate. Haemocyte has long cytoplasmic strands associated with the particles.

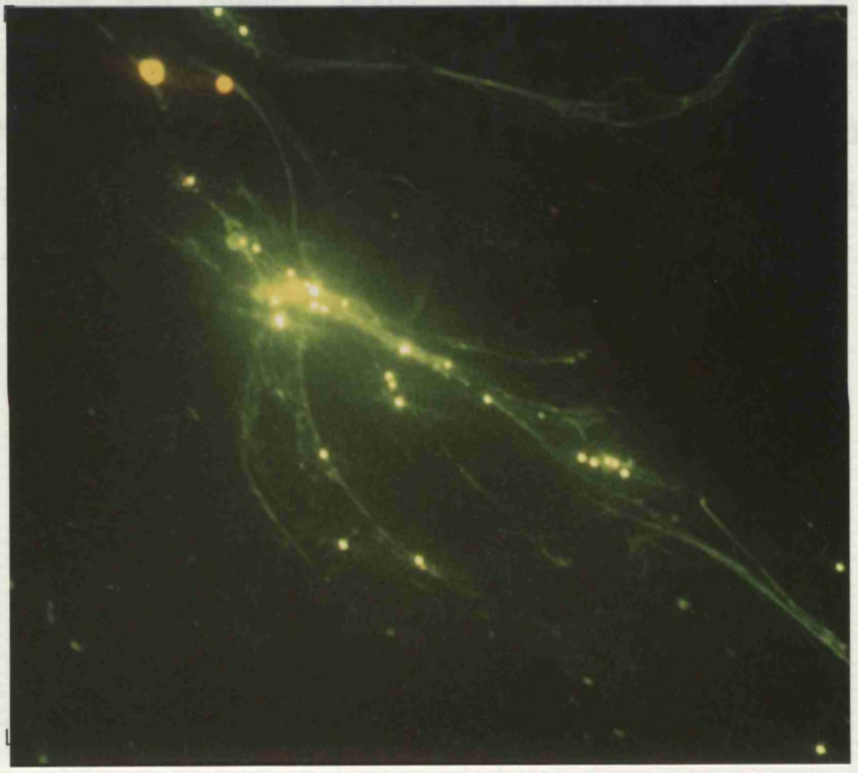
Bar = 10 μm

FIGURE 54

a



b



when challenged with bacteria. There was no correlation found between cells staining positve for WGA and particle uptake. Staining with fluorescein diacetate revealed the presence of non-specific esterases in the majority of cells. The haemocytes that remained unstained with this reagent appeared degranulated and were possibly dead cells.

The single B. nasutus inoculated with bacteria and bled 2 h later, appeared to have a higher number of haemocytes. A cell count was not carried out but large numbers of cells, often in clumps, were observed. When the haemocytes were treated with fluorescein diacetate not all cells were able to liberate the fluorescein by esterase action (figure 55). Some of the plasmatocytes in the preparation appeared to have thick pseudopodia (figure 56), possibly akin to the 'funnel-like' pseudopodia observed in the haemocytes of Biomphalaria glabrata, which produce this type of pseudopodium after treatment with lectins and incubation with erythrocytes. (Schoenberg and Cheng, 1980). The bacteria used for this inoculum had been previously stained with ethidium bromide as a fluorescent probe which emits light at 616 nm (red). The fluorescence was discernible at a very low level, with the granulocytes exhibiting the highest intensity (figure 56).

FIGURE 55

Phase (a) and fluorescence (b) micrographs of haemocytes from B. nasutus 1h after inoculation with E. coli. Stained with fluorescein diacetate.

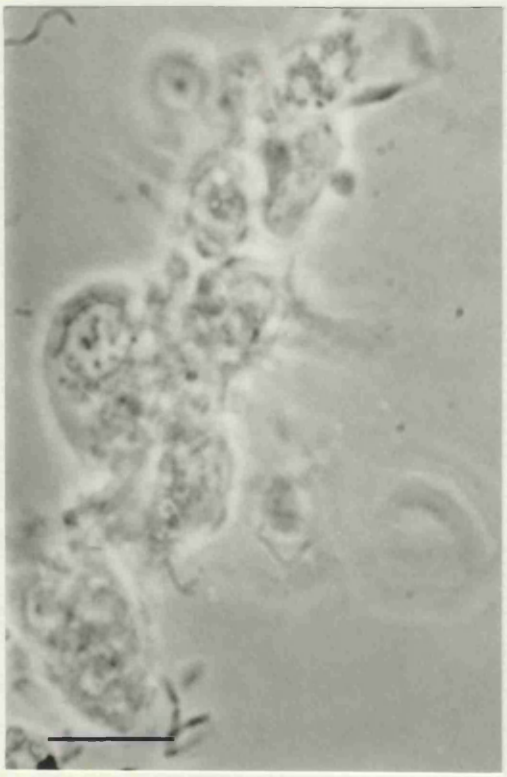
FIGURE 56

Phase (a) and fluorescence (b) micrographs of a single plasmatocyte from B. nasutus after inoculation with E. coli showing thick pseudopodia.

Bar = 10 μm

FIGURE 55

a



b

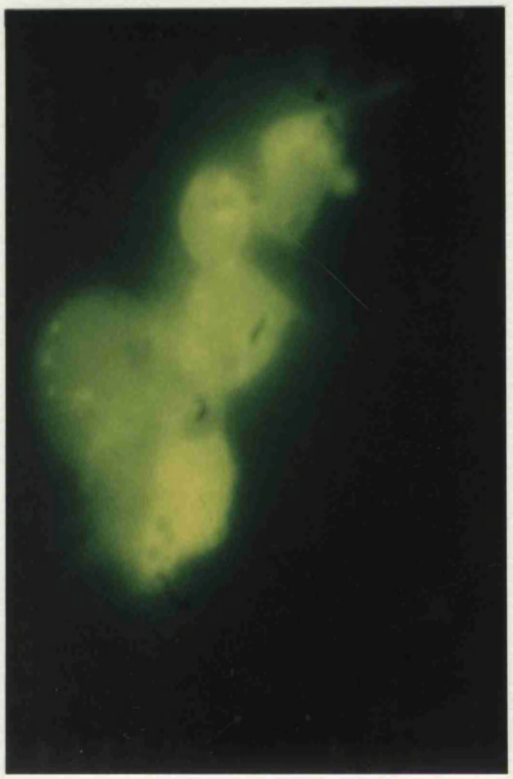
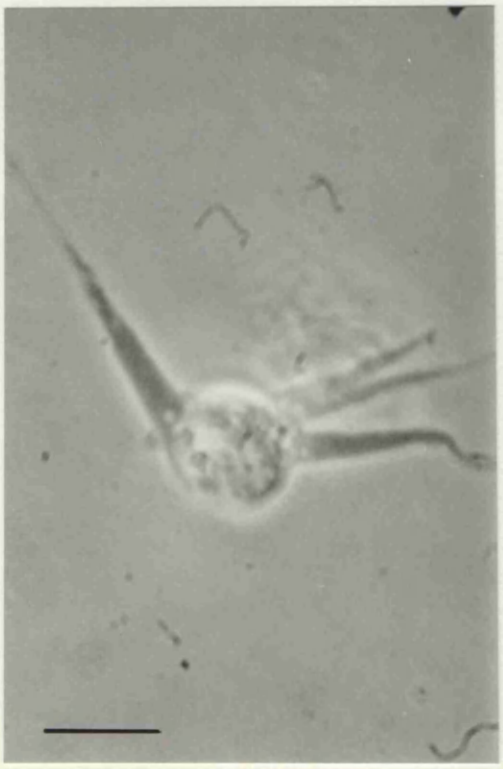


FIGURE 56

a



b

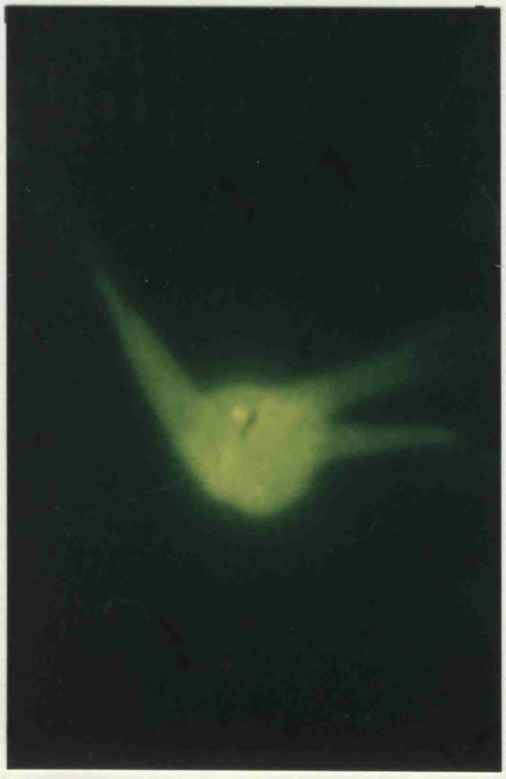


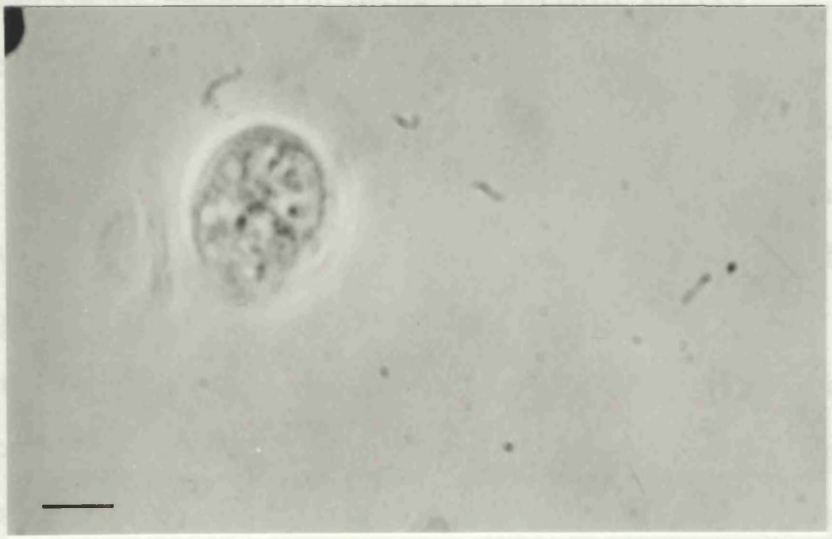
FIGURE 57

Phase (a) and fluorescence (b & c) micrographs of single granulocyte from B. nasutus after inoculation with E. coli. Haemocyte stained with fluorescein diacetate (b) but also emitting red fluorescence indicating presence of bacteria (c).

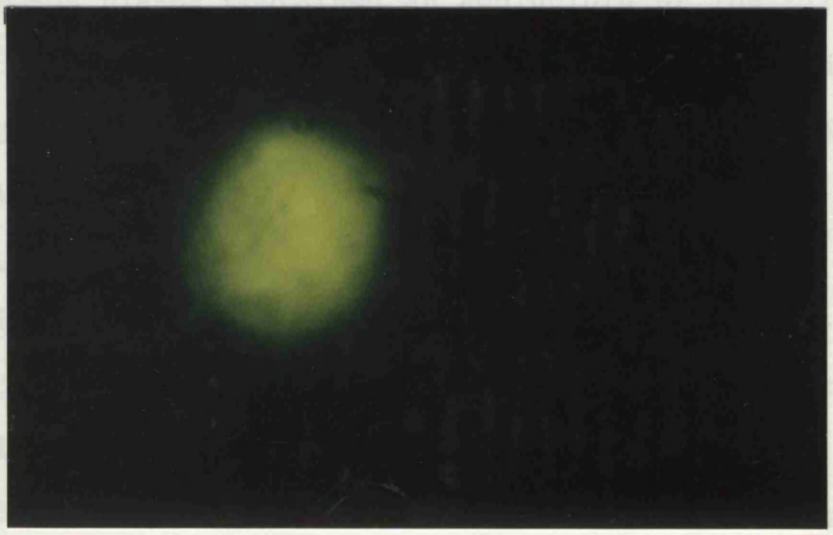
Bar = 5 μm

FIGURE 57

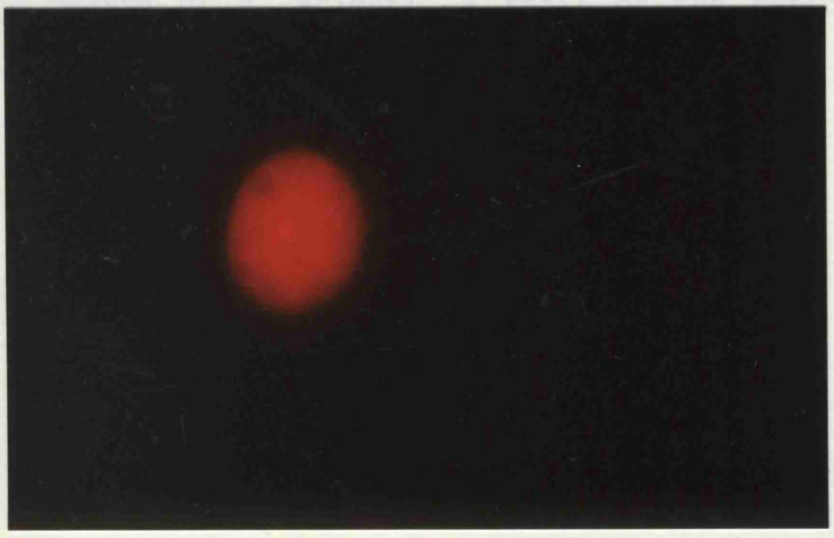
a



b



c



3. HAEMMAGGLUTININS

(a) Presence of Haemagglutinins in Bulinid Snails.

(i) Interspecific variation within the genus Bulinus.

A survey using plasma from different species of bulinid snails revealed the presence of haemagglutinins to human blood types A and B in five of the nine species tested. Species and haemagglutination titres are given in table 10.

There appears to be little correlation between the presence of haemagglutinins and the snail species groups. Three of the five species of Africanus snails tested possessed an agglutinin/agglutinins for types A and B red blood cells. B. globosus plasma contained an agglutinin of low titre for type B cells only. It is not known whether this agglutinin is binding to the terminal galactose residues that distinguish this blood group.

B. tropicus and B. natalensis are diploid snails belonging to the truncatus/tropicus complex and their plasma did not agglutinate the red blood cells. However the tetraploid snail, B. truncatus, did have a low titre agglutinin but these data are insufficient to assume a correlation of agglutinin with ploidy.

The plasma of B. wrighti, a snail of the reticulatus group, showed no agglutinating activity.

TABLE 10

HAEMAGGLUTINATION OF HUMAN RED BLOOD CELLS BY THE PLASMA OF BULINID SNAILS

	KNOWN SCHISTOSOME COMPATIBILITY	AGGLUTINATION TITRE Human A	Human B
Africanus group			
B. globosus	*S. haematobium*		
	S. mattheei		
	S. intercalatum	-ve	1:2
	S. bovis		
	S. leiperi		
B. jousseaummei	*S. haematobium*	1:16	1:128
B. nasutus	*S. haematobium*	1:65536	1:32768
B. africanus	*S. haematobium*		
	S. mattheei		
	S. intercalatum	1:2048	1:2048
	S. bovis		
	S. leiperi		
B. abyssinicus	*S. haematobium*	-ve	-ve
Truncatus/Tropicus complex			
B. tropicus	*S. margrebowiei*	-ve	-ve
B. truncatus	*S. haematobium*		
	S. margrebowiei	1:2	1:2
	S. bovis		
B. natalensis	*S. haematobium* ?	-ve	-ve
	S. margrebowiei		
Reticulatus group			
B. wrighti	All schistosomes of the *S. haematobium* group.	-ve	-ve

(Snail classification after Brown, 1980).

(ii) Intraspecific variation within the species Bulinus nasutus.

The individual plasma samples from B. nasutus snails showed little variation in haemagglutinating activity and the titres achieved were similar to those produced in other experiments. The small amount of variation that did occur was probably due to experimental error rather than snail to snail variation. The results are represented in table 11. No differences were observed between the adult and juvenile snails. All human blood types tested (A1, A2, B and O) were agglutinated, with type A2 generally giving the highest titres. These erythrocytes differ from those of type A1 by having fewer type A determinants this gives a further indication that the agglutinin is not recognising major ABO blood group determinants.

TABLE 11

HAEMAGGLUTINATING ACTIVITY OF THE PLASMA OF *B. NASUTUS* SNAILS

	BLOOD GROUP			
	A1	A2	O	B
ADULT SNAILS				
1	1:65536	1:65536	1:32768	1:32768
2	1:65536	1:131072	1:32768	1:32768
3	1:32768	1:262144	1:65536	1:32768
4	1:131072	1:131072	1:32768	1:32768
JUVENILE SNAILS				
1	1:131072	1:131072	1:32768	1:32768
2	1:65536	1:131072	1:32768	1:32768
3	1:65536	1:65536	1:65536	1:32768
4	1:65536	1:131072	1:32768	1:32768

(b) Haemagglutinin Induction

B. nasutus snails are resistant to infection by S. margrebowiei miracidia. Snail plasma was tested 2 h and 24 h post exposure to these miracidia. There was no elevation of haemagglutinin titre, indicating that the agglutinin is not inducible, at least not within 24 hours of challenge. However, since the snails are not kept under sterile conditions it is not known whether this represents the constituent level of production or whether the snail immune system has been triggered by pathogenic organisms living in the snail trays. It is also possible that the miracidia did not enter the snails since these snails were not sacrificed and tested for the presence of encapsulated schistosomes.

(c) Red cell specificity

Red blood cells from sheep, rabbit, rat and mouse were tested for agglutination with B. nasutus plasma. The agglutination titres are given in table 12.

TABLE 12

HAEMAGGLUTINATION OF VERTEBRATE RBCS WITH *B.NASUTUS* PLASMA

RBC source	Haemagglutination Titre
Sheep	1:4096
Rabbit	1:32768
Rat	1:8192
Mouse	Variable

The plasma agglutinated erythrocytes from all the species of vertebrate tested i.e. sheep, rabbit, rat and mouse as well

as human. The haemagglutinin titres varied but were all high when compared with purified lectins. For example Con A agglutinates a 2% w/v suspension of human erythrocytes at a dilution of 1:16 and the blood group A specific agglutinin purified from Helix pomatia is active at a dilution of 1:1000 (Sigma chemicals specifications).

Haemagglutination results using mouse red blood cells gave varying results and the tests were extended to include erythrocytes from different strains of mice. The mice available in the laboratory were inbred strains of known major histocompatibility recombinant haplotypes. Mice are one of the few animals to display these determinants on the surface of their red cells. The results of these tests are given in Table 13.

TABLE 13

HAEMAGGLUTINATION OF RED CELLS FROM DIFFERENT STRAINS OF MOUSE

Mouse Strain	MHC H-2 haplotype	Haemagglutination Titre
CBA	k	1:4096
Balb/c	d	-ve
B10SCN	b	-ve
B10D2	g_i	-ve
Athymic	d	-ve
T/O	polymorphic	1:512

B. nasutus plasma only agglutinated erythrocytes from CBA mice with the H-2 k haplotype and the outbred Tyllers Original strains. Unless the RBCs from the other species of vertebrate tested carry antigenic determinants that are structurally similar to mice H-2 k MHC molecules, it is unlikely that the agglutinin is specific for these MHC antigens. It is not known

how these strains of mice differ from one another in aspects other than their MHC molecules.

(d) Divalent Cations.

Haemagglutinating activity for human A2 erythrocytes was found to be independent of the requirement for divalent cations since the agglutination titres remained unaltered in the presence of EGTA and EDTA. This is also the case for some agglutinins found in other molluscs, namely Achatina, (Mitra and Sakar, 1988) and Biomphalaria glabrata (Boswell and Bayne, 1984). However two other agglutinins found in the haemolymph of Biomphalaria glabrata are dependent on divalent cations (Gilbertson and Etges, 1967; Stein and Basch, 1979).

(e) Haemagglutination inhibition tests

Experiments to determine the carbohydrate-binding specificity of the B. nasutus haemagglutinin by inhibition with sugars were largely unsuccessful. The panel of common carbohydrates used failed to produce any marked reduction in agglutination titre. All agglutination titres were somewhat lower in the test wells when compared with the control but this is probably the result of steric hindrance and not specific interactions. The results are shown in Table 14.

Two of the sugars used in this inhibition study, galactose and N-acetyl galactosamine did have some inhibitory effect but only when the plasma was at a dilution of 1:4096. At this dilution inhibition by non-specific interactions cannot be ruled out. Also the methylated anomers of galactose had less of an inhibitory effect. However it is of interest to

note that all human erythrocytes have surface galactose residues, as ascertained by ricin staining (Kabat, 1976) and the only larval form of the schistosome S. margrebowiei to bind the agglutinin is the miracidium (see results section 3 f) whose predominant surface sugar is galactose (results section 1 c).

TABLE 14

HAEMAGGLUTINATION INHIBITION ASSAY

	Agglutination Titre
B. nasutus plasma (control)	1:32768
Inhibiting Sugars (100mM)	
Glucose	1:16384
Galactose	1:4096
Mannose	1:16384
Fucose D	1:16384
Fucose L	1:16384
Mannose + glucose	1:16384
Methyl α-D galactoside	1:8192
Methyl ß-D galactose	1:8192
Methyl α-D mannoside	1:32768
N-acetyl glucosamine	1:16384
N-acetyl galactosamine	1:4096
Sialic acid	1:16384
Fetuin (1mg ml^{-1})	1:32768

(f) Haemagglutinin absorption by larval schistosomes

B. nasutus plasma was incubated with freshly hatched miracidia derived from macerated mouse liver, in vitro transformed sporocysts and the newly shed cercariae of S. margrebowiei. The plasma was subsequently tested for

haemagglutinating activity with human erythrocytes type A_2. The results are shown in Table 15 and figure 58.

Approximately 300 of the different larval stages were used in each test, the liver tissue was included because the miracidial preparation was not entirely free from contamination with this tissue. The liver was used as a 20% v/v suspension, far in excess of that found contaminating the miracidia.

The haemagglutinating activity of B. nasutus plasma was lost after incubation with S. margrebowiei miracidia indicating that the agglutinin had been adsorbed onto the surface of this larval stage. There was also some reduction in haemagglutination titre with the liver tissue control but only to a titre of 1:16384. The sporocysts and cercariae did not absorb agglutinin and the haemagglutinating activity remained unaltered.

In a further experiment freshly collected haemolymph from five B. nasutus snails was incubated with approximately 10^3 S. margrebowiei miracidia. The agglutinating activity was completely abrogated by this treatment (figure 59).

A sample of the plasma, post absorption, was fractionated using polyacrylamide gel electrophoresis and compared with the same plasma pre-absorption. After silver staining the gel, a doublet was discernible in the lanes of pre-absorbed plasma that was absent in those post absorption (figure 60). These bands corresponded to an apparent M_r of approximately 75,000 and 81,000.

FIGURE 58

Haemagglutination assay: Absorption of B. nasutus haemagglutinin by S. margrebowiei larval schistosomes.
B. nasutus haemolymph used at an initial concentration of 1:1024 and tested against 2% v/v solution of human A_2 erythrocytes.

A & B Cercariae

C Miracidia

D & E Sporocysts

F Liver tissue

G Haemolymph control

Wells 11 & 12 in each row are negative controls, PBSA and erythrocytes only

FIGURE 59

Haemagglutination assay: Absorption of B. nasutus haemagglutinin by S. margrebowiei miracidia.
B. nasutus haemolymph used undiluted and tested against 2% v/v solution of human A_2 erythrocytes.

A Doubling dilution series of haemolymph after incubation with miracidia

B Doubling dilution series of B. nasutus haemolymph

C PBSA and erythrocytes only

FIGURE 58

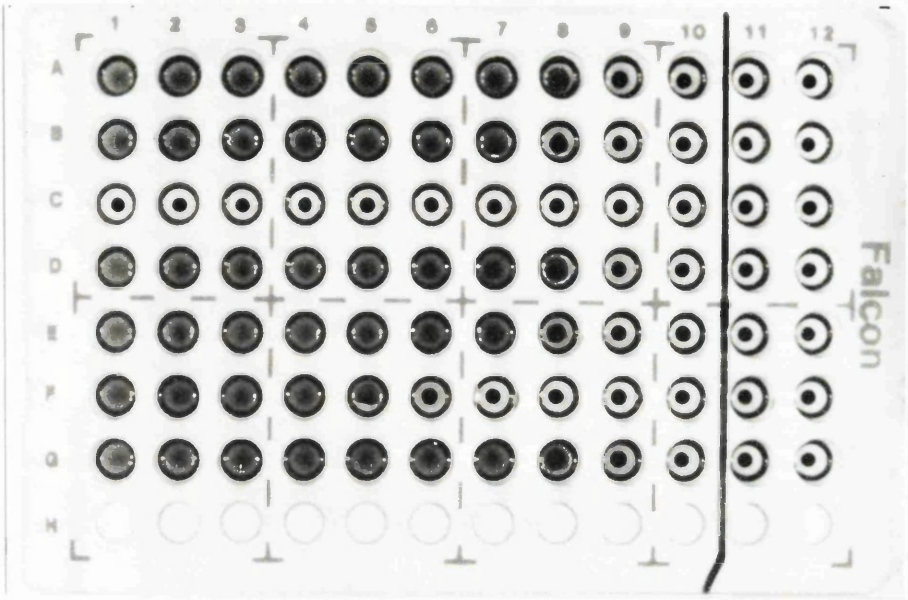


FIGURE 59

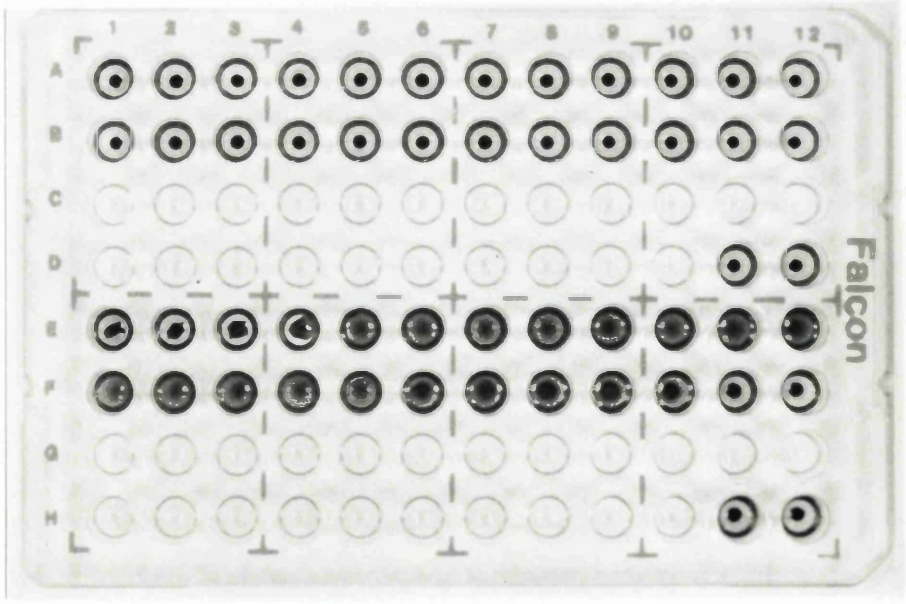


FIGURE 60

Comparison of B. nasutus haemolymph before (lanes 2 and 4) and after (lane 3) incubation with S. margrebowiei miracidia. Haemolymph proteins separated using SDS-PAGE and silver stained.

FIGURE 60

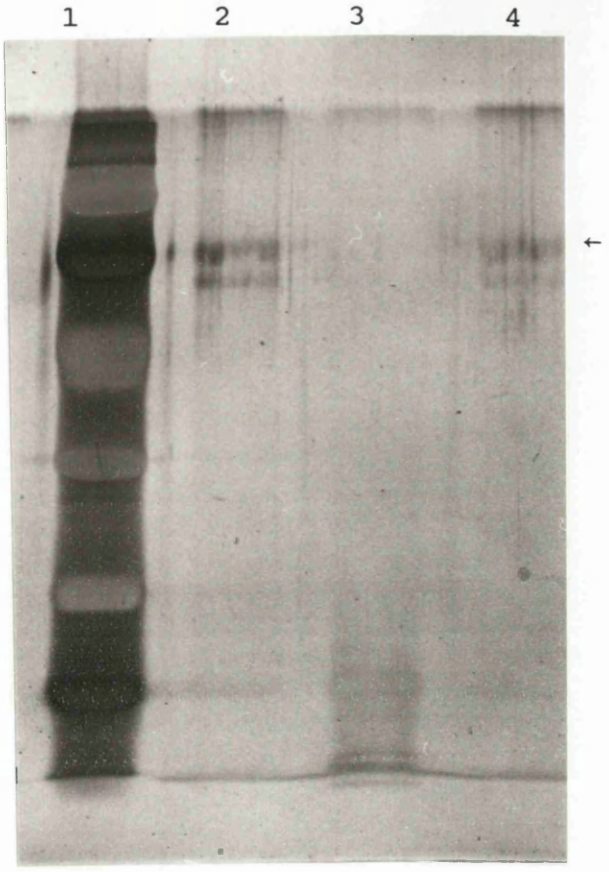


TABLE 15

HEAMAGGLUTINATION ABSORPTION

	Haemagglutination Titre
B. nasutus plasma (control)	1:131072
Larval Schistosomes	
Miracidia	-ve
Sporocyst	1:131072
Cercariae	1:131072
Liver tissue	1:16384

The agglutinin also absorbed onto the surface of the ciliate Tetrahymena (figure 61) and therefore was possibly recognising an ubiquitous ciliary component. However when the experiment was repeated using miracidia from the schistosome S. mansoni the haemagglutination titres remained the same as the controls (figure 62).

(g) Fractionation of B. nasutus plasma using Fast Protein Liquid Chromatography (FPLC).

The plasma of B. nasutus snails was fractionated using fast protein liquid chromatography. On elution from the column, only one peak was detected by absorption spectrophotometry (at 280nm). This peak corresponded to fractions 15-19 and an estimated M_r of 88,000 (figure 63). Similar results were obtained with B. natalensis plasma (figure 64). A comparison of the plasma protein profiles obtained using SDS-PAGE indicated that a high degree of homology exists between the plasma of these two species of snail (figure 65).

All fractions eluted from the column were tested for agglutinating activity. The agglutinating activity was found in fractions 16-21 i.e. of an estimated M_r of 76,000-86,000. The agglutinin is, therefore, similar in size to the snail haemoglobin or there is a loose association between the two proteins. More stringent methods of fractionation are required to resolve this problem.

FIGURE 61

Haemagglutination assay: Absorption of B. nasutus haemagglutinin by the ciliate Tetrahymena (A). B. nasutus haemolymph used at an initial concentration of 1:200 and tested against 2% v/v solution of human A_2 erythrocytes. (B) Haemolymph control

FIGURE 62

Haemagglutination assay: Haemolymph tested after incubation with S. mansoni miracidia against 2% v/v solution of human A_2 erythrocytes

A B. nasutus haemolymph at a dilution of 1:400

B Negative control, no haemolymph

C & D Haemolymph post incubation with miracidia

FIGURE 61

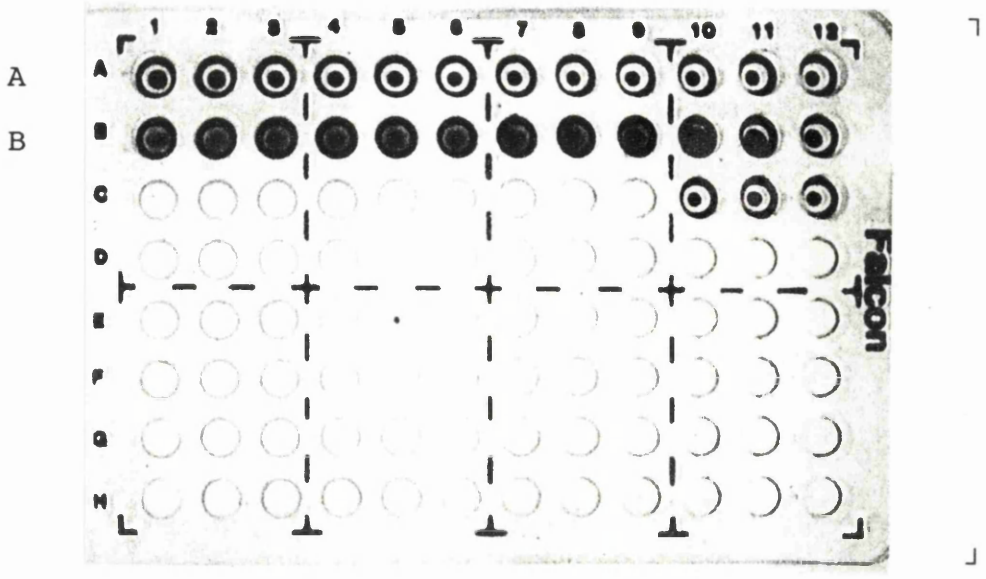


FIGURE 62

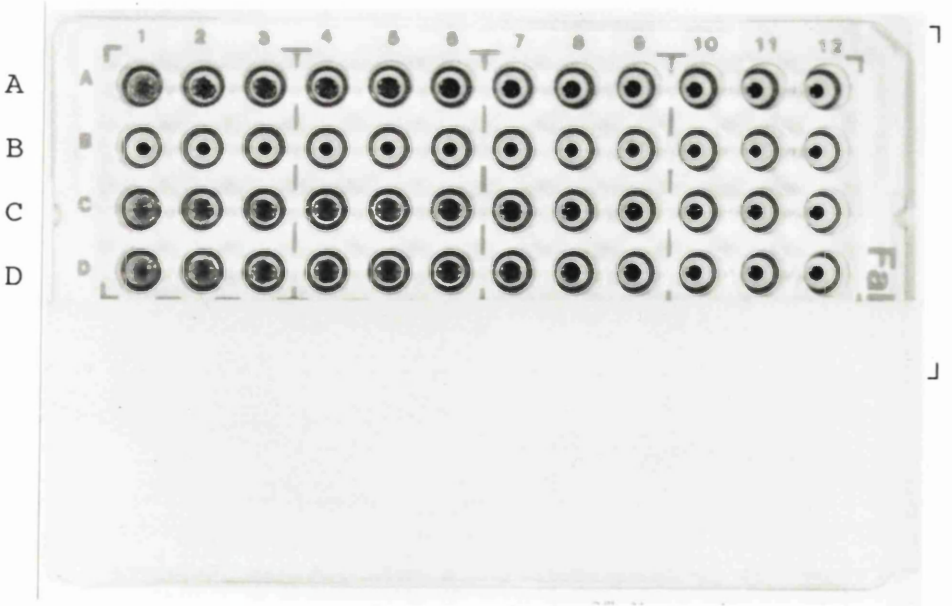


FIGURE 63 B. nasutus haemolymph

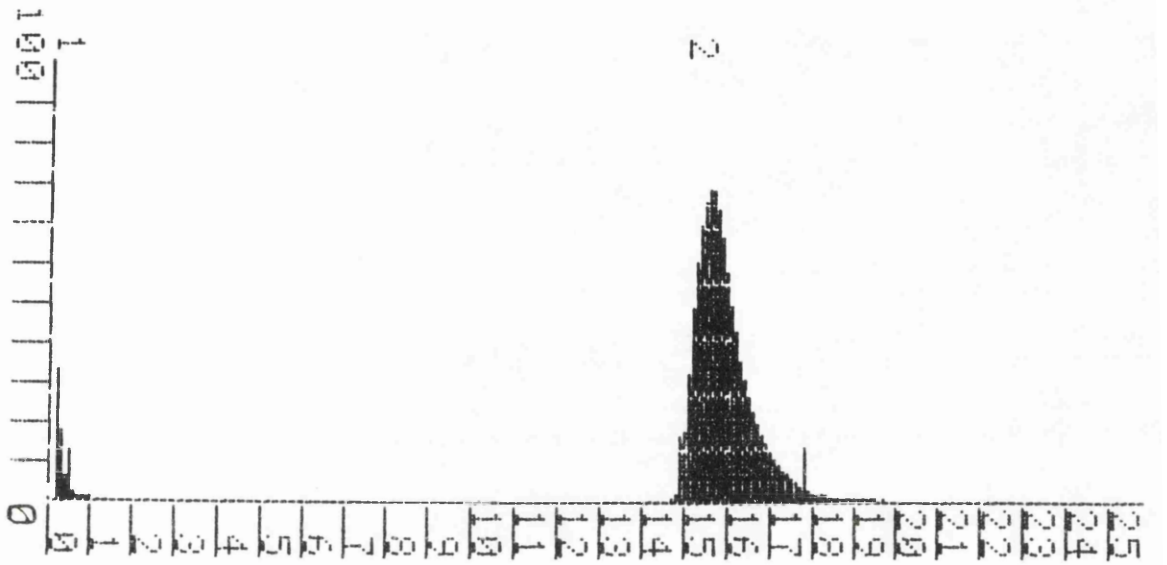
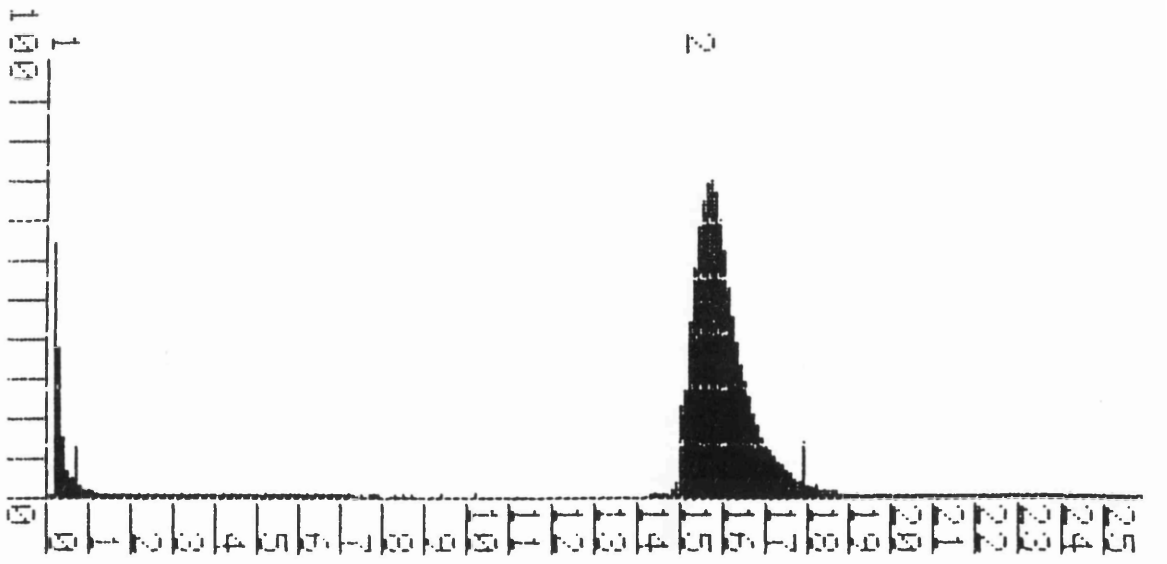


FIGURE 64 B. natalensis haemolymph



ORDINATE = Fraction number

ABSCISSA = Absorbance (arbitary scale)

FIGURE 65

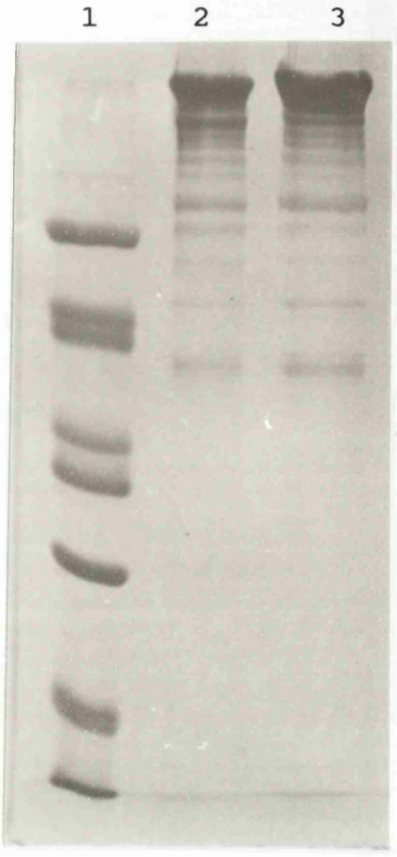
Comparison of the haemolymph proteins of B. natalensis and B. nasutus using SDS-PAGE.

Lane 1 Molecular weight markers

Lane 2 B. natalensis

Lane 3 B. nasutus

FIGURE 65



1. Schistosomes

The intramolluscan stages of the schistosome life cycle include the miracidium, mother sporocyst, daughter sporocyst and the cercaria. The stages are morphologically and functionally distinct but each are in intimate contact with the snail's immune system. In order to survive, the parasite has to either avoid being recognised as foreign or actively disarm the host's defence effector mechanisms. Schistosomes do not appear to interfere with the defence mechanisms of their molluscan hosts. This can be inferred from co-infection studies using B. glabrata and two strains of S. mansoni which show that the compatible strain is able to develop in close proximity to the second (incompatible) strain being encapsulated (Kassim and Richards, 1979). However, it has been noted that there is a breakdown in innate resistance if the snail is heavily infected (Lie, 1982).

In contrast, trematode parasites belonging to other genera do appear able to interfere with the snail immune system. The co-infection of B. glabrata (strain 10-R2) with the echinostome E. paraensei and S. mansoni, allows the development of S. mansoni where it would normally be encapsulated (Lie, Jeong and Heyneman, 1981).

There is also evidence to suggest that the immune system of snails belonging to the genus Bulinus can be compromised when infected with amphistomes. The bulinid snail B. tropicus was not previously regarded to be a suitable host for S. bovis

but recent evidence has shown that if these snails are infected with amphistomes the schistosome can develop successfully (Southgate, Brown, Warlow, Knowles and Jones, 1989). This is of epidemiological importance since a breakdown in host/parasite specificity would aid the spread of schistosomiasis.

The strategy for survival adopted by schistosome larvae appears to be immune evasion, in that the snail should fail to recognise the parasite as foreign. Immune evasion may occur through the ability of schistosomes to synthesise and display antigens that mimic molluscan host molecules. This strategy requires the genetic make-up of the schistosomes to encode surface glycoproteins that specifically resemble snail antigens. To account for schistosome/snail specificity the parasite would have to 'tailor' its surface antigens to fit its selected intermediate host. Presenting the 'wrong' surface antigens would result in the parasite being recognised as foreign and the larvae being encapsulated. However, this seems unlikely as transplantation experiments have failed to show snails capable of xenogenic recognition. This has proved to be true for snails of the genera Biomphalaria (Tripp, 1961), Lymnaea (Sminia, Pietersma and Scheerboom, 1973) and Bulinus (Brisson, 1971). The transplantation experiments indicate that snails do not have species-specific self antigens. If the schistosomes were mimicking an antigen common to all the snails of a genus, then there would be no parasite/host specificity on a species or population level.

The mechanism of molecular mimicry would require that the major surface antigens of the four intramolluscan stages be identical. Alternatively they differ, with each stage mimicking a different snail antigen. Investigations into the major surface glycoproteins of S. mansoni larvae revealed that dramatic changes occurred when the miracidium transformed into the sporocyst (Yoshino, Cheng and Renwrantz, 1977). This led to the hypothesis that the transformation allowed the parasite to change its surface antigens and prompted the synthesis of mimicked antigens (Yoshino and Boswell, 1986). This was tested subsequently in experiments employing polyclonal antibodies raised in rabbits against snail haemolymph components. The antibodies were used as probes to determine whether miracidia or sporocysts shared antigens. The sporocysts were cultured in vitro free from snail components. The results showed a strong cross-reactivity with miracidia (Yoshino and Cheng, 1978; Yoshino and Bayne, 1983) and to a lesser extent with sporocysts (Yoshino and Bayne, 1983). Antibodies raised against S. mansoni miracidia were also found to cross-react with B. glabrata haemocytes and haemolymph components (in Yoshino and Boswell, 1986), as did those raised against sporocysts (Bayne and Stephens, 1983).

Similar cross-reactivity experiments were carried out on the avian schistosome Trichobilharzia ocellata and its molluscan host Lymnaea stagnalis. Anti-miracidial antibodies cross-reacted to haemolymph components, the reciprocal cross was also positive, anti-haemolymph antibodies bound to the miracidia but there was no cross-reaction to either mother or

daughter sporocysts (van der Knaap, Boots, Meuleman and Sminia, 1985).

There is little evidence to suggest that the shared antigens offer protection to the parasitic larvae. Yoshino and Bayne, (1983) found a greater degree of mimicked antigen sharing in compatible relationships than incompatible ones but a direct correlation could not be made as the antiserum was raised in different rabbits. The anti-sporocyst antibody used by Bayne and Stephens, (1983) bound equally well to haemocytes from both resistant and susceptible strains. Using the techniques of Western blotting, Bayne, Boswell and Yui (1987) found that the cross-reactivity of antigenic determinants was due to a widespread sharing of epitopes between parasite and snail. Since the antibodies are raised in rabbits, it is not surprising that the epitopes that prove to be the most immunogenic are those of common invertebrate origin. This is a reflection of the evolutionary distance between rabbits and both schistosomes and snails. An example of a common invertebrate glycoprotein proving immunogenic in vertebrates is a glycoprotein (gp38) which forms part of a 115 kD molecule secreted by adult worms of S. mansoni. Mammalian host antibodies to gp38 have been implicated in the immunity to superinfection observed in rats, as the epitope is also displayed on the surface of cercariae and early schistosomula (Capron, Dessaint, Capron, Ouma and Butterworth, 1987). Further studies using anti-gp38 antibodies revealed that the epitope is expressed on miracidia and eggs of S. mansoni, haemolymph components of B. glabrata and makes up part of the

keyhole limpet haemocyanin molecule (Dissous, Grzych, and Capron 1986). It is unlikely that such determinants represent an attempt by the parasite to avoid recognition by the molluscan host but represent a conserved molecular species of important, if unknown, function.

An alternative strategy for the evasion of the snail host response is the passive acquisition of host molecules. This strategy plays an important role in immune evasion in the mammalian host. The schistosome miracidia are non-feeding organisms that obtain energy through the metabolism of their endogenous glycogen reserves. Once within the snail host the miracidia transform into sporocysts and nutritional uptake is across the newly formed syncytial tegument. The mature tegument of both mother and daughter sporocyst is specialised for nutrient absorption and is covered in microvilli (Jourdane and Théron, 1987). S. mansoni sporocysts that have been transformed in vitro and susequently placed in snail haemolymph rapidly acquire snail host antigens (Bayne, Loker and Yui, 1986). The observation by Stein and Basch (1979) that an agglutinin, in the plasma of susceptible B. glabrata bound to the surface of miracidia and sporocysts led to the hypothesis that the larvae selectively bound such molecules to avoid detection (Stein and Basch, 1979). The interactions of haemagglutinins with schistosome larvae is discussed more fully in the section on haemagglutinins. Western blot analysis of the acquired antigens indicate that their uptake is non-selective (Bayne and Loker, 1987). The covering of the

sporocyst with any antigen of host origin would act as a mask by obscuring the parasite specific antigens.

Successful encapsulation reactions are initiated within 24h of the parasite entering the snail host, i.e. before the maturation of the tegument and, presumably, before the acquisition of any host antigens. This would explain the continued loss of resistance observed in B. glabrata infected with both S. mansoni and E. paraensei. E. paraensei larvae immunocompromise the snails and allow the development of S. mansoni in resistant snails. When irradiated echinostome larvae are used in these experiments, they are unable to transform and die early in the sporocyst stage. Non-irradiated S. mansoni larvae survive the death of the echinostomes and are not encapsulated. These data were interpreted as the ability of S. mansoni sporocysts to develop their own ability to interfere with the immune system (Lie, Jeong and Heyneman, 1980a). An alternative view is that the echinostome protected the S. mansoni larvae while they were vunerable, as miracidia and early sporocysts. By the time the echinostomes had died the mature S. mansoni sporocysts had acquired host antigens and were no longer at risk from immune attack. It follows that the intramolluscan stages most risk from the snails immune system are the miracidia, the early sporocysts and the cercariae. It is not known (nor was it even considered) how these stages escape the immune response in compatible snails.

The miracidia, sporocysts and cercariae of S. margrebowiei were studied in an attempt to characterise their major surface antigens and thereby look for equivalent or

stage-specific changes. A study of miracidial transformation was also undertaken to provide sporocysts free from snail components and without the use of complex media. Miracidia/sporocyst transformations have been achieved with other schistosome species, namely the avian schistosome, Trichobilharzia ocellata (Mellink and van den Bovenkamp, 1985); Schistosoma japonicum and S. mansoni (Voge and Seidel, 1972); S. mansoni, (Basch and DiConza, 1974; Samuelson, Quinn and Caulfield, 1984).

The transformation of T. ocellata and S. mansoni miracidia by Mellink and van den Bovenkamp, (1985) and Basch and DiConza, (1974) was accomplished using a complex solution of amino acids, salts, vitamins and sugars supplemented by serum. Mellink and van den Bovenkamp, (1985) used the basic medium of Basch and DiConza, (1974) and sought to improve the overall performance of the media by varying the constitiuents. They noted that cessation of swimming and loss of ciliated plates could be effected by many different salt solutions and complex synthetic media, with the change in osmolality (from <10 mOsm to 110 mOsm) appearing to be the crucial factor. However they concluded that faster and more generalised shedding required the presence of serum at an optimal concentration of 20% by volume. Earlier Voge and Seidel (1972) had concluded that the osmolality of the media was important and found that salt solutions of 200m Osm caused the immediate cessation of swimming but could not sustain plate shedding. The length of time the miracidia were incubated in the salt solutions was not given. They found that full transformation

required culture medium with added serum. Samuelson, Quinn and Caulfield, (1984) repeated these experiments using S. mansoni and found that plate shedding could be achieved in different media including PBSA, they also found that placing miracidia in amino acid or vitamin solutions did not effect transformation.

In studying the transformation of S. margrebowiei in vitro I found that transformation could be divided into two stages. The first stage is manifested by the cessation of ciliary beating and the sloughing of the ciliated plates. The second phase involves both the development of the surface tegument for its function in nutritional uptake and the pronounced elongation of the sporocyst.

The first stage can be triggered by an increase in the salt concentration from that found in freshwater to values approaching mammalian physiological saline. These results were similar to Samuelson, Quinn and Caulfield, (1984) in that S. margrebowiei miracidia lost their ciliated plates in PBSA but this was also achieved by the addition of NaCl to the water in which the eggs had hatched. Schistosome eggs will not hatch in 145 mM NaCl, but do so on contact with fresh water. Freshly hatched miracidia when returned to 145mM will transform into sporocysts. In vivo the miracidium enters the snail then migrates to the loose connective tissue in the head/foot (Kinoti, 1971). Here the miracidia would be bathed in haemolymph whose ionic concentration probably initiates ciliated plate shedding.

In vitro there is a time lag between the loss of plates and the expansion of the syncytial tegument to cover the sporocyst. This can be visualised by staining with fluorescent lectins. WGA and Con A stain only the intercellular ridges in early sporocysts but later stain the entire surface. In an ultrastructural examination of the transformation of T. ocellata in vivo, Meuleman, Lyaruu, Khan, Holzmann and Sminia (1978) observed that the intercellular ridges expanded under the ciliated plates so that the underlying muscle layer was not exposed. It is therefore possible that in in vitro transformation where the miracidia are in solution the transferring of culture vessels from incubator to microscope may produce mechanical jarring that dislodge the ciliated plates prematurely.

After the loss of the ciliated plates the new sporocysts do not survive unless they are given a nutrient medium. As mentioned earlier, complex growth media is usually used to achieve this end. However the reason for wanting to transform the miracidia without the involvement of snail components was to investigate the native surface of the sporocysts. It was felt that the sporocyst surface tegument would take up and display factors present in the medium, especially serum, in a similar manner to their acquisition of host plasma antigens. The sporocysts of S. mansoni have been shown to acquire these antigens within minutes of being transferred from culture medium to snail plasma (Bayne, Loker and Yui 1986). Later stage sporocysts were therefore prepared by incubation with basic salt solution (Chernin, 1963) without sugars but with

the addition of human haemoglobin, since adult schistosomes are known to utilise the haemoglobin moiety from human erythrocytes (Foster and Bogitsh, 1986). Although snail temperatures are usually in the range of 20-30°C, miracidia were transformed at 37°C with no detrimental affect. The increased temperature increased the rate of transformation.

Having achieved the transformation of miracidia without snail components and without sera, the surface of the intramolluscan forms were compared. The predominant surface carbohydrates and proteins were investigated. The results of these studies showed that the larvae had surface carbohydrate and protein molecules specific for their stage in the life cycle. Comparisons with reports on the surface carbohydrates of S. mansoni reveal that differences exist between the intramolluscan larvae of S. margrebowiei and S. mansoni.

Yoshino, Cheng and Rewrantz, (1977) studied the surface alterations with transformation of miracidia to sporocysts using a mixed agglutination assay. The larvae were fixed for 8h in glutaraldehyde, incubated with lectin for 1h, after washing the larvae were challenged with human erythrocytes known to be agglutinated by the lectin. The differences in protocol make direct comparisons difficult. This is complicated by the fact that, apart from Con A, different lectins were used. The lectins and their sugar specificities, together with a comparison of the results is given in Table 16. S. mansoni miracidia were found to have determinants for Con A, Dolichos biflorus (Db) and eel agglutinin. The Db determinants were at a lower concentration than Con A or eel

agglutinin. In contrast S. margrebowiei miracidia did not bind Con A, nor did they bind Asp pea (Asp pea has a similar binding specificy to eel agglutinin i.e. α-L-fucose). Db was not used with S. margrebowiei larvae. The two lectins that specifically bound to S. margrebowiei miracidia were ricin, that bound to the cilia and PNA that was localised to the basement membrane of the ciliated plates, neither lectin was tested with S. mansoni larvae. The adherence of erythrocytes in the mixed agglutination assay make it difficult to discern the exact location of the lectin determinants. Yoshino, Cheng and Rewrantz, treated the miracidia with pronase prior to re-testing with the lectins. This treatment caused the loss of Con A determinants but the eel aggluinin and Db determinants remained intact. They concluded that the pronase sensitive Con A determinants were glycoproteins and those for eel agglutinin and Db were glycolipids. The presence of glycolipids on the miracidial surface may account for the difficulty experienced in labelling the surface proteins of S. margrebowiei miracidia.

On transformation to sporocysts, the S. mansoni larvae lost the determinants for eel agglutinin but retained those for Con A and Db. It was found that the pattern of Db binding varied from being randomly distributed to predominantly at one end of the sporocyst. The extent of sporocyst tegument formation in these cultures is not known. S. margrebowiei sporocysts have determinants for both the lectins Con A and WGA. The binding patterns achieved with these lectins could be used to monitor the maturation of the sporocyst tegument. The

lectins bound to the intercellular ridges of the early sporocysts and later came to cover the entire surface. S. mansoni miracidia were transformed in vitro by incubation in culture media either with the addition of foetal calf serum or with glucose as a serum substitute. They were washed x 5 prior to fixation but if the tegument had by this time (18h) begun to take up nutrients components of the serum or glucose might be expressed as surface molecules. Although the methods and lectins differed in these two studies it does appear that the predominant sugar displayed on the surface of the miracidia differ in these two species of schistosome.

TABLE 16

COMPARISON OF THE MAJOR SURFACE CARBOHYDRATES EXPRESSED ON THE SURFACE OF *S. MANSONI* AND *S. MARGREBOWIEI* as ascertained by lectin binding

SCHISTOSOMA MANSONI

LECTIN	SUGAR SPECIFICITY	LARVAE
Db	α-D-gal-NAc	miracidia
Eel agglutinin	α-L-fucose	miracidia
Con A	α-D-mannose/ α-D-glucose	miracidia + sporocysts

SCHISTOSOMA MARGREBOWIEI

LECTIN	SUGAR SPECIFICITY	LARVAE
WGA	(D-glc NAc)2Glc NAc	sporocysts
Con A	α-D-mannose/ α-D-glucose	sporocysts
PNA	ß-D-gal(1-3)-D-galNAc	miracida (basement membrane)
Ricin	ß-D-gal	miracidia (cilia)
Asp pea	α-L-fucose	cercariae

Chiang and Caulfield (1988) examined the glycocalyx of both cercariae and miracidia of S. mansoni by staining with ruthenium red or ferrocyanide-reduced OsO_4. They found that the miracidial glycocalyx to be antigenically similar to that of the cercariae but was a much thinner layer. Although present on the miracidial plates and lost on transformation to sporocyst, its distribution was restricted to the base of the cilia and it was not found on the cilia themselves. The presence of a glycocalyx has not been demonstrated with S. margrebowiei miracidia or cercariae. Since PNA binds to the base of the cilia it is possible that it is binding to this glycocalyx.

Further differences in surface carbohydrates are apparent in the cercariae of these two species. Using lectins to stain the surface of S. mansoni cercariae, Linder (1985) found the major surface carbohydrates to be galactose and N-acetyl galactosamine. Both these moities were only found on the body of the cercariae with no lectin binding the tail (Linder, 1985). The only lectin to bind S. margrebowiei cercariae was Asp Pea that recognises L-fucose residues. These differences in lectin binding patterns may be species specific for schistosomes and could be exploited to provide a simple method of testing the species of the present the cercariae of different species of schistosome are distinguished by passaging through mice or hamsters in order to obtain adults or eggs. Alternatively the cercariae are treated with silver nitrate to stain the papillae and examined microscopically. The number and arrangement of the papillae are used as

differentiating characteristics between species (Short and Kuntz, 1976). The method is time consuming and requires considerable expertise to accomplish.

The novel use of biotin to label proteins still resident in the plasma membrane proved successful. It also demonstrated that the 3 larval stages of S. margrebowiei expressed surface proteins with differing electrophoretic mobilities. Further characterisation of the proteins is required in order to ascertain whether they are stage specific proteins or one protein with stage specific modifications.

2. Haemocytes

So far there has been little evidence for the existence of lytic factors, analogous to the haemolysins of insects, in the haemolymph of gastropods. Thus the immune response of gastropod molluscs to invasive pathogens is confined to the cellular reactions of phagocytosis and encapsulation. Investigations into the cellular aspects of the immune system of snails can be divided into three areas:-

(i) quantitative assessment of haemocyte number.

(ii) haemocyte function.

(iii) haemocyte sub-populations.

(i) Quantitative assessment of haemocyte number.

An estimate of haemocyte number in 'resting' snails is required in order to evaluate the impact of experimentally manipulated conditions. The data also serve as a standard to compare with other species of snail. Such estimates are difficult as the immune status of the snails, prior to

experimental manipulation is unknown. Snails are not kept in sterile conditions and the aquaria provide ideal habitats for ciliate protozoa and rotifers and may harbour potentially pathogenic bacteria. This may account for the wide variation in haemocyte number observed between individual bulinid snails. This variation was revealed by an experiment designed to assess the effect of schistosome invasion of B. natalensis and B. nasutus. The average haemocyte count for B. natalensis and B. nasutus was $1 \cdot 9 \times 10^5$ cells ml^{-1} and $6 \cdot 2 \times 10^4$ cell ml^{-1} respectively. These values are comparable to those found in other gastropod molluscs (Table 17).

TABLE 17

THE NUMBERS OF CIRCULATING HAEMOCYTES FOUND IN GASTROPOD MOLLUSCS

Mollusc	number of cells ml^1	Reference
Helix pomatia	$4 \cdot 9 \times 10^5$	Renwrantz, Schäncke, Harm, Erl, Liebsch & Gercken. (1981).
Helix pomatia	$3 \cdot 7 \times 10^5$	Renwrantz and Cheng (1977)
Helix pomatia	$2 \cdot 0 \times 10^5$	Bayne (1974)
Lymnaea stagnalis	$3 \cdot 6 \times 10^5$	Van der Knaap, Meuleman and Sminia (1987)
Lymnaea stagnalis	$8 \cdot 0 \times 10^5$	Van der Knaap, Tensen, Kroese and Boerrigter-Barendsen, (1982)
Lymnaea stagnalis	$1 \cdot 4 \times 10^6$	Dikkeboom, Van der Knaap, Meulman and Sminia (1984)
Biomphalaria glabrata	$1 \cdot 8 \times 10^5$	Stumpf and Gilbertson (1978)
Biomphalaria glabrata	$1 \cdot 7 \times 10^5$	Noda and Loker (1989).
Bulinus nasutus	$6 \cdot 2 \times 10^4$	This report
Bulinus natalensis	$1 \cdot 9 \times 10^5$	This report

When haemocyte counts were carried out on individual snails the results ranged over 2 orders of magnitude, from $3 \cdot 6 \times 10^4$ to 146×10^4 cells ml^{-1}. Such difficulties may be overcome

by using pooled haemolymph samples from many snails, differences in these average counts can then be assessed. Experiments of this kind were not carried out on bulinid snails due to the lack of experimental material. However, the results of such experiments have been reported in the literature. These have shown that the number of haemocytes present in the haemolymph at any one time is dependent on both immunological and environmental factors.

The injection of bacteria into snails causes a reduction in the number of circulating haemocytes (reviewed by Bayne, 1983). For L. stagnalis, the haemocyte number falls from $8 \cdot 0 \times 10^5$ cells ml^{-1} to $5 \cdot 0 \times 10^5$ cells ml^{-1} 1 hour post injection (van der Knaap, Tensen, Kroese and Boerrigter-Barendsen, 1982). On the other hand the number of circulating haemocytes tends to increase on subjection to schistosome larvae. Even in some compatible schistosome/snail combinations, the presence of parasite larvae can cause an elevation in the number of haemolymph haemocytes. A doubling of the haemocyte number has been recorded for B. glabrata 2h post exposure to S. mansoni miracidia (Stumpf and Gilbertson, 1978). Renwrantz and Cheng (1977) found that merely wounding snails can produce a sixfold increase in the number of circulating haemocytes within 4 h.

Temperature can also affect the number of circulating haemocytes, B. glabrata kept at 22°C had an average haemocyte count of $1 \cdot 75 \times 10^5$ cells ml^{-1} whereas the cell count for snails kept at 27°C was $3 \cdot 5 \times 10^5$ cells ml^{-1}. Temperatures higher than 27°C caused a decrease in cell number (Stumpf and Gilbertson, 1978). The snails were maintained at the

experimental temperature for 3 days prior to cell counts being taken but work with another gastropod mollusc illustrates how quickly these changes can occur. Transferring limpets (Patella vulgata) from aquaria at 5°C to 25°C caused a ninefold increase in the number of haemocytes in the haemolymph within 4h (Davies and Partridge, (1972).

What is apparent from these data is that the fluctuations in haemocyte numbers occur too rapidly to be accounted for by mitosis of the circulating haemocytes. This indicates that areas of the snail body, other than the haemolymph are used for the storage or accumulation of haemocytes.

This can also be demonstrated by calculating the number of haemocytes that would be required to mount an encapsulation response to schistosome larvae. B. nasutus are capable of withstanding infection by S. margrebowiei. If the total number of haemocytes was taken as the average for this snail i.e. $6 \cdot 2 \times 10^4$ cell ml^{-1} then it would follow that the total number of haemocytes in a snail with 50μl of haemolymph (an estimation based on the fact that only 15μl of haemolymph can be withdrawn from a snail using normal bleeding techniques) would be 3,100. This would only be sufficient to encapsulate two sporocysts based on the following calculation:-

Assuming that the sporocyst is an ellipse of revolution (about the major axis) with semi-minor axis a and semi-major axis b

Then from the electron micrograph

$$a = \tfrac{1}{2} \times 38\mu m = 19\mu m$$

$$b = \tfrac{1}{2} \times 100\mu m = 50\mu m$$

The surface area of the sporocyst

$$S = 4\pi ab$$

then $$S = 1{\cdot}19 \times 10^4 \ \mu m^2$$

Given that the surface area of a flattened, glass adherent haemocyte is $77\mu m^2$, then a capsule 10 layers thick would require 1,550 haemocytes.

It appears that activation of the immune system causes haemocytes to either be released into the haemolymph or sequestered from it. The cellular sites of haemocyte accumulations have been investigated by the following methods.

Bayne (1974) using radioactively labelled bacteria, followed the fate of haemocytes of B. glabrata after bacterial challenge. He found that the haemocytes accumulated in the digestive gland. This finding was confirmed by Renwrantz, Schäncke, Harm, Erl, Liebsch and Grecken (1981) for Helix pomatia. These authors also noted clusters of haemocytes in the foot muscle and attached to the lining of blood sinuses.

Histological examination of B. truncatus and B. africanus by Kinoti (1967) revealed the presence of haemocytes throughout the connective tissue and blood sinuses of these snails, with the highest concentration occurring in the connective tissue of the mantle. One particular area, adjacent to the heart in the posterior mantle Kinoti describes as lymphoid tissue, since it consists of clusters of haemocytes surrounding a blood sinus (Kinoti, 1967). This tissue appears to be equivalent to the amoebocyte (=haemocyte) producing organ of B. glabrata described by Lie, Heyneman and Jeong (1976) A similar structure has been also been identified in

Lymnaea truncatula by Rondelaud and Barthe (1982). Infection of the snails with trematode larvae causes an enlargement of this organ (Kinoti, 1967; Lie, Heyneman and Jeong, 1976; Rondelaud and Barthe, 1982). This organ may play a role in haemocyte storage as well as production.

The rapid increase in the number of circulating haemocytes seen after wounding, miracidial invasion, or temperature increase is presumably due to a release of haemocytes from storage areas into the haemolymph. The question arises as to whether the examination of haemocytes, taken from a sample of haemolymph from a snail whose immune response is not activated, can be assumed to represent a comprehensive population of cells. It has also been documented that cells other than circulating haemocytes are involved in the defence reactions of gastropods (Sminia and van der Knaap, 1986). These cells include antigen trapping cells that line the blood sinuses and are thought to bear carbohydrate receptors for foreign particles (Renwrantz, Schäncke, Harm, Erl, Liebsch and Grecken, 1981). Other cell types are known as fixed phagocytes, they are present throughout the connective tissue, and can be sub-divided into reticulum cells and pore cells (Sminia, van der Knaap and Kroese, (1979).

Snail haemocytes can be sub-divided on morphological grounds into two distinct categories: the plasmatocytes and the granulocytes. Plasmatocytes are large cells (> 15μm) that readily flatten on glass substrates. Granulocytes do not adhere well to glass and remain small and spherical (< 8μm). During counts of haemocytes of bulinid snails the numbers of

cells belonging to each category were noted. Generally, snails with haemocyte counts above the average had a higher proportion of granulocytes. These granulocytes were often seen in clumps of about thirty cells.

The majority of data cited in the literature do not distinguish the type of haemocyte responsible for raised haemocyte counts but changes in haemocyte number and type were monitored for B. glabrata infected with the echinostome E. paraensei (Noda and Loker, 1989). The parasite induced a slow and sustained rise in haemocyte number. The haemocytes responsible for this elevation in number were granulocytes (described as small round cells) and partially spread plasmatocytes (termed, granulocytes).

The raised haemocyte numbers in L. stagnalis caused by the presence of T. ocellata daughter sporocysts (prior to this stage there was no difference in haemocyte number between infected and control) was found to be due to an increase in the number of plasmatocytes, often in aggregates (van der Knaap, Meuleman and Sminia, 1987).

(ii) Haemocyte function.

The functional role of plasmatocytes and granulocytes is, as yet, undetermined. Dikkeboom, van der Knaap, Meuleman and Sminia (1984) and Sminia and Barendsen (1980) consider the granulocytes to be immature plasmatocytes. Granulocytes were distinguished from plasmatocytes by their small size, a non-lobulated nucleus with a high nucleus to cytoplasm ratio. Granulocytes were also found to have fewer cytoplasmic granules and were thought to be rich in RNA, since they

readily stained with pyronin. These characteristics were taken as indicative of a lack of differentiation and yet this description would also be applicable to functionally mature vertebrate lymphocytes. It appears that this deduction is based on the observation that the haemolymph of juvenile snails have a higher proportion of granulocytes than is found in the haemolymph of adults. Juvenile L. stagnalis are susceptible to infection by the trematode T. ocellata whereas adults are not. However both adults and juvenile snails readily eliminate bacteria from the circulation (Dikkeboom, van der Knaap, Meuleman and Sminia, 1985) and whole blood taken from juvenile snails is capable of the in vitro killing of S. mansoni sporocysts (Dikkeboom, Bayne, van der Knaap and Tijnagel, 1988).

The increase in number of granulocytes in B. glabrata, post infection with E. paraensei, is taken as an indication of a reduction in immunocompetence in these snails (Noda and Loker, 1989). Echinostomes have been shown capable of immunocompromising their hosts. B. glabrata, normally resistant to S. mansoni, can be rendered susceptible if co-infected with E. paraensei (Lie, Heyneman and Richards, 1979). The strain of B. glabrata used by Noda and Loker was a strain normally susceptible to S. mansoni infection and thus a direct comparison cannot be made. The elevation in granulocyte number could be due to the presence of developing parasites in the snail tissues, activating a wound response. This has been noted for other compatible snail/trematode combinations (Stumpf and Gilbertson, 1978) though the

morphology of the haemocyte responsible for the increase was not recorded.

My own observations on the functions of these two cell types indicate that the granulocytes are immunocompetent cells. Experiments on in vitro phagocytosis indicate that the granulocytes were the first cell type to bind both latex spheres and bacteria. It is difficult to assess, at the light microscope level, whether cells have internalised the particles or whether they are simply bound to the surface membrane. However surface binding is a pre-requisite to internalisation and the observed association of particles and granulocytes indicates their involvement in clearance mechanisms. The interactions between haemocytes and particles were carried out in suspension and subsequently transferred to glass coverslips for microscopy. This is in contrast to other in vitro phagocytosis experiments that have been reported in the literature. The procedure traditionally used is to prepare monolayers of haemocytes on glass coverslips. The haemolymph is pipetted onto coverslips, left for 15-25 mins to settle, then rinsed with saline. Monolayers are then overlaid with a suspension of particles and observed (Sminia, van der Knaap and Edelenbosch, 1979; Abdul-Salam and Michelson, 1980; Schoenberg and Cheng, 1982; Yoshino and Granath, 1985; Dikkeboom, van der Knaap, Meuleman and Sminia, 1985). Granulocytes do not attach well to glass substrates and readily lyse on contact with the air. The production of monolayers, therefore introduces a bias, favouring the glass adherent plasmatocytes

and the phagocytic role of the granulocytes has not been adequately assessed.

The haemocyte types involved in the process of encapsulation have received little attention. Ultrastructural observations of capsules produced in response to laboratory infections of L. stagnalis with T. ocellata sporocysts identified a sole morphological type of haemocyte, the plasmatocyte (van der Knaap and Meuleman, 1986). In a similar study of Lymnaea palustris challenged with Fasciola hepatica larvae the sporocysts were encapsulated by two morphologically distinct types of haemocyte, namely plasmatocytes and granulocytes (McReath, 1979). It was noted that the association of the granulocytes with the sporocyst preceeded that of the plasmatocytes. From this it is clear that the examination of mature capsules may give a false impression of cell-type involvement and that time course studies are essential for the evaluation of the encapsulation response. It is possible that the granulocytes have been overlooked in histological studies of mature capsules, either because they have lysed, or because of the difficulty in identifying cell types in sections following paraffin wax embedding.

Experimental studies on the dynamics of encapsulation are available only for certain insects. For example, the encapsulation reaction of the tobacco budworm, Heliothis virescens, has been investigated in some detail. Glass rods were implanted into the haemocoel, removed at various time intervals and prepared for SEM. Early capsules (15 min.) were found to be composed, almost entirely of granulocytes. Later

(1h) both plasmatocytes and granulocytes were observed (Davies and Vinson, 1987).

Cell-cell interactions in immune reactions are not unknown in invertebrates. The production of haemolysins by the defence cells of the sea star, Asterias rubens, requires the involvement of three distinct cell populations (Leclerc, Brillouet, Luquet and Binaghi, 1986). There is also evidence, from the crustacea, of a cell-type that can be triggered to degranulate and lyse, the semi-granular cells. Interactions with foreign molecules cause the degranulation of these cells and components that activate the pro-phenol oxidase cascade can trigger cell lysis (Smith and Söderhall, 1986).

If the molluscan encapsulation reaction followed the same pattern then the granulocytes would represent the first haemocyte type to associate with the target. Degranulation and/or lysis of the granulocytes could then provide a second signal to promote the attachment of plasmatocytes. This may provide an explanation for the 'benign' association of haemocytes observed in compatible parasite/snail combinations. The migration of miracidia through the host tissues and the degeneration of sloughed ciliated plates could initiate a non-specific wound healing response. Plasmatocytes would accumulate in the vicinity of the parasite and ingest the damaged tissues. This would be distinct from the specific encapsulation response requiring the involvement of granulocytes.

(iii) Haemocyte sub-populations

The plasmatocytes and granulocytes are easily distinguished as two morphologically distinct haemocyte populations but only after their attachment to glass substrates. Difficulties arise when attempting to categorise haemocytes on the basis of their morphology since this is dependent on the experimental techniques employed. During the in vitro phagocytosis experiments, occasionally small cells were seen with extremely long cytoplasmic processes (90+μm), which appeared to have 'trapped' the latex spheres. Whether these cells were plasmatocytes, granulocytes or a distinct sub-set was not known. In addition, the inoculation of bacteria into the foot of B. nasutus produced haemocytes with thick pseudopodia, a character not hitherto seen in cell preparations. The presence of 'funnel-like' pseudopodia on haemocytes has been reported by Renwrantz, Yoshino, Cheng and Auld (1980). The haemocytes were isolated from B. glabrata and Bulinus truncatus haemolymph and could be induced to form these pseudopodia by treatment with the lectin WGA and subsequent challenge with vertebrate erythrocytes. The erythrocytes were internalised through these pseudopodia.

Other problems arise when attempting to categorise the haemocyte populations, in that degenerating cells may give spurious results. The haemocytes of intermediate morphology reported by Dikkeboom, van der Knaap, Meuleman and Sminia (1984) were flattened cells, lacking filopodia but with marginal spikes. These cells were occasionally observed in haemocyte preparations from bulinid snails but since they

always stained with propidium iodide were no longer considered to be intact cells.

To overcome the difficulties outlined above it would be useful to have specific markers for haemocyte populations. Lectins have been widely used in this context and prove very useful in distinguishing between morphologically similar cell types. For example the lectin from Dolichos biflorus only binds human erythrocytes belonging to the A blood group and phytohaemagglutinin binds to T but not B-lymphocytes.

The results of lectin staining of bulinid haemocytes revealed that the populations of plasmatocytes and granulocytes could be further sub-divided by their cell surface markers. The lectin wheat germ agglutinin bound to the surface of about 10% of both the granulocytes and the plasmatocytes. Therefore there are at least 4 categories of haemocytes in these snails. The other lectins used in this study, either did not label any of the haemocytes (eg Asp pea, SBA), or stained them all (Con A).

Lectin staining of haemocytes have been assessed for three other snail species: L. stagnalis (Dikkeboom, Tijnagel and van der Knaap, 1988); B. glabrata (Yoshino, 1983); H. pomatia (Renwrantz and Cheng, 1977). The results of these studies, together with the results from the 2 species of Bulinus are given in table 18.

Con A was found to stain the haemocytes of all the species. L. stagnalis haemocytes have ligands for Asp pea and WGA that were absent from the haemocytes of B. glabrata and H. pomatia. The haemocytes from both B. glabrata and H. pomatia

TABLE 18

LECTIN STAINING OF SNAIL HAEMOCYTES

LECTIN	SUGAR SPECIFICITY	[a]L. stagnalis	[b]B. glabrata	[c]H. pomatia	[d]B. natalensis	[d]B. nasutus
Asp pea	α-L-fucose	+	-	-	-	-
SBA	α-D-gal NAc	-	-	-	-	-
PNA	ß-D-gal(1-3)-D-galNAc	-	-	0	-	-
Con A	α-D-glu/α-D-mann	+	+	+	+	+
WGA	[GlcNAc(ß-1-4)]2GlcNAc	+	-	-	+/-	+/-
Ricin	ß-D-gal	+/-	+	+	0	0

[a] Dikkeboom, Tijnagel and van der Knaap, 1988

[b] Yoshino, 1983

[c] Renwrantz and Cheng, 1977

[d] This report.

0 experiment not done

stained with ricin, whereas for L. stagnalis only 10% of the haemocytes from adult snails bound this lectin. There was a higher proportion of ricin positive haemocytes from juvenile snails (24%). However since the total number of haemocytes increases with age it is possible that the number of ricin positive haemocytes remains constant. The morphology of the ricin positive haemocytes was not given. Ricin staining of the bulinid haemocytes was attempted but the results were inconclusive. No staining was seen with B. natalensis haemocytes but with B. nasutus some 70% of the plasmatocytes were stained. However the staining was faint and not easily distinguishable from control specimens that had been incubated in the presence of ß-D-galactose. Further experiments need to be carried out, possibly using a double labelling method to enhance the fluorescent signal eg. biotinylated ricin and FITC-avidin.

Similar problems arise with the assessment of haemocyte populations using lectins as were present with the functional assessment of these cells. Snail haemocytes, in particular the granulocytes, proved to be fragile cells that would readily lyse on contact with the air. In addition the granulocytes did not adhere well to glass substrates. In the studies on L. stagnalis, B. glabrata and H. pomatia cited above the haemocytes were prepared as monolayers of cells, washed and fixed prior to staining. This procedure markedly reduces the number of granulocytes available for subsequent staining. Neither were any measures taken to ensure that the haemocyte surface membranes remained impermeable to the lectins used.

The methods employed for lectin staining of bulinid haemocytes were designed to minimise the problem of cell lysis and granulocyte attachment. Haemocytes were used 'live' in order that membrane integrity could be assessed by the exclusion of the dye propidium iodide. Such procedures are not altogether satisfactory because of the additional problems of receptor capping and internalisation. Lectins, being multivalent molecules, can bind to more than one surface receptor and cross-link them. In motile cells, like the snail haemocytes, the cross-linking of surface receptors induces an aggregation of the receptors at one pole of the cell. The receptors are subsequently internalised in a process known as 'receptor-mediated endocytosis'. The process is time and temperature dependent. The snail haemocytes were therefore labelled in suspension for 15 min at 4°C prior to being plated onto glass coverslips (at room temperature). This may be insufficient time for saturation of the surface receptors with lectins.

Future investigations into haemocyte sub-populations would be better served using the techniques of flow cytometry. This has the advantage over more traditional methods in that cells are both labelled and analysed in suspension, without the requirement of cell washing since background fluoresence can be readily filtered out. Two fluorescent dyes with different spectral properties can be used simultaneously eg propidium iodide and fluorescein so that live cells can be distinguished from dead ones. Altering the gating parameters would allow the haemocytes to be evaluated on size as well as

staining characteristics. Despite the fact that plasmatocytes in suspension would be spherical, they could still probably be differentiated from granulocytes on the basis of size. The results of WGA staining of B. natalensis haemocytes using the flow cytometer correlated well with those obtained with the alternative method. This indicates that flow cytometry would be suitable for use with snail haemocytes. It also confirms the presence of a subset of haemocytes that waterborne parasites that differ from the rest of the population in having surface receptors for this lectin.

Differences in cell surface receptors can denote differences in cellular function and/or ontogeny. Sminia (1981) and van der Knaap and Meuleman (1986) maintain that only one type of haemocyte exists in gastropod molluscs. They account for the differences in morphology and surface antigens as being a reflection of the different stages of cellular differentiation. It is possible that the WGA+ve granulocytes are immature WGA+ve plasmatocytes. This would still leave unexplained the lack of WGA receptors on 90% of the haemocytes. An alternative theory would be that the two WGA+ve subsets interact with one another, possibly via haemolymph agglutinins, to bring about a specific immune response. The discovery of a lectin that binds to a proportion of bulinid haemocytes can be used in future experiments designed to couple haemocyte function with cell staining characteristics.

3. Haemagglutinins

Snails differ in their susceptibility to schistosomiasis in a species specific manner but the basis of this resistance is not known. Two questions arise in connection with snail/parasite interactions: how do schistosomes in compatible interactions avoid being encapsulated and what signals trigger the encapsulation of the same parasite in resistant snails. The answers to both lie in the snail's ability to discriminate between self and nonself.

The encapsulation reaction is carried out by haemocytes and it is possible that these cells carry membrane bound receptors for foreign molecules. This would enable haemocytes to bind to the surface of the parasites. However, there is evidence from studies on B. glabrata that humoral factors also play a role in mediating the cellular defence response. Experiments show that the haemocytes of susceptible snails are intrinsically capable of encapsulation reactions. This is apparent in the bulinid snails as most snails are susceptible to one parasite but resistant to another. For the schistosome/snail combination, S. mansoni and B. glabrata the same is true, there being different strains of parasite and snail. In double infection experiments when B. glabrata snails are co-infected with 2 different strains of S. mansoni, the snail haemocytes encapsulate the larvae belonging to one strain of parasite whilst the other develops normally (Kassim and Richards, 1979). Haemocytes from susceptible snails are also capable of the in vitro encapsulation of S. mansoni sporocysts but only when incubated with haemolymph from

resistant snails (Bayne, Buckley and DeWan, 1980; Loker and Bayne, 1982). The lectin Con-A is able to substitute for the haemolymph factor. Pre-incubation of the sporocysts with Con-A induces the normally benign haemocytes to become cytotoxic (Boswell and Bayne, 1985). Plus the inoculation of resistant plasma into susceptible snails renders these snails resistant (Granath and Yoshino, 1984).

The identity of the haemolymph factors is not known. Examination of the haemolymph of planorbid snails shows that the most abundant protein, accounting for 97% of the total, is haemoglobin (Figueiredo, Gomez, Heneine, Santos and Hargreaves, 1973). A comparison of the plasma proteins of different strains of B. glabrata (Bayne, Boswell, Loker and Yui, 1985) and B. nasutus, as compared with B. natalensis show no strain specific differences in protein profiles. Present in the haemolymph and albumin glands of many species of snail are haemagglutinins. This ability to agglutinate vertebrate erythrocytes can be interpreted as a self protein binding to a nonself cell surface, and has led to much speculation on the nature and function of these molecules, but most of the evidence for an immune function is, as yet, circumstantial.

The importance of plasma for the uptake of foreign particles by defence cells in vitro and in vivo has been widely reported, as have the presence of haemagglutinins (reviewed by Coombe , Ey and Jenkin, 1984). However, there is the possibility that factors other than the haemagglutinins are responsible for this enhanced phagocytic uptake, e.g. proteolytic enzymes modifying the surface of cells and

exposing haemocyte binding sites (Coombes, Ey and Jenkin, 1984). Such an explanation does not account for all of the observations but has to be borne in mind when assessing the data. It also emphasises the importance of using purified agglutinins in experiments on the defence reactions of snails.

Indirect evidence of the importance of agglutinins is demonstrated by the ability of Con-A to substitute for haemolymph in enhancing the phagocytic uptake of yeast cells by the haemocytes of B. glabrata (Schoenberg and Cheng, 1982). The haemolymph of the snail Helix pomatia is capable of agglutinating various species of bacteria. One species Aeromonas formicans is not agglutinated and infection with this bacteria proves fatal to the snails (Bayne, 1982).

The use of purified agglutinin by Renwrantz and co-workers has established that agglutinins are responsible for opsonisation, at least in two instances. An agglutinin was isolated and purified from the haemolymph of the mussel, Mytilus edulis. In vitro experiments with mussel haemocytes demonstrated that yeast, coated with agglutinin caused an enhanced uptake of the yeast by the haemocytes (Renwrantz and Stahmer, 1983). A purified lectin was also used in in vivo experiments with H. pomatia. Snails were injected with human erythrocytes to absorb the haemagglutinin from the haemolymph. They were then given a second dose of erythrocytes and the rate of clearance of these cells was markedly reduced. This blockade was reversed if the challenge dose was previously incubated with a sub-agglutinating concentration of purified agglutinin (Harm and Renwrantz, 1980).

Agglutinin molecules, free in the body fluids or as membrane glycoproteins bind to carbohydrates. Carbohydrates are constituents of surface glycoproteins and glycolipids of self and nonself cells. Hence molecules that are able to bind to carbohydrates can function in self cell signalling and nonself recognition. Examples of self cell signalling by agglutinins can be found in organsims as evolutionary distant as the protozoa and the vertebrates. Mating in Chlamydomonas (a single celled algae) is mediated by species specific sexual agglutinins that induce cell contact and fusion (Snell, 1976). Cellular aggregation in sponges is facilitated by "aggregation factors" which are species-specific (Müller and Müller, 1980). The presence of lectins in the embryonic and adult tissues of vertebrates is thought to be related to a function in tissue organisation (Barondes, 1981).

The nonself recognition properties of agglutinins include the surface lectin molecules of pathogens (eg. viruses) that mediate the recognition and attachment to carbohydrates on the surface of their target cells (Gold and Balding, 1975), the beneficial identification of nitrogen fixing bacteria by agglutinins on plant root cells (Schmidt, 1978), plus the examples given above where purified agglutinins are able to enhance the activities of defence cells of molluscs.

Agglutinins have more than one binding site and their binding to specific cell surface receptors may result in the cross-linking of these receptors. Alternatively, if the receptors are on different cells, agglutinins link the cells together. The cross-linking of cell surface receptors causes

their re-distribution which, in turn, can act as a signal for cellular activation, cell division and receptor mediated endocytosis. This form of signalling is especially apparent in cells of the vertebrate immune system. Usually the triggering molecules are antigens, either directly or after opsonisation but similar results are obtained using heterologous lectins. Phytohaemagglutinin, from kidney beans, specifically cross-links the receptors on T-lymphocytes and induces mitosis. The lectin Con-A binds to polymorphonuclear cells causing the production of superoxides by triggering the respiratory burst (Morrell, 1984). Phagocytosis can be regarded as a form of receptor mediated endocytosis: if the lectin has also bound to an antigen then the lectin is able to act as an opsonin. This has been demonstrated by the enhanced phagocytic uptake of yeast cells by vertebrate macrophages when the yeast is coated with Con-A (Sharon, 1984).

In vertebrate immune systems, nonself recognition is the property of molecules that belong to the immunoglobulin superfamily, namely the MHC molecules and antibodies. The ability of lectins to trigger the components of the vertebrate immune system has led to the theory that invertebrate agglutinins are analogous to and possibly ancestral to the vertebrate antibodies.

Antibodies can act as inducible humoral opsonins. Invertebrate agglutinins include examples that are both inducible and non-inducible. Examples of inducible lectins include the Lumbricus terrestris agglutinin (Cooper, Stein and Wodjani, 1984) and a haemolymph agglutinin from the lepidopteran

Anticarsia gemmatalis (Pendland and Boucais, 1985). The agglutinins are present at low levels in the haemolymph but show an increase in titre when the organism is infected with microorganisms. The cellular and molecular interactions required to produce this increase in synthesis are not known. Antibodies are generally not detected in the blood of vertebrates until after exposure to antigen and their synthesis requires the complex interactions of cells and soluble mediators.

Lectins and agglutinins are defined as proteins or glycoproteins that bind to carbohydrate molecules. Antibodies are capable of binding to carbohydrates but T-cell dependent antibody production requires the presence of protein. The reason the protein component is important, in vertebrates, is due to the involvement of MHC class II molecules, which are essential for the presentation of antigen to T-helper (T_h) cells. The initial step in antibody formation is the phagocytic or pinocytic uptake of antigenic material by specialised cells, the antigen presenting cells (APCs). These cells which include the macrophages and follicular dendritic cells are found throughout the body but predominantly in the lymphoid organs. They show enhanced phagocytic uptake if the antigen is opsonised but otherwise phagocytosis is non-discriminatory and it is still not fully understood how self is distinguished from nonself. The antigen is then processed by being degraded intracellulary and short peptide sequences are complexed with the class II molecules of the MHC. The MHC molecule with its bound peptide is displayed on

the surface of the APC for recognition by T_h cells via the T-cell receptor (TCRαß). The APC-Th interaction is facilitated by other cell surface molecules, eg CD4 molecule on the T_h cell binds to MHC class II. The interaction activates the T_h cells to multiply, forming a clone of memory cells and activated T-cells. The activated T_h cells synthesise and secrete lymphokines which stimulate the B-cells into proliferation and antibody secretion.

The binding of peptide to MHC is also essential for the cytotoxic T-cell response. All nucleated somatic cells carry self determinants in the form of class I MHC molecules. Like the class II loci, the loci encode the class I antigens are highly polymorphic ensuring that the individuals in an outbred population have a distinct set of 'self' antigens. Viral infection of cells results in the expression of proteins of viral origin. The MHC class I molecules bind to these proteins and subsequently express them on the cell surface. The complex is recognised by T-cells of the Tc subset (cytotoxic T-cells). The binding of T-cell to classI + antigen results in the killing of the infected cell.

The MHC class I and II molecules belong to the immunoglobulin superfamily. Other members of this superfamily include the T cell receptor, immunoglobulins themselves and the surface molecules that aid cell-cell interactions, CD4 and CD8. This gene family arose out of gene duplication and modification from an ancestral gene with Thy-1, a surface marker of mice T-cells, a possible expression of this gene. To date there are three examples of invertebrate proteins

belonging to the Ig superfamily. A glycoprotein in squid axons (Williams, Tse and Gagnon, 1988); fasciclin II, a glycoprotein implicated in nerve growth cone guidance in grasshoppers (Harrelson and Goodman, 1988) and the product of the ama gene from Drosophila whose function is not known but is expressed on mesodermal and neural cells during early development (Seeger, Haffley and Kaufman, 1988). In B. glabrata, anti-Thy 1 antibody has been shown to cross-react with molecules on the cell surface of haemocytes (Yoshino, 1983). The immunoglobulin superfamily may have evolved because of the ability of these molecules to bind to peptides as well as sugars (either foreign or self), as opposed to the agglutinins and lectins that bind carbohydrates (either foreign or self).

The MHC involvement in the T_h dependent antibody response effectively restricts the production of antibodies to proteins or glycoproteins. Though a subset of T-cells is known to exist where the usual T-cell receptor, TCR$\alpha\beta$, is replaced by a different receptor, designated TCR δ. Strominger (1989), suggests that this receptor binds specifically to a sub-set of MHC molecules, class Ib that does have carbohydrate recognition functions. However, the immune response of vertebrates does have other strategies for dealing with pathogens of a predominantly carbohydrate nature. Investigations into these strategies may prove more fruitful in finding similarities with the invertebrate immune response.

Antibodies can be produced in a T-cell independent manner, carbohydrate molecules bind directly to the surface of B-cells and cross-link the surface receptors. Cross-linking of

the receptors activate the B-cells into antibody production, this is mainly an IgM response and has no memory component. Present in the serum of vertebrates are a number of proteins that make up the complement system. The system can be activated in one of two ways, by the classical or the alternative pathways. The result of activation of complement is the production of lysins and opsonins. Microbial and plant polysaccharides activate the complement system via the alternative pathway. C3b is a potent, short-lived opsonin, generated by the cleavage of the third component (C3) of complement. Macrophages and PMNs have receptors for C3b and the Fc portion of antibodies, these cells are responsible for the clearance of the opsonised particles. The alternative pathway of complement activation is thought to pre-date the classical pathway and therefore represent an ancient highly efficient opsonising system (Coombes, Ey and Jenkin, 1984).

Many microorganisms possess anti-phagocytic surface factors and cannot be phagocytosed unless opsonised. An example of one such microorganism is the capsulated gram-positive bacterium, Streptococcus pneumoniae, whose polysaccharide capsule serves to inhibit phagocytosis. The opsonins IgM, IgG and C3b are all produced to the capsule but in this instance do not act as opsonins. These bacteria are only opsonised by the binding of C-reactive protein (CRP) to the capsular surface. CRP is an agglutinin found in trace amounts in normal human sera. The site of synthesis of this protein are the hepatocytes of the liver and inflammatory stimuli cause a 1000 fold increase in its synthesis. CRP is a

powerful opsonin that is able to bind to the capsular surface of S. pneumoniae and similar bacteria causing them to be phagocytosed by PMNs (Drutz and Graybill, 1984). Investigations into CRP have revealed that macrophages also have receptors for this protein and it is able to activate the complement pathway by binding factor C1q (Mold, Du Clos, Nakayama, Edwards and Gewurz, 1982). Although CRP was first discovered through its binding to S. pneumoniae it binds to a number of other bacteria, including S. aureus and E. coli. CRP also binds snail galactans but detailed studies on the binding specificities have shown that despite the fact that it appears to bind to carbohydrates, especially D-galactose, the binding is actually directed towards phosphate groups that are minor constituents of these polysaccharides (Soelter and Ulhenbruck, 1986).

CRP is a member of a family of proteins known as pentraxins so called because they are made up of individual sub-units (each approximately 20-30 kDa) that combine to form pentameric discs, with occasionally two discs linked face to face (Maudsley, Baltz, Munn, Buttress, Herbert, Feinstein and Pepys, 1987) The Ca2+-dependent binding is either directed towards foreign particles (eg CRP) or towards self (eg serum amyloid protein). Pentraxins are well conserved through evolution and have been isolated from a wide variety of vertebrate species, differences occur between species with respect to glycosylation and number of sub-units (5 or 10) (Maudsley, Baltz, Munn, Buttress, Herbert, Feinstein and Pepys, 1987). Pentraxins have also been found in

invertebrates, the agglutinin limulin from the horseshoe crab, Limulus polyphemus, has significant sequence homology with human CRP, but it is a much larger molecule with a hexameric structure (Robey and Liu, 1981; Maudsley *et al.* 1987). Limulin binds to N-acetylated-D-hexosamines and has affinity for galactans but can be isolated by the same methods used for the isolation of CRP, ie. by exploiting its affinity for phosphorylcholine (Robey and Liu, 1981) the binding may, therefore, prove to be towards the phosphate groups only. Another agglutinin from invertebrates has proved to be a member of the pentraxin family, the tridacins, isolated from tridanid clams (Uhlenbruck, Solter and Janssen, 1983). It is possible that other invertebrate agglutinins will prove to have structural similarities with the vertebrate pentraxins. Structural analysis at either the gene or amino acid level is required to resolve this point, since the antigenic determinants may differ. Human SAP (serum amyloid protein) is a pentraxin that shows strong sequence homology with CRP and yet there is no antigenic cross-reactivity between these two proteins.

There are other humoral factors that are present in the vertebrate immune system, that are essential for the regulation of the immune response. These include the cytokines and lymphokines that are produced by one cell type and influence the behaviour of a second cell type. Such a reaction can be seen with T-cell help directed towards B-cells which is mediated through lymphokines. There is currently much interest in these factors and the information may prove valuable in

providing a greater understanding of immune responses in invertebrates.

Non-self recognition molecules in vertebrates and invertebrates have evolved from molecules that function in self recognition. In the vertebrates the evolution of the immunoglobulin domain has resulted in the potential production of 10^{12} different antibodies. Even if the haemolymph of invertebrates contains more agglutinins than is currently known it does not seem likely that their diversity will equal that of antibodies. However, as pointed out by Vasta and Marchalonis (1983), a single agglutinin is able to bind to a number of different carbohydrate molecules, albeit with different affinities. This may not provide the range of antigen recognition of the vertebrates but may be sufficient to deal with the commonly encountered pathogens. The binding of carbohydrate to agglutinin is dependent on the overall isomeric configuration of the molecule. It would follow that the 'better the fit' of foreign carbohydrate the more immunogenic it would be. It is possible that micro-organisms and parasites whose antigenic surface produced low affinity binding with agglutinin could still elicit an immune resonse.

The subsequent binding of agglutinin to haemocyte could occur in the following ways:-

i) The attachment of agglutinin to pathogen could induce a conformational change in the agglutinin molecule thereby exposing a binding site specific for haemocyte receptors.

ii) Cleavage of the agglutinin after binding to pathogen. The cleaved portion is then able to act as a second messenger for the activation of haemocytes.

It has also been postulated that agglutinins bind to foreign particles with high affinity and self cells with low affinity. Localised increases in agglutinin concentration would then serve to increase the binding to haemocytes. Renwrantz (1986) described how this could be achieved theoretically. Low affinity binding of agglutinin to haemocyte receptor would mean a high dissociation constant and the equilibrium of the equation would be shifted to the left.

receptors-agglutinin -----> receptors + agglutinin

According to the law of mass action, a correlation exists between the concentrations of the participating molecules. Increasing the concentration of one of the molecules will shift the equilibrium in the opposing direction.

[receptors-agglutinin] -----> [receptors] + [agglutinin]

Patching and capping of haemocyte receptors would further contribute to localised increases in receptor concentration (Renwrantz, 1986). Subsequent activation of haemocytes could occur through mechanisms similar to those seen in the vertebrate immune system, the agglutinin acting as a direct opsonin, prompting a form of receptor mediated endocytosis or the agglutinin cross-linking the haemocyte surface receptors (or complexed agglutinin binding to receptor) inducing the production of cytolytic or chemotactic factors eg. superoxides.

An argument against agglutinins playing a role in invertebrate immunity has been their apparent absence in many of the species investigated. However, negative results have to be viewed with caution because of the insensitivity of the assessing assay. Agglutinins are detected by their ability to clump together vertebrate red blood cells. The test is straightforward and reproducible but it is dependent on the carbohydrates displayed on the surface of the erythrocytes used. The haemolymph of Helix pomatia does not agglutinate human type A erythrocytes unless they have been enzyme treated. Enzyme treatment exposes a carbohydrate moiety previously obscured and the haemolymph of H. pomatia changes its status from being negative for agglutinins to being positive (Nielson, Koch and Drachmann, 1983). The undetected presence of agglutinins may account for the lack of strain/species specific differences observed in the polyacrylamide gels of the haemolymph proteins of closely related snails. The plasma proteins from different strains of B. glabrata have been compared using polyacrylamide gel electrophoresis. The strains differed in their resistance to infection by S. mansoni and in their ability to agglutinate vertebrate erythrocytes. No differences were observed in the protein profiles of the haemolymph from these snails (Bayne, Boswell, Loker and Yui, 1985). A similar result was obtained when the plasma proteins of B. natalensis and B. nasutus were compared. This indicates the possiblity of there being a family of agglutinins, all with similar molecular weights but differing in their binding specificities.

Agglutinins may also be present but a levels that cannot be detected in haemagglutination assays. Renwrantz, (1986) calculated that a sub-agglutinating concentration of H. pomatia albumin gland agglutinin (an agglutinin with high affinity for human blood group A receptor) would contain 7·5 x 10^{12} molecules per litre. He considers that this concentration would be sufficient to still be functional in an immune response. Snails have an open circulatory system and the albumin gland is in direct contact with the haemolymph. It is thought that lectins synthesised in the albumin gland are slowly released into the haemolymph (Renwrantz, 1986).

One of the original reasons for investigating the haemolymph and albumin glands of snails for the presence of haemagglutinins, was to assess their potential usefulness in taxonomy. The presence and type of haemagglutinin might prove to be a useful non-destructive chemical character in the difficult field of snail taxonomy. However it was found that, for many snails, the presence of haemagglutinins, although specific for populations of snails, did not follow the species boundaries as defined by morphological characteristics (Pemberton, 1974; Michelson and Dubois, 1977).

The haemolymph from nine species of Bulinus was tested for the presence of agglutinins against human erythrocytes. Haemagglutinins were found in five of these species. Previously it had been reported that a population of B. truncatus from Egypt, had an agglutinin for type A erythrocytes. Two other populations of B. truncatus, also from Egypt, failed to exhibit any agglutinating activity for human

erythrocytes (Pemberton, 1974; Michelson and Dubois, 1977). The B. truncatus used in this study was isolated from Malawi and had a low level agglutinin (1:2) for both blood types A and B. Three populations of B. globosus had also been tested for haemagglutinins, from Ghana, Nigeria and South Africa, only the snail colony that originated in S. Africa showed any agglutinating activity, at a low level (the cell type was not recorded) (Pemberton, 1974; Michelson and Dubois, 1977). The haemolymph of B. globosus snails from Malawi was tested for haemagglutinating activity and at a dilution of 1:2 was able to agglutinate human type B red blood cells. The haemagglutinins found in the haemolymph of the B. jousseaummei, B. nasutus and B. africanus snails have not been reported elsewhere. Only single snail colonies were used, it is therefore not known whether differences exist on a population level within these species.

There appears to be little correlation between presence of agglutinins for human erythrocytes of type A and B and schistosome compatibility. However if the agglutinin is playing a role in the defence of the snail against parasite invasion then the correlation would be with resistance and not compatibility. Snails of the genus Bulinus serve as intermediate hosts for schistosomes of the S. haematobium group. Within this broad delineation there exists levels of compatibility based on geographical strains of schistosome and local populations of snails. Following the hypothesis that all snails possess agglutinins then the binding affinities of the agglutinins would reflect the local pathogens. The biological

cost of haemagglutinin synthesis would mean that it would only be maintained if it conferred a selective advantage on the local population of snails. Correlations between the presence of agglutinins/haemagglutinins and snail populations may become apparent if 'resistance profiles' were constructed. This would require the challenging of single colonies of snails with schistosomes endemic to their area. Alternatively the development of an agglutinin/parasite binding assays may resolve this situation.

The isolation of different populations of B. glabrata, followed by genetic inbreeding has resulted in the production of a number of different strains of snail that differ in their susceptibility to S. mansoni. The haemolymph of these snails has been investigated for haemagglutinins and five have been isolated from different strains (Stanislawski, Renwrantz and Becker, 1976; Michelson and Dubois, 1977; Stein and Basch, 1979; Jeong, Sussman, Rosen, Lie and Heyneman, 1981; Boswell and Bayne, 1984). There appears to be no correlation between presence of agglutinin and resistance to schistosome infection. One haemagglutinin has been implicated in defence reactions against echinostome larvae. The agglutinin binds to glutaraldehyde fixed, trypsinised calf erythrocytes and was found in 9 different strains of B. glabrata both resistant and susceptible to S. mansoni. One strain (M-RLc) was found to have an increased expression of the agglutinin when infected with the echinostome E. lindoense. The rise in titre occurred at a time when there was an increase in the redial population of this trematode. Further experiments showed that a rise in

titre could be induced by exposing the snails to irradiated E. lindoense miracidia. The rise in haemagglutinin titre coincided with the destruction of challenge doses of the echinostome and was accompanied by an increase in haemocyte number (Jeong, Sussman, Rosen, Lie and Heyneman, 1981).

The haemagglutinin isolated and purified from PR-B snails (susceptible to S. mansoni infection) was specific for human blood group A erythrocytes. FITC labelled agglutinin was found to bind to the surface of miracidia, sporocysts and cercariae of S. mansoni but not to the surface of haemocytes. It was postulated that the haemagglutinin binding to larval schistosomes may mask them from attack by haemocytes (Stein and Basch, 1979). In experiments to discover whether plasma factors could protect sporocysts from encapsulation S. mansoni sporocysts were incubated with plasma from susceptible snails. They were subsequently used in in vitro encapsulation experiments with haemocytes from resistant snails, no protection was observed (Loker and Bayne, 1982). The agglutinin from the PR-B snails may be directed towards bacteria or other pathogens, with eg. fixed phagocytes operating as effector cells. The B. glabrata strains of snail have specifically bred to differ in their resistance to infection by S. mansoni, but the basis of the susceptibility is not known. Snails could, for example, have 'lost' haemocyte receptors for agglutinin binding.

The miracidia-immobilising substances reported by Lie, Jeong and Heyneman (1980) are possibly agglutinins. They are found in the haemolymph of B. glabrata snails infected with

echinostomes. The binding is either directed to miracidia of the infecting echinostome or, in some cases, to a different echinostome species. Miracidia-immobilising substances stop ciliary beating and cause the transformation of miracidia into sporocysts. However these changes occur when the ionic concentration of the solution is increased. The differences seen between species of echinostome and length of infection could be due to the more generalised effects of parasitism on the constituents of the haemolymph than on the specific induction of haemolymph factors that adhere to the miracidia.

As mentioned earlier, the function of agglutinin cannot be assessed unless purified agglutinin is used in experimental procedures. The high titre haemagglutinin of B. nasutus snails was investigated in order to characterise it and attempts were made to purify it. The haemagglutinin appeared to be independent of either age of the snail or its immune status. However it is difficult to ascertain the immune status of snails kept in non-sterile conditions. An increase in titre, post miracidial invasion, would have shown the agglutinin to be inducible and indicated its importance in defence reactions against S. margrebowiei parasites. Invertebrate agglutinins have been shown to be either inducible eg. Lumbricus terrestris (Cooper, Stein and Wodjani, 1984); or non-inducible eg. Helix pomatia. The H. pomatia agglutinin is a non-inducible lectin, produced by the digestive gland of this snail (in Klein, 1982). If the same were true of the haemagglutinin from B. nasutus it would account for the

observation that the presence and titre of the agglutinin remained constant whilst the haemocyte number fluctuated.

In order to understand the nature of the binding site of the haemagglutinin, it was tested against a panel of different vertebrate red blood cells and in the presence of inhibitory sugars. B. nasutus haemagglutinin had the ability to agglutinate erythrocytes from divergent sources, human, sheep, rat, rabbit and mouse. With the mice, only the outbred strain Tyllers Original Swiss and the CBA strain (k haplotype) were recognised by the haemagglutinin. The results of these experiments indicate that it is not the distinguishing features of the human blood group antigens or the mouse MHC antigen that binds the agglutinin. However it is possible that the haemolymph of B. nasutus contains more than one haemagglutinin that recognise different erythrocytes. Experiments were not carried out to test this.

In a different approach to binding site specification a panel of simple sugars was used in an attempt to inhibit the agglutination reaction. The sugars used failed to produce any marked inhibition of agglutinating activity. This could be due to a number of reasons. It is possible that the panel of sugars didn't include the 'correct' molecule. Alternatively the binding site may require the appropriate sugar to be presented in a complex configuration before inhibition of the binding site can be accomplished. Other invertebrates have haemagglutinins that are not inhibited by simple sugars, the oyster Crassostrea virginica (Cheng, Marchalonis and Vasta 1984), and the earthworm Lumbricus terrestris (Cooper, Stein

and Wodjani 1984). In the case of C. virginica this appears to be due to the complexity of the binding site. The agglutinin specifically binds sialic acid residues but only when presented in the form of complex glycoproteins such as bovine submaxillary mucin or fetuin. Free sialic acid does not inhibit agglutination (Cheng, Marchalonis and Vasta, 1984). The binding site of the plant lectin Wheatgerm Agglutinin (WGA) consists of 3 sub-sites able to bind a sequence of three ß-D(1-4)- linked N-acetyl-D-glucosamine units, the binding is not inhibited by N-acetyl-D-glucosamine monomers (Goldstein, Hammarstrom and Sundblad, 1975). Limulin from horseshoe crabs was also considered to require the presentation of complex sugars to inhibit binding (Robey and Liu, 1981). However, more recent work on the binding specificities of CRP, described earlier, raise the possibility of this agglutinin binding to phosphate groups that are minor constituents of complex polysaccharides (Soelter and Uhlenbruck, 1986).

The binding specificities of B. nasutus agglutinin require more detailed study including the testing with more complex glycosylated molecules and phosphorylcholine. Though it is doubtful that the agglutinin is a member of the pentraxin family as haemagglutination was not Ca2+ dependent. The low level of inhibition achieved with galactose could be further investigated by either attempting to inhibit agglutination using a complex carbohydrate with terminal galactose residues, or by treating erythrocytes with galactosidase to ascertain whether this treatment abolished heamagglutination. If more than one haemagglutinin was present

then inhibition would have been more marked if combinations of simple sugars were used. Alternatively the nature of the binding site could be assessed by inhibition with the Fab fragments of monoclonal antibodies directed against specific epitopes on the erythrocyte membrane.

The haemagglutinin of B. nasutus snails failed to agglutinate or immobilise S. margrebowiei larvae, however it did bind to the surface of the miracidia. This was demonstrated by the failure of B. nasutus plasma to agglutinate red blood cells after its incubation with the miracidia. Incubation with either sporocysts or cercariae did not alter the agglutinating activity of the plasma. The presence of the agglutinin on the surface of miracidia was also visualised by biotinylation. The predominance of glycolipids or the highly glycosylated nature of the ciliary membrane proteins, made the cilia resistant to biotinylation. The labelling of proteins using activated biotin was repeated after the miracidia had been incubated with B. nasutus haemolymph. On this occasion the cilia were labelled with biotin and gave a strong fluorescence image when stained with FITC-avidin.

If the agglutinin plays an opsonising role in the encapsulation reaction, then binding to the surface of the miracidium would ensure a rapid response to invasion by this parasite. Snails differ in the timing of the onset of an immune response. A snail with 'strong' resistance encapsulates parasites within three hours of tissue penetration (Bayne, 1983). This suggests that the encapsulation reaction, of

snails exhibiting strong resistance, is directed towards the miracidium and not the sporocyst. Ultrastructural examination of S. mattheei invasion of B. truncatus indicate that in this snail/schistosome combination it is the miracidia and not the sporocysts that are encapsulated (Kinoti, 1971). Further evidence of the miracidial stage being important in host response mechanisms has come from work carried out by Kechemir and Combes (1982). The snail Planorbarius metidjensis is resistant to infection by the schistosome S. haematobium. When sporocysts of this schistosome (taken from the susceptible host B. truncatus) are surgically implanted into the resistant snail they are not encapsulated and are able to develop into patent infections. The sporocyst stage of S. margrebowiei did not bind agglutinin. This suggests that once a miracidium loses its ciliated plates it will no longer be recognised. This hypothesis could be tested by transforming the miracidia in vitro then inoculating the newly transformed sporocysts into B. nasutus snails.

The in vitro experiments on the encapsulation of S. mansoni parasites with haemocytes from B. glabrata have been carried out using sporocysts. The interaction of haemocytes and miracidia has not been documented. It is of interest to note that the main carbohydrate groups on the surface of the miracidia of S. mansoni are L-fucose (as ascertained through eel agglutinin binding) and D-glucose/D-mannose (Con A binding). On transformation to sporocyst the L-fucose residues are lost but the Con A determinants remain (Yoshino, Cheng and Renwrantz, 1977). Con A was shown able to substitute for the

'resistance factor' present in the haemolymph of resistant snails thereby inducing haemocytes from susceptible snails to become cytotoxic (Boswell and Bayne, 1985). The resistance factor, which as yet has not been isolated, may prove to have similar binding specificities to Con A and therefore be reactive against the miracidium and the sporocyst. B. nasutus haemolymph was incubated with S. mansoni miracidia but with no reduction of agglutination titre post incubation. B. nasutus snails are known to be resistant to infection by S. mansoni. Whether this is due to an inability of the parasite to develop or due to a positive encapsulation reaction is not known. If there is an encapsulation reaction then it may be directed to the early sporocyst stage, which was not tested by haemagglutination assay, or there may be another agglutinin present in the snail haemolymph undetected by the assay system used.

The haemolymph, before and after absorption with S. margrebowiei miracidia was compared using polyacrylamide gel electrophoresis. The lane corresponding to the haemolymph before absorption had two bands corresponding to an apparent M_r of 75,000 and 80,000 that were absent from the lanes of post absorption haemolymph. These bands may correspond to the haemagglutinin but it is possible that other molecules from the haemolymph may also bind to the miracidial surface. It is also possible that apparent similarities on this one dimensional gel may obscure protein differences. This could be resolved if the haemolymph proteins were separated on the basis of size and isoelectric point (two-dimensional

electrophoresis). However an estimation of molecular weight was also made by comparing the miracidia with absorbed haemolymph against native miracidia. In this case the surface proteins of the miracidia and the miracidia plus agglutinin were biotinylated prior to their separation with gel electrophoresis. Visualisation of the proteins with labelled avidin revealed a single band difference corresponding to an apparent M_r of 61,000. Other, more subtle differences may have present and not detected. In a third approach, to approximate the size of the agglutinin molecule, native B. nasutus haemolymph was fractionated using FPLC. Here the agglutinating activity was found in those fractions that corresponded to a M_r 85,000 -90,000. In each of these methods the main problem was not knowing whether the haemagglutinin was, either binding to the surface of the miracidia alone or whether other proteins, undetected by haemagglutination tests, were also present. The same was true for the separation using FPLC. The fractions showing agglutinating activity were very close to, and overlapped with, the haemoglobin fractions. It is possible that the snail haemoglobin forms an association with the agglutinin which causes their co-migration and co-absorption.

The purification of B. nasutus haemagglutinin may be achieved by the discovery of an inhibitory carbohydrate, this would enable the separation of agglutinin from the haemolymph by affinity chromatography. Alternatively, the use of the FPLC could be extended by using the ion exchange and chromatofocusing facilities, manipulating the variables to separate the haemoglobin from the haemagglutinin.

A protective role for haemolymph agglutinins still lacks definitive proof but many of the observed phenomena can be explained if these molecules were to have an immune function. If the binding of agglutinin to the surface of the schistosome were to act as a trigger for haemocyte activation then the resistance/susceptibility to schistosomes could be due to the range and binding specificities of the agglutinins in each population of snails. The genes controlling the characteristics of the agglutinins would be polymorphic, which would account for the variation observed on the population level of snail resistance and the differences between individual snails.

The inbred strains of B. glabrata exhibit different susceptibility patterns to infection by S. mansoni: Type I, non-susceptible at any age; Type II, juvenile susceptible, adult resistant; Type III, susceptible at any age; Type IV, juvenile susceptible, adult variable (Richards, 1984). The Type IV snails became resistant at the onset of egg-laying and the resistance was lost when egg-laying ceased. Many, but not all, of these snails also formed 'atrial amoebocytic accumulations' during the egg-laying period (Richards and Minchella, 1987). The atrial amoebocytic accumulations, were first described by LoVerde, Gherson and Richards (1982) as a manifestation of reaction against self. Haemocytes would accumulate in the heart area and occasionally attack the heart tissue. The authors suggested that the inbreeding had resulted in a defect in the snail recognition system. The later observation that the presence of atrial amoebocytic

accumulations prevented S. mansoni infection led to the view that transient non-susceptibility could operate as a 'low cost' alternative to complete resistance. However, they point out that since only 1% of B. glabrata are infected in nature there would be a low level of selection pressure for this host strategy, especially if a high proportion of snails developed heart defects as a result (Richards and Minchella, 1987).

The different patterns of snail susceptibility may be the result of the agglutinins being under polymorphic gene control. Inbred strains of B. glabrata, resistant to infection would then be the result of the selection of a gene(s) encoding an agglutinin with binding specificities for S. mansoni miracidia and/or sporocysts (Type I). In susceptible snails (Type III) a different allele of the gene encodes an agglutinin with different binding sites that are unable to bind to the parasites. Type II and Type IV snails have haemolymph agglutinins that do not bind to parasites but albumin gland agglutinins that do. The presence of agglutinins in the albumin gland and egg masses of snails has been commonly noted. The function of these agglutinins is not known, though it is possible that they may serve to protect the snail embryos. Snails have an open circulation system and it has been suggested that albumin gland agglutinins are released into the haemolymph (Řewrantz, 1986). The amoebocytic accumulations in some Type IV snails may be the result of the agglutinin cross-reacting to carbohydrate epitopes on the surface of cardiac cells. The maintenance of resistance in type II snails after egg laying could be due to the

persistance of the agglutinin in the haemolymph or that the site of synthesis is not the albumin gland and metabolic changes occurring with maturation trigger the synthesis of S. mansoni specific agglutinins.

In the snail/schistosome combination of Lymnaea stagnalis and Trichobilharzia ocellata the juveniles are susceptible to infection whereas the adults are resistant. The haemocytes of snails in these two age groups differ in that juvenile snails have fewer haemocytes with a higher proportion of granulocytes (Dikkeboom, van der Knaap, Meuleman and Sminia, 1984). It was suggested that the differences in the haemocytes of adult and juvenile snails may account for the differences in susceptibility to this trematode (Dikkeboom, van der Knaap, Meuleman and Sminia, 1984). However, juvenile snails are capable of eliminating bacteria from the haemolymph at a similar rate to adult specimens (Dikkeboom, van der Knaap, Meuleman and Sminia, 1985) and juvenile L. stagnalis haemocytes are capable of encapsulating S. mansoni sporocysts (Dikkeboom, Bayne, van der Knaap and Tijnagel, 1988). It has been demonstrated that the plasma of adult, but not juvenile, L. stagnalis contain an opsonin which enhances the phagocytic uptake of rabbit erythrocytes in haemocytes from both adult and juveniles (Dikkeboom, van der Knaap, Meuleman and Sminia, 1985). The plasma of adult and juvenile L. stagnalis contains a haemagglutinin for sheep erythrocytes. The titre of this haemagglutinin is lower (1:80) in juvenile snails than it is in adults (1:160) (Dikkeboom, van der Knaap, Meuleman and Sminia, 1985). If this agglutinin bound to the surface of

pathogens with a similar affinity to that observed with sheep red blood cells, then a titre of 1:80 would be sufficient for it to have an opsonic role (see Renwrantz, 1986). Interactions of agglutinins/opsonins with trematode larvae have not been carried out but one hypothesis would be that the sheep erythrocyte haemagglutinin is capable of binding to S. mansoni, but not T. ocellata, larvae, whereas the opsonin present in the haemolymph of adult snails was able to bind to T. ocellata larvae and trigger their encapsulation.

If, as suggested by Vasta and Marchalonis (1983), the agglutinin is able to bind to more than one pathogen, then the snail immune system might, on occasions be seen to make an 'inappropriate' response. An example of this can be seen with the S. haematobium infection of B. truncatus. In this parasite/snail combination the schistosome develops with no activation of haemocytes until the cercariae begin to break free of the daughter sporocysts. The haemocytes are then activated and daughter sporocysts whose tegument has been ruptured, are occasionally encapsulated (Kinoti, 1967). The cercarial output may be reduced by such reactions but it has little overall benefit for the snail. The presence of an agglutinin specific for the cercarial stage of the parasite life cycle is therefore probably the result of its shared antigenic cross-reactivity to another invasive pathogen.

Molluscan agglutinins vary in their characteristics: they can either be constitutive or inducible; the binding can be dependent on divalent cations or not and the site of synthesis can vary. The L. stagnalis agglutinin described by van der

Knaap and Meuleman (1986) is synthesised by the haemocytes. H. pomatia agglutinin is synthesised by the digestive gland (Klein, 1982) and many agglutinins are produced by the albumin gland (eg. Michelson and Dubois, 1977). The haemolymph of snails may contain an example from each of the separate sources of synthesis and their functions may differ. Some agglutinins may be triggered by a change in the divalent cation concentration possibly as a consequence of wounding, with others acting independently of cation concentration, binding directly to the surface of pathogens. The binding site of the agglutinin molecule must be able to bind to the surface determinants of non-self cells but be prevented from binding to self cells. Extensive research on the agglutinins from the chelicerates has shown that all the species so far studied have lectins that bind sialic acid and this sugar is absent from the tissues of these animals (Vasta and Marchalonis, 1984). Alternatively, the agglutinins do bind to self cells but with a low affininty. Agglutinins that bound with high affinity to self determinants would have to be suppressed. Coombe, Ey and Jenkin (1984) suggest that phagocytosis of self cells with bound agglutinin may be prevented by a second signal, a 'don't kill' signal given by the self cell. The surface determinant responsible for the self signal must be a common epitope to account for the lack of allogeneic reactivity and limited xenogeneic reactivity to tissue implants exhibited by snails (reviewed by Bayne, 1982).

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ABBREVIATIONS

ADCC	Antibody dependent cell-mediated cytoxicity
APC	Antigen presenting cell
Asp Pea	Asparagus pea
BM(NH)	British Museum (Natural History)
BSA	Bovine serum albumin
CBSS	Chernin's balanced salt solution
Con A	Concanavalin A
CRP	C-reactive protein
Db	Dolichos biflorus
FCTW	Fish conditioned tap water
FPLC	Fast protein liquid chromatography
I-CAM	Intercellular adhesion molecule
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
N-CAM	Neural cell adhesion molecule
SDS	Sodium dodecyl sulphate
PBSA	Phosphate buffered saline
PI	Propidium Iodide
PNA	Peanut agglutinin
RBC	Red blood cell
SBA	Soybean agglutinin
PAGE	Polyacrylamide gel elctrophoresis
SEA	Soluble egg antigen
Th	T helper cell
Tc	Cytotoxic T cell