HAEMOLYMPH PROTEINS AND THE SNAIL IMMUNE RESPONSE

A thesis submitted for the degree of Doctor of Philosophy

in the University of London

by

ROBERT ADAM HARRIS

DEPARTMENT OF BIOLOGY

UNIVERSITY COLLEGE LONDON

1990

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Anterior end of Schistosoma bovis showing oral and ventral suckers.

For
Mum and Dad

ABSTRACT

In this dissertation, the presence of a haemagglutinin of vertebrate erythrocytes in the cell-free haemolymph of the freshwater snail *B. nasutus* 1214 has been confirmed. This agglutinating activity could be adsorbed out of the haemolymph by incubation with human erythrocytes or by miracidia, but not sporocysts or cercariae of *Schistosoma margrebowiei*. The agglutinating property is abrogated by heating the haemolymph to 50°C, and by prior digestion with pepsin or trypsin, indicating the proteinaceous nature of the agglutinin(s). Agglutinating activity could be inhibited by porcine mucin, but not by single monosaccharides or disaccharides, or by combinations of these carbohydrates.

In attempts to isolate the haemagglutinin factor(s), several conventional protein isolation procedures were employed but because of the intrinsic problems presented by the small volumes of haemolymph available these were unsatisfactory. However, success came with the adoption of a novel combination of affinity chromatography using concanavalin A-sepharose 4B followed by FPLC gel filtration using Superose 6 (Pharmacia). By this procedure the haemagglutinating property of the haemolymph was shown to reside in a single polypeptide. This haemagglutinin has an M_r of 135k (reduced) and is a glycoprotein.

Glycoproteins of similar M_r were isolated from other species of *Bulinus* using an identical purification protocol. Similarities in the polypeptide backbones of these proteins were demonstrated by Cleveland mapping. An antiserum raised in Balb-C mice to *B. truncatus* 1521 135k protein showed cross-reactivity within the genus *Bulinus*, being restricted to the **truncatus/tropicus** species complex.

Following infection with larval schistosomes, and upon mechanical wounding, alterations in the levels of plasma proteins were observed. The 135k proteins were not detectable in haemolymph during the early stages of infection. Staining of frozen sections of schistosome-infected *Bulinus natalensis* 272 with the 135k antiserum indicated that the 135k molecules had bound to the invading parasites. This is evidence to support the hypothesis that the 135k haemagglutinin acts as a recognition factor in the defence mechanisms of *Bulinus spp.*

Studies of snail haemocytes have revealed physiological and functional similarities between haemocyte populations of different snail species. The nature of the buffer utilised in *in vitro* haemocyte studies has been shown to be of importance, although (SSS) (developed specifically for haemocytes of *L. stagnalis*) has proved efficient for haemocytic populations of both aquatic and terrestrial species. Lectin and antibody markers have been utilised in attempts to define haemocytic subpopulations, and haemocytes from different species have been shown to possess identical surface determinants for these markers. The production of reactive oxygen intermediates (ROI's) by snail haemocytes was investigated, and shown to vary among the populations tested.

ACKNOWLEGEMENTS

I would like to express my gratitude to my supervisors T M Preston and V R Southgate, for their instruction and guidance throughout the course of this project. I acknowledge the SERC for funding my studentship.

I would like to acknowledge Cöen Adema with whom I collaborated with for the main haemocyte study, to Dr W P W van der Knapp for overseeing this collaboration, and to Elly van Deuketom-Mulder for technical assistance. I acknowledge the support of the British Council which funded my visit to Amsterdam.

Many thanks are due to the staff of the Experimental Taxonomy Unit, Natural History Museum, and in particular to Mike Anderson and Jill Lines for provision of parasite and snail material.

There are many persons in the Biology Department at UCL who have helped me with my endeavours in the laboratory, and I would particularly like to acknowledge Dr Benny Chain, Dr Durward Lawson and Julia Burne for their assistance.

In the preparation of the thesis, I would like to thank Helen Wilson for allowing me to be a general nuisance in her office, and to Professor Martin Raff and Dr Leon Nawrocki for reading through the various stages of my thesis.

I would like to thank my family and friends for their support and encouragement and for countless discussions with my "Mergers Mates" during the last three years.

Finally, I would like to acknowledge Dr Taff Jenkins of the Biology Department, Portsmouth Polytechnic, for introducing me to the world of Parasitology.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS.

BSA	bovine serum albumin
Con A	Concanavalin A
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid
FITC	fluoresceinisothiocyanate
FPLC	Fast Protein Liquid Chromatography
g	gravity
KCN	potassium cyanide
LDCL	Luminol Dependent Chemiluminescence
M_r	relative molecular mass
NBT	nitrobluetetrazolium
PBSA	phosphate-buffered saline
PNA	peanut agglutinin
RKB	red kidney bean agglutinin
SBA	soybean agglutinin
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
SSS	sterile snail saline
TBS	tris-buffered saline
Tris	Trizma base
WGA	wheatgerm agglutinin
w/v	weight/volume
v/v	volume/volume

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

Schistosomiasis is a parasitic disease of the tropical and neotropical regions of the world, where 600 million people live in endemic areas, and an estimated 200 million are currently infected. It is one of six parasitic diseases which led to the formation of the Special Programme for Research and Training in Tropical Diseases (TDR) by the World Health Organization (WHO), the United Nations Development Programme (UNDP) and the World Bank in 1975, in an attempt to control the parasites and treat their victims (Maurice, 1985).

The causative agents of the disease are schistosomes, a group of platyhelminth blood flukes of the sub-class Digenea, order Strigeatoiida. They are characteristically dioecious, a unique feature within the Digenea whose all other members are hermaphrodite. Eighteen species of schistosomes have been identified, five of which are important parasites of humans, namely *Schistosoma mansoni, S. japonicum, S. haematobium, S. intercalatum* and *S. mekongi* (Table 1). Other mammals which serve as hosts of *Schistosoma spp.*, include cattle and sheep for *S. bovis,* pigs for *S. incognitumn* and antelopes for *S. margrebowiei.* Interest in schistosome species with non-human definitive hosts has heightened in recent years in relation to the concept of heterologous immunity, according to which persons exposed to attempted infection by cercariae of non-human species can acquire immunity to other species primarily infecting humans (Taylor, Nelson and Smith, 1973). Also, the prevalence and incidence of schistosomiasis in cattle in Africa is probably even higher than in humans, and is of obvious economic and agricultural importance as many serve as domestic livestock.

The snail genus *Bulinus* (gastropod molluscs, sub-class Pulmonata, family Planorbidae) is more widespread in Africa than *Biomphalaria*, which is uncommon

TABLE 1 DETAILS OF *Schistosoma* SPECIES, THEIR SNAIL HOSTS, DISTRIBUTION AND THEIR DEFINITIVE MAMMALIAN HOSTS
(From Rollinson and Southgate, 1987)

SPECIES	SNAIL HOST GENUS	DISTRIBUTION	DEFINITIVE HOSTS
***Schistosoma haematobium* group**			
Schistosoma haematobium (Bilharz, 1852; Weinland, 1858)	*Bulinus*	Africa	Primates
Schistosoma intercalatum (Fisher, 1934)	*Bulinus*	Africa	Primates
Schistosoma matthei (Veglia & Le Roux, 1929)	*Bulinus*	Africa	Artiodactyla
Schistosoma bovis (Sonsino, 1876; Blanchard, 1895)	*Bulinus* *Planorbarius*	Africa	Artiodactyla
Schistosoma curassoni (Brumpt, 1931)	*Bulinus*	Africa	Artiodactyla
Schistosoma margrebowiei (Le Roux, 1933)	*Bulinus*	Africa	Artiodactyla
Schistosoma leiperi (Le Roux, 1955)	*Bulinus*	Africa	Artiodactyla
***S. mansoni* group**			
Schistosoma mansoni (Sambon, 1907)	*Biomphalaria*	S.America Caribbean Africa Madagascar	Primates Rodentia
Schistosoma rodhaini (Brumpt, 1931)	*Biomphalaria*	Africa	Rodentia Carnivora
Schistosoma edwardiense (Thurston, 1963)	*Biomphalaria*	Africa	Artiodactyla
Schistosoma hippopotomi (Thurston, 1963)	?	Africa	Artiodactyla
***S. indicum* group**			
Schistosoma indicum (Montgomery, 1906)	*Indoplanorbis*	India S.E.Asia Sri Lanka	Artiodactyla
Schistosoma spindale (Montgomery, 1906)	*Indoplanorbis*	India S.E.Asia Sri Lanka	Artiodactyla
Schistosoma nasale (Rao, 1933)	*Indoplanorbis*	India Sri Lanka	Artiodactyla
Schistosoma incognitum (Chandler, 1926)	*Lymnaea* *Radix*	India S.E.Asia	Rodentia Carnivora Artiodactyla
***S. japonicum* group**			
Schistosoma japonicum (Katsurada, 1904)	*Oncomelania*	China Japan Phillipines Taiwan Indonesia	Primates Rodentia Carnivora Perissodactyla Artiodactyla
Schistosoma mekongi (Voge, Bruckner & Bruce, 1978)	*Tricula*	S.E.Asia	Primates Carnivora
Schistosoma sinensium (Pao, 1959)	*Tricula*	S.E.Asia	Rodentia
Schistosoma malayensi	*Robertsiella*	Malaya	Primates

or not found in extensive areas in the east and west. Some species of *Bulinus* thrive in seasonally filled pools, a habitat not usually exploited by *Biomphalaria.* As the intermediate hosts of *S. haematobium* are species of *Bulinus*, this parasite is present in a greater area of Africa than *S. mansoni*, and urinary schistosomiasis is transmitted under a wide variety of conditions (Brown, 1980). About forty species of *Bulinus* have been described (Table 2). These species are sub-divided into four species groups by a classification based primarily on shell and mesocone morphology. Species members of the same species group are regarded as being more closely related than members of different species groups. Each of the four species groups are represented throughout much of Africa (Figures 1, 2 and 3), however, isolates of a particular species may be distributed over a great geographical distance (Figure 4), and thus interactions with *Schistosoma spp.* may differ for different isolates of the same species. depending on the geographical distributions of the parasite species.

The great success of these parasites may be attributed to certain aspects of their biology, such as their complex life cycles which allow for a great reproductive potential. The life cycle (Figure 5) is indirect, involving a definitive vertebrate host and a freshwater molluscan intermediate host. Paired adult worms live *in copula* in the hepatic portal vein, mesenteric veins or veins of the vesical plexus (depending on species) in the vertebrate host, producing eggs, which rather than the adults themselves, are the cause of the greatest pathological damage. This is due to eggs becoming trapped in tissues, particularly the liver, where cellular responses are mounted against substances produced by the developing miracidia, the first larval stage, within the egg. The resulting encapsulation response results in a granuloma, which becomes a localised point of necrosis. Clinical manifestations include splenomegaly, liver hypertrophy, haematuria and urticaria, the disease often proving

TABLE 2 CLASSIFICATION OF *Bulinus spp.* INTO SPECIES GROUPS
(after Brown, 1980)

B.forskalii group -	*B. bavayi*
	B. beccarii
	B. camerunensis
	B. canescens
	B. cernicus
	B. crystallinus
	B. forskalii
	B. scalaris
	B. senegalensis
B.reticulatus group -	*B. reticulatus*
	B. wrighti
B.africanus group -	*B. abyssinicus*
	B. africanus
	B. hightoni
	B. globosus
	B. jousseaumei
	B. nasutus
	B. obtusispira
	B. obtusus
	B. ugandae
	B. umbilicatus
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

FIG. 1 DISTRIBUTION OF AFRICANUS GROUP SPECIES

B. abyssinicus
B. africanus
B. hightoni
B. globosus
B. jousseaumei

B. nasutus
B. obtusispira
B. obtusus
B. ugandae
B. umbilicatus

FIG. 2 DISTRIBUTION OF FORSKALII GROUP SPECIES

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

FIG. 3 DISTRIBUTION OF TRUNCATUS/TROPICUS COMPLEX SPECIES

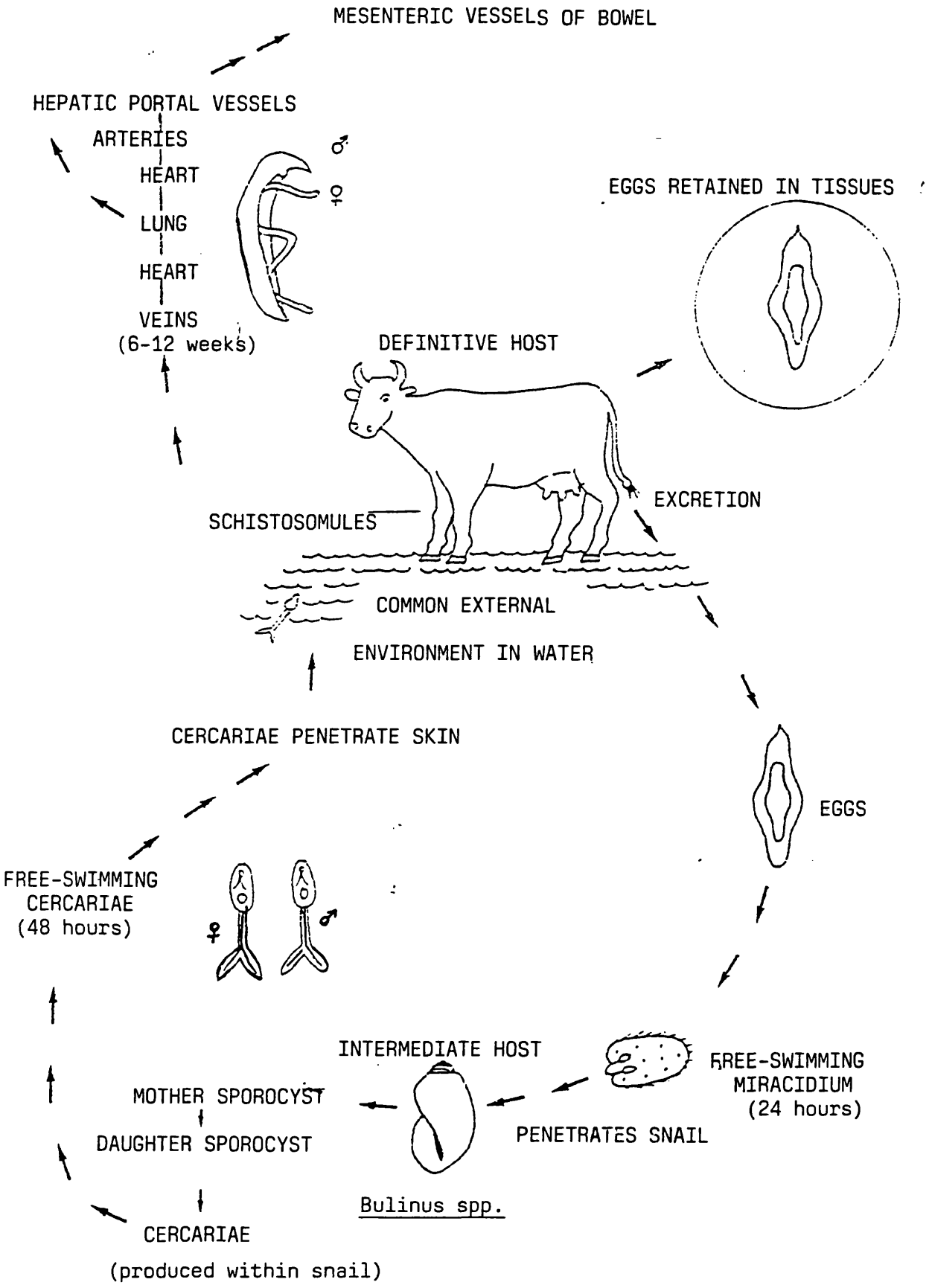
B. angolensis
B. coulboisi
B. guernei
B. hexaploidus
B. liratus
B. natalensis
B. nyassanus
B. octoploidus
B. permembranaceus
B. succinoides
B. transversalis
B. trigonis
B. tropicus
B. truncatus

FIGURE 4 DISTRIBUTION OF *B. umbilicatus* AND *B. nasutus*

B. umbilicatus
1. Mauritania; Kiffa
2. Mali; near Mopti
3. Niger; Gaya
4. Nigeria; Yau
5. Chad; Wadi Tourba
6. Sudan; Legarek district

B. nasutus
7. Uganda; Lango district
8. Kenya; Kitui
9. Tanzania; Shinyanga
10. Tanzania; Mbarali
11. Tanzania; Tunduma

FIGURE 5 LIFE CYCLE OF Schistsoma bovis



fatal if untreated. The release of eggs follows a circadian rhythm, and these are voided in either the faeces or urine, depending on species. If the fully embryonated eggs reach freshwater, the osmotic changes encountered induce the release of the activated miracidium, the first larval stage (Kusel, 1970). This is a free-swimming larva capable of rapid motion. A combination of phototactic, geotactic and chemotactic responses results in location of a snail intermediate host, these responses having been developed in direct relation to the behaviour of the host snails (Takahashi, Mori and Shigeta, 1961; Mason and Fripp, 1977; Chernin, 1970).

Miracidia possess a number of apical glands, the secretions of which aid penetration of the exposed snail tissues. If penetration is successful, the miracidium sheds its external ciliated plates and becomes a mother sporocyst, the second larval stage. These vermicular stages greatly increase parasite numbers by a form of asexual reproduction involving budding of germinal cells. The resultant daughter sporocysts also undergo a round of reproduction in the digestive gland of the snail, producing the cercariae, the final larval stage. These free-swimming larvae are released according to defined circadian rhythms when mature, again in response to environmental taxis. The cercariae locate and penetrate the skin of a vertebrate host, facilitated by a number of apical secretions. The propulsive tail is lost and the trilaminate outer tegument becomes heptalaminate, a characteristic of this immature adult stage, the schistosomulum (Hockley and McLaren, 1973). Schistosomula migrate via the lymphatic and venous systems to the lungs, where they begin to mature into schistosomes (Crabtree and Wilson, 1986). Migration to the hepatic portal vein is followed by a further migration once fully mature, to the mesenteric veins or veins of the vesical plexus, depending on species.

Water is the key element that links humans and snails in the cycle of

transmission. The heaviest human infections arise in those caught up in agriculture and domestic activities of washing, cooking, animal watering or water recreation. As the birthrate of the Third World increases so the demand for water intensifies, and so levels of infection are likely to increase in kind.

Strategies for the control of a disease such as schistosomiasis involve breaking the cycle of parasite transmission. To this end, a number of approaches have been adopted. Molluscicides have been utilised for a number of years in an attempt to reduce the size of snail populations. Ideally, such chemicals must be safe, non-toxic, stable in storage, snail-specific, simple to apply and cost-effective, hence niclosamide is used in preference to copper sulphate. Total snail eradication by application of molluscicides over large areas would require large quantities of chemicals, and those countries in most need are least able to afford their use. Consequently, molluscicide application is used only as part of larger control programmes.

Efficient environmental management of water resources has been effective in limiting the size and distribution of snail populations, thereby reducing levels of parasite transmission. Methods used in such schemes have included the introduction of sprinkler irrigation systems in place of open canals, the periodic removal of vegetation from canals and streams, direct manipulation of water levels and lining irrigation ducts with cement or rubber. Bad management and planning of agricultural irrigation schemes has led to the increased distribution of snails, and duly of the disease. This point has been illustrated in construction of the Aswan Dam in Egypt. The Nile is more than 2000 miles long but the irrigation ditches that now utilise the constant flow of water are fifteen times that length. Egypt's entire population, 45 million people, live along the Nile and half now have schistosomiasis.

The provision of fresh water supplies, better sanitation and laundry facilities has

been successful in reducing human contact with natural water sources, as shown in the St.Lucia project. However, over the extensive global areas that would be involved, widespread provision of fresh water is economically unsound. Health education is a cheap and effective control strategy, and has proved rewarding with the younger generations of at-risk populations.

A number of anti-schistosomal drugs have been developed and employed over the years (e.g. oxamniquine, metrifonate and praziquantel), for the treatment of infected individuals with adult flukes. These drugs have often proved to be species-specific, and both natural resistance and hybridization between species are inherent problems in such control programmes. Praziquantel is the only available chemotherapeutic agent active against all schistosome species, but is a relatively expensive treatment and not economically viable given the economic status of the affected populations.

Much current research centres on the immunology of the disease and the development of vaccines directed against the immature schistosomula and fully mature adults. As yet, no vaccine is commercially available. Adult flukes have developed effective strategies by which they are able to evade the vertebrate hosts' immune responses. Evasion is achieved during the transformation between the cercaria and the schistosomulum and involves the acquisition of host antigens during maturation (Clegg, Smithers and Terry, 1971; Bout, Capron, Dupas and Capron, 1974). This is a very effective survival strategy, and until the processes involved have been fully elucidated the development of a protective vaccine seems a distant prospect. However, given the level of our understanding of vertebrate immunology, it is likely that the mechanisms of host antigen acquisition will be determined, and this should serve to speed vaccine production.

Many aspects of the biology of schistosomiasis are in need of further elucidation. One such area encompasses the immunological relationships between the parasites and their intermediate snail hosts. Approximately 5-10 thousand species of digenetic trematodes are estimated to exist, the majority of which utilise gastropods as obligatory first intermediate hosts (van der Knaap, Loker & Meuleman, 1990). One hallmark of digenean-snail interactions is the relatively stringent degree of specificity they exhibit. This specificity has the important practical effect of limiting medically important trematodes, such as schistosomes, to geographic areas occupied by compatible snails. The functional basis of the phenomenon of resistance/susceptibility and compatability is not fully understood. As far as is known, there is no selection of compatible individual snails by a miracidium - presumably it is only when the schistosome encounters the internal defence system of its intermediate host that the phenotype of the snail with respect to susceptibility/non-susceptibility is revealed (Sullivan and Richards, 1981). The outcome of a snail-schistosome encounter is determined not only by the innate response capacity of the snail with respect to the miracidium, but also by the influences of other factors, including other parasites, on that innate capacity (Lie, 1982), as well as physiological constraints which an unsuitable host may provide.

The degree of compatability between schistosomes and snails is determined to a large extent by the interaction of the parasite's infectivity genes and the host's resistance genes. As a result, the outcome of miracidium-snail encounters in natural situations will vary, depending on the genetic constitution of the participants (Basch, 1976). Richards (1975a,1975b) presented the first evidence that infectivity is genetically-based, with at least one such factor being sex-linked. Further evidence is

illustrated by the extension of intermediate host ranges by hybrid schistosomes (Rollinson and Southgate, 1985). Natural hybridization has been observed between the human parasite *S. haematobium* and the cattle parasite *S. mattheei* in South Africa, involving the intermediate host *B. globosus*; between the human parasites *S. haematobium* and *S. intercalatum* in West Africa, involving the respective intermediate hosts *B. rohlfsi* and *B. forskalii*; and between the sheep parasite *S. curassoni* and the cattle parasite *S. bovis* in West Africa, involving the respective intermediate hosts *B. umbilicatus* and *B. guernei* (see Southgate and Rollinson, 1987). Richards (1984) noted four susceptibility patterns in his studies using the *S. mansoni/Biomphalaria glabrata* system - (1) non-susceptible at any age; (2) juvenile susceptible, adult non-susceptible; (3) susceptible at any stage; (4) juvenile susceptible, adult variable. Snail strains susceptible to one strain of schistosome may be resistant to others. Unfortunately, the precise nature of the genes and associated products responsible for these processes remains enigmatic.

In compatible hosts primary sporocysts lodge in the head-foot, tentacles or mantle collar, (Pan, 1965; Schutte, 1975). Secondary sporocysts migrate to the digestive gland, where they cause remarkably little damage to the digestive epithelium, but they extract nutrients from, and expel wastes into, the host's haemolymph (blood). The resultant effects on the host's metabolism, growth, reproduction and survival are intimately interrelated to host and parasite survival. Most damage is incurred by the snail host during the migration and release of the final larval stage, the cercaria (Pan, 1965; Schwanbek, Becker and Rupprecht, 1986).

The invasion of snail tissues by these larval stages may elicit defence responses by the host. The systems used by snails to combat invading organisms are preferably termed an "internal defence system" rather than an "immune system", as lymphocytes,

immunoglobulins and specific secondary responses to antigen are lacking (i.e. innate and not adaptive responses). Defence responses in mammalian systems have been well-characterised, but those of invertebrates are appreciably less well understood. The vertebrate defence system has received extensive attention, and many of its operative intrinsic processes have been elucidated. The system is divided into two functional divisions, innate defence mechanisms and the adaptive immune system. Innate responses act as a first line of defence against infectious agents and most potential pathogens are checked before they establish an overt infection. If these first defences are breached the adaptive immune system is employed. The adaptive system produces a specific antibody-mediated reaction to each infectious agent, which normally results in its eradication, and memory cells are produced so that a faster response can be enacted on subsequent challenge with the same antigens (i.e. reinfection). The innate and adaptive immune responses consist of a variety of cellular and humoral effectors distributed throughout the body (Table 3).

Recognition of foreign antigens is achieved by lymphocytes. These are subdivided into T and B subsets, the former being involved in cell-mediated immunity, and the latter in antibody production. A given lymphocyte will only recognise a single epitope, and thus highly specific responses can be elicited. Epitope recognition is achieved through specific T-cell receptor-major histocompatability complex interaction. Once activated, lymphocytes undergo a clonal expansion so that many copies of the activated cells are formed. Some of these clones are retained as memory clones, while others are immediately involved in cytotoxic (T cell) and antibody production (B cells) activities. Due to this immense replication and the destructive nature of the responses, immune responses must normally be subject to strict and specific controls. Such activation and regulatory processes involve a number

TABLE 3 .FUNCTIONAL DIVISIONS OF THE VERTEBRATE DEFENCE SYSTEM

	INNATE DEFENCE SYSTEM	ADAPTIVE IMMUNE SYSTEM
	Response not improved by repeated infection	Resistance improved by repeated infection
SOLUBLE FACTORS	lysozyme, complement, lymphokines pentraxins	antibody, cytokines
CELLS	phagocytes, natural killer cells	T & B lymphocytes
RESPONSES	phagocytosis, opsonization inflammation	opsonization, cytotoxicity hypersensitivity

of humoral factors, including lymphokines and antibody. These controls must be specific not only for the antigen but for the type of immune response elicited. This enables a choice to be made among such responses as T-cell mediated cytotoxicity and antibody responses of one or more of the several isotypes which are available.

It is apparent in this summary that vertebrate immune responses are varied, involve a multiplicity of cellular and humoral effectors, and that specific interactions are carefully regulated. Our understanding of the nature of these processes extends to the molecular level. By contrast, research into invertebrate defence systems is still in its infancy. The invertebrate defence system also comprises both cellular and humoral responses which co-operate, both in the process of self/non-self discrimination and mediating defence against non-self. Much research has been conducted using molluscs, and in particular, schistosome/snail interactions have been a main focus of study.

The molluscan defence system is able to deal with an enormous influx of microorganisms, as well as metazoan parasites such as schistosomes. The skin and shell body wall constitute the primary lines of defence to infection by other organisms. Thereafter the "haemocytes", a group of morphologically and functionally heterogenous blood cells, comprise the principal line of cellular defence (Malek and Cheng, 1974; Michelson, 1975). Evidence for the participation of humoral factors has also been shown (Yoshino, Cheng and Renwrantz, 1977; Stein and Basch, 1979; Bayne, 1980: Loker and Bayne, 1982).

In resistant snails, defence responses are mounted against invading miracidia, and even in compatible infections, extensive cellular responses may be seen against large numbers of miracidia, sporocysts or cercariae in a localised site (Kole, 1979; Pan, 1965; Schutte, 1975). The cellular responses involve phagocytosis, encapsulation

and nodule formation (LoVerde, Gherson and Richards, 1982). These processes are usually directed against non-self particulate soluble materials, although reaction to "self" can occur in many molluscs. Responses to "self" are usually due to damaged tissues, sequestered stimulatory molecules appearing on normal tissues, or defects in soluble recognition factors failing to discriminate self/non-self. These defence processes consist of numerous activities; each one must be triggered to start and be regulated thereafter.

Phagocytosis is a vital part of vertebrate defence mechanisms, and is even more important in invertebrates which lack lymphoid tissue and classic adaptive immunity (Cushing, 1967). Phagocytosis involves adhesion of blood cells to the "target" material and its subsequent ingestion. The populations of cells reported to be involved in phagocytosis varies within the gastropod molluscs; for example, granulocytes in the oyster *Crassostrea virginica* and macrophages in the mussel *Mytilus edulis.* In spite of the fact that numerous studies have been conducted on blood cells of gastropods, there is still no agreement on the question of how many cell types that can be distinguished (e.g. George and Ferguson, 1950; Cheney, 1971; Sminia, 1972; Cheng and Auld, 1977). The adoption of terms used in vertebrate haematology has created much confusion, so for the purposes of this thesis, I have used the following definitions. In molluscs three types of defence cell exist: antigen-trapping cells, fixed phagocytes (reticulum cells and pore cells) and highly phagocytic haemocytes (Sminia, 1981). The antigen trapping cells line blood vessels and blood spaces, and play an important role in the first phase of clearance of foreign particles. After trapping, the foreign particles are not engulfed by these cells, but are phagocytosed by circulating haemocytes. There are two types of fixed phagocytes, reticulum cells (Reade, 1968; Bayne, 1973; Sminia van der Knaap &

Kroese, 1979) and pore cells (Sminia & Boer, 1973), which lie closely apposed to one another. Reticulum cells display numerous cell processes closely associated with connective tissue fibrils, and ingest a wide range of injected foreign substances (Sminia, 1981; Sminia & Boer, 1973). To date, the precise functional differences between reticulum cells and circulating haemocytes are undetermined, but only the latter cell type posseses the enzyme peroxidase. In contrast to reticulum cells and haemocytes, pore cells have a selective endocytic capacity, apparently selecting on the nature of the particles which can be ingested (e.g. high affinity for proteins). Their capacity to digest the incorporated material is low, so that it is thought that pore cells are probably mainly involved in digestion and turnover of self-proteins.

The major phagocytic cell type in molluscs is the haemocyte. These cells move freely in the open circulatory system and are able to migrate into the loose connective tissue (and *vice versa*), thereby allowing a continuous surveillance within these compartments. The ability to react to chemotactic stimuli gives direction to haemocyte migration (Schmid, 1975; Howland & Cheng, 1982). Some investigators (Cheng, 1981; Fisher, 1986) consider that haemocytes appear to comprise a heterogeneous population of cells, while others are of the opinion that the range of haemocytes is much smaller, and that the differential morphology and spreading behaviour constitute different developmental stages of one haemocyte type (Sminia, van der Knaap & van Asselt, 1983; Sminia & van der Knaap, 1986; Bachere, Chagot & Grizel, 1988). Surprisingly, the location of haemopoesis is still debatable, but one opinion is that a "haemopoetic organ" exists and is part of the connective tissue in the heart-kidney region. In this region, stem-like haemopoetic cells have been reported for *Biomphalaria glabrata* (Lie, Heyneman & Yau, 1975; Jeong, Lie & Heyneman, 1983; Joky, Matricon-Gondran & Benex, 1985) and *Lymnaea truncatula*

(Rondeland & Barthe, 1981), but conclusive evidence is lacking as yet. Mitosis frequently occurs in these areas, especially in trematode-infected specimens (Lie, 1982; Sullivan, Cheng & Howland, 1986), but haemocytes in the circulation and in other tissue areas retain their capacity to divide. The number of circulating haemocytes varies among gastropod species, and also among specimens (Sminia, 1972; Renwrantz, 1979, Sminia, 1981), and is influenced by age (Dikkeboom, van der Knaap, Meuleman & Sminia, 1985), heart-rate, temperature and chemical stimuli (Abdul-Salam & Michelson, 1980b; Wolmarans & Yssel, 1988; Cheng, 1988). The number of circulating haemocytes also increases after wounding, blood extraction, and challenge with particulate matter, bacteria or trematodes (van der Knaap, Sminia, Kroese & Dikkeboom, 1981; Bayne, 1973; Cheng & Rifkin, 1970; Sminia, Pietersma & Scheerboom, 1973; van der Knaap, Meuleman & Sminia, 1987; Abdul-Salam & Michelson, 1980a). Whether these rapid, temporary fluctuations in haemocyte numbers are due to the formation or the migration of haemocytes from one compartment to another, has not been determined.

Haemocytes show functional heterogeneity. They are involved in phagocytosis and encapsulation (and killing), produce humoral defence factors and are also engaged in tissue repair and nutrition (Fisher, 1986; Sminia, 1981). Whether a single or a heterogenous population of haemocytes exists that is involved in these different functions has never been conclusively shown, despite the use of separation techniques (Bachere *et al*, 1988) or lectin and monoclonal antibody markers (Yoshino, 1981; Yoshino, 1983; Yoshino & Granath, 1983; Yoshino & Granath, 1985; Dikkeboom, van der Knaap, Maaskant & de Jonge, 1985; Dikkeboom, Tijnagel & van der Knaap, 1988). Activities of molecules at the surface of haemocytes are decisive in determining whether or not adherence and recognition will occur. Factors produced

by haemocytes and released into plasma are implicated in these processes. These soluble factors, enzymatic or non-enzymes, may kill targets before haemocyte engulfment, or following phagocytosis or encapsulation.

A variety of plasma factors, other than lytic/killing and recognition molecules, may support the cellular activities by operating independently or in conjunction with haemocytes. Indeed, for some molluscan species phagocytosis of a given particle may be possible only in the presence of plasma factors, e.g. uptake of formalized yeast and sheep red blood cells (SRBCs) by haemocytes of *Helix aspersa* (Prowse and Tait, 1969). In contrast, haemocytes of *Biomphalaria glabrata* phagocytose formalized SRBCs (Abdul-Salam & Michelson, 1980b), human erythrocytes (Schoenberg and Cheng, 1980a), live yeast cells (Schoenberg and Cheng, 1982) and heat-killed yeast (Fryer and Bayne, 1989) in the absence of plasma factors. Such factors may be involved in the chemotaxis of phagocytic cells, as demonstrated in the response of *Viviparus malleatus* to *Staphylococcus aureus* (Schmid, 1975), and *Crassostrea virginica* to live/dead bacteria.

The encapsulation response of molluscs is highly dynamic and active, and differs in response to different stimuli. Encapsulation is a necessary defence reaction to invasive organisms which are too large to be phagocytosed by haemocytes (e.g. schistosome larvae). The initial haemocyte response following infection probably involves directed movement towards the parasite larvae, although definite evidence for such a response is lacking. Once haemocytes are recruited into the vicinity of the sporocyst, contact has to be established with the parasite's tegument. In some host-parasite combinations, although haemocytes are found adjacent to the sporocyst, there is a failure to establish contact (Lie, Jeong & Heyneman, 1982), probably due to the surface of the sporocyst tegument. *In vitro*, haemocytes of *Biomphalaria*

glabrata bind to *S. mansoni* sporocysts, but not to *Echinostoma paraensi* sporocysts. Experimental modulation of the sporocyst tegument can also either reduce or enhance binding (Loker, Boston & Bayne, 1989). Once contact is established, haemocytes flatten and form a continuous layer by extending processes into all irregularities of the surface of the material being encapsulated. The capsule strengthens due to interdigitation of pseudopodia of adjacent haemocytes. Several layers of haemocytes contribute to the resultant capsule, which prevents further locomotion of larval schistosomes. Hydrolytic enzymes may then be released in destructive responses. In the most resistant molluscs, encapsulation and consequent destruction of invasive organisms occurs in the peripheral tissues (Lie, Hyeneman & Richards, 1977). In less resistant individuals, the encapsulation process may occur in the deeper tissues. The destructive response in highly-resistant gastropods is active in just 24 hours, and by 4 days post-infection, no trace of an invading parasite is seen.

The encapsulation response need not be destructive, however; instead, there may be a benign association of haemocytes with the parasite surface, e.g. the nematode *Angiostrongylus cantonensis* is encapsulated but not destroyed by haemocytes of *Biomphalaria glabrata* (Harris and Cheng, 1975). In these compatible infections fewer haemocytes are involved, but it must be noted that the benign haemocytes still retain the same cytotoxic potential as resistant ones, following pretreatment with Concanavalin A for instance (Boswell and Bayne, 1985). Benign haemocytes have been shown to aid the situations of certain parasites they encapsulate (e.g. haemocytes of *Lymnaea catascopicum* with *S. douthitti* and haemocytes of *Bulinus guernei* with *S. haematobium),* by moving their glycocalices away from contact with the parasite surface (Krupa, Lewis and del Vecchio, 1977).

Assuming that encapsulating haemocytes have been provoked to a cytotoxic state,

killing may then take place, relying on several effector mechanisms, including non-oxidative and oxidative processes. Humoral factors are produced by haemocytes and released into the haemolymph; apposition of target and haemocyte is not a prerequisite for the release of these factors, which include lysosomal enzymes, bacteriostatics and bacteriocidins. The activity of lysozyme in haemolymph is inducible, increased levels having been noted following injection of bacteria and infection with nematodes (Cheng & Rodrick, 1975; Cheng, Chorney & Yoshino, 1977a,1977b; Takashi, Mori & Nomura, 1986). A bacteriostatic factor which inhibits *Staphylococcus saprophyticus* multiplication has been observed in *L. stagnalis* plasma. Proteins which can not only inhibit bacterial growth but lyse mouse tumour cells, are secreted by *Aplysia juliana* (Kamiya, Muramoto, Goto, Sakai & Yamazaki, 1989). Molluscs are also capable of producing molecules that kill certain bacteria (Cushing, Evans & Evans, 1971; Mori, Murayama, Kanno, Nakamura, Ohira, Kato & Nomura, 1984), and viricidal activity has been demonstrated in *Eledone cirrosa* (Li, Prescott & Johnes, 1962).

Molluscan haemocytes also possess cytotoxicity mechanisms that are only active when haemocyte and target are apposed. Such an interface can be formed extracellularly in encapsulation reactions, or as in phagocytosis, initially on the outside of the haemocyte and then intracellularly. Lysosomal enzymes, including lysozyme (Cheng, Rodrick, Foley & Koehler, 1975; Cheng & Downs, 1988), acid and alkaline phosphatases, non-specific esterases, peroxidase (Sminia & Barendsen, 1980), aminopeptidase (Mohandas & Cheng, 1985; Cheng, Lie, Heyneman & Richards, 1978; Cheng, Howland, Moran & Sullivan, 1983), phospholipase C (Yoshino, 1988), beta-glucuronidase (Harris & Cheng, 1975), ATP-ase and alpha-naphthylacetate esterase (McKerrow, Jeong & Beckstead, 1985) may cause degradation of a target

either extracellularly when the enzymes are released into the cleft between target and encapsulating haemocyte (Dikkeboom, Bayne, van der Knaap & Tijnagel, 1988), or intracellularly when lysosomes fuse with phagosomes (Cheng & Cali, 1974). An oxidative killing mechanism also appears to be in operation. In mammalian leucocytes, contact with non-self leads to increased oxygen consumption due to NAD(P)H-oxidase activity in the plasma membrane. The enzyme transforms molecular oxygen to reactive oxygen intermediates (ROI's); superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (OH^-) are all produced, and are potent killing effectors. Cytotoxicity is exerted through enzyme inhibition, reaction with unsaturated lipids that damage the membrane and yield toxic unsaturated fatty acid aldehydes and alkyl radicals (Kappus, 1985), or genetic damage (Meneghini, 1988). Generation of superoxide was first shown in haemocytes of *L. stagnalis* (Dikkeboom, Tijnagel, Mulder & van der Knaap, 1987; Dikkeboom, Mulder, Tijnagel & Sminia, 1985), and has also been observed in haemocytes of *Biomphalaria glabrata* (Shozawa, 1986), *Helix aspersa, Planorbarius corneus* and *Pomacea canaliculata* (Shozawa, 1989; Dikkeboom, van der Knaap, van der Bovenkamp, Tijnagel & Bayne, 1988; Shozawa, Suto & Kumada, 1987). Haemocyte production of hydrogen peroxide was first shown in the bivalve *Patinopecten yessonensis* (Nakamura, Mori, Inooka & Nomura, 1985), and has also been shown for *L. stagnalis* and *P. canaliculata* (Shozawa, 1989; Dikkeboom *et al*, 1987; Dikkeboom *et al*, 1985; Showaza *et al*, 1987). When viewed with the electron microscope, portions of the tegument of sporocysts are closely surrounded by haemocyte processes and appear to be undergoing phagocytosis (Bayne, Buckley & DeWan, 1980a). Thus in addition to putative oxidative and non-oxidative cytotoxicity, a mechanical mode of killing may be operative. Pseudopodia of resistant haemocytes may directly destroy

the syncitial tegument, a structure needed for both nutrient aquisition and maintenence of osmotic balance (van der Knaap *et al*, 1990).

The haemocyte is, therefore the key element in the molluscan defence response. Following bacterial injection an associated initial drastic decrease in the level of circulating haemocytes occurs as phagocytic and encapsulative processes proceed (van der Knaap, *et al*, 1981(b); van der Knaap, *et al*, 1987). It takes only an hour for this highly efficient gastropod defence response to clear bacteria from the circulation. The ambient temperature, also, has been shown to influence the rate of bacterial clearance in the sea hare *Aplysia californica*, by influencing the total number of circulating haemocytes (Pauley, Krassner & Chapman, 1971). Starvation and pH may also both modulate the rate of bacterial clearance.

In gastropods, some recognition can occur in the absence of humoral factors, as non-self particles can be phagocytosed by some species in plasma-free conditions. The initial interaction between a haemocyte and a foreign object may be strongly influenced by physicochemical properties of the participants. However, a more fine-tuned discrimination between self, effete self and non-self would seem to require molecules specialised for recognition. Humoral factors are very important in recognition events. In response to bacterial or metazoan infections, levels of plasma lysozyme, alkaline phosphatase and aminopeptidase increase (*Biomphalaria glabrata* - Cheng *et al*, 1978; Cheng *et al*, 1977a, 1977b; Lie, Heyneman and Jeong, 1976; *Lymnaea stagnalis* - Sminia, 1972, 1980; *Patella vulgata* - Cooper-Willis, 1979). Levels of an anti-bacterial substance, a bactericidin, can be induced in abalones (Cushing *et al*, 1971), levels of bacterial agglutinins have been induced in oysters (Hardy, Fletcher and Olafsen, 1977) and opsonizing properties have been proposed by others (Renwrantz and Mohr, 1978; Sminia, Van der Knaap and Edlenbosch, 1979).

Parish (1977) and others postulated the role of humoral factors in foreign recognition in molluscs. A role in snail/schistosome interactions is suggested by showing that haemocytes from susceptible snails encapsulate sporocysts of *S. mansoni* in the presence of cell-free plasma of resistant snails (Bayne *et al*, 1980a, 1980b; Loker and Bayne, 1982). Conversely, susceptible snail plasma fails to protect sporocysts from attack by resistant haemocytes (Loker and Bayne, 1982).

Haemocytes free of plasma factors from resistant snails, encapsulate and sometimes damage *S.mansoni* sporocysts. However, haemocytes from susceptible snails attach to but do not damage sporocysts. Therefore, both haemocyte types have the ability to recognise the parasite as foreign. Cell-free plasma fractions alone,either from susceptible or resistant snail species, have no detectable effect on parasites (Bayne, 1980). The greatest destructive response occurs when haemolymph and haemocytes are both present. This evidence suggests that humoral factors may facilitate parasite destruction through surface interactions with haemocytes and the parasite.

There appears to be a definite role for plasma factors in snail/parasite interactions, but this is not necessarily as a recognition factor. In resistant snails, these factors may serve to inactivate sporocyst lytic factors, or in susceptible snails such factors may interfere with the haemocytes ability to encapsulate and destroy the parasite by masking recognition sites (Stein and Basch, 1979). Plasma host antigens have been shown to be present on sporocyst surfaces within 3 hours of infection, selected peptides being acquired (Bayne, Loker and Yui, 1986). Most plasma antigens are acquired selectively, shown by the antigen concentration taken up. An altered antigenicity due to the acquisition of snail-derived peptides may contribute to parasite defence evasion. While this process may be serving to mask recognition

sites, it might otherwise be serving to mark such sites. By the hypothetical action of an agglutinin or opsonizing lectin present in the plasma (i.e recognition role), resistant plasma may initiate a cell-mediated cytotoxic response against fixed sporocysts.

Agglutinins are multivalent substances that bind to the surfaces of cells and particles, causing their aggregation. They occur on viral and bacterial surfaces, in primitive and advanced plants as well as in both vertebrate and invertebrate defence systems (Gold and Balding, 1975). They are either membrane - bound or free in solution, and occur primarily as lectins (carbohydrate binding proteins). Not all agglutinins are lectins however. Lipids such as oleic acid, dioleolyl phosphatidic acid and gangliosides show haemagglutination activity (Stone, 1946; Tsivion and Sharon, 1981). Unlike lectins, lipoidal agglutinins are insensitive to temperature (Russel, Rodriques and Lai, 1983) and to saccharide inhibitors, but are more active against glutaraldehyde - fixed erythrocytes than fresh erythrocytes (Tsivion and Sharon, 1981). The aggregation of normal erythrocytes of different sources by an agglutinin is termed "panagglutination", and that of altered, defective or otherwise abnormal erythrocytes is "polyagglutination". With such a sensitive assay system, panagglutination can be caused by bacterial contamination of serum test samples, or by synthetic detergents used to clean laboratory equipment (Landsteiner, 1947; Jandl and Simmons, 1957; Renton and Hancock, 1957; Bird, 1971). Care therefore has to be taken when discussing the apparent presence and activity of agglutinating molecules.

The capacity of direct binding of specific invertebrate cell types to specific erythrocyte types may be mediated by agglutinins. Agglutinins have been identified in a number of invertebrate species. Camus (1899) and Noguchi (1903) first

recognised their presence in serum of the lobster *Homarus americanus.* Cantacuzene (1912, 1918) identified others in *H. gammarus, Eupagurus pridezuxii, E. pernhardus* and the spider crab *Maja squinado.* The specificity of serum agglutinins was investigated by Tyler and Metz (1943) in the spider lobster *Panulirus interruptus,* and shown to involve a protein. The presence of sugar-binding, thermolabile and pH-sensitive humoral agglutinins was first recorded in *H. americanus* by Cornick and Stewart (1973). Hall and Rowlands identified two lectins, showing heterogeneity within a species (1974a,b).

A list of species of invertebrate which are known to possess haemagglutinins in their body fluids is shown in Table 4. The list is very extensive, encompassing a large range of phyla and classes, but is certainly not complete. It is immediately apparent that the Arthropoda and the Chordata have been the most extensively studied phyla. This probably reflects the large volumes of body fluids which can be extracted (relatively easily) from sizeable specimens of these, as opposed to alternative phyla. While the range of invertebrate species showing haemagglutinating activity is extensive, the number of agglutinins which have been fully characterised is very restricted, as seen in Table 5. Gastropod molluscs, malocostracan crustaceans and insectan uniramians encompass all species for which characterization has been reported. Even within this list it is apparent that complete characterization is still lacking for haemagglutinins of certain species, given the few criteria utilised in the review.

The erythrocyte specificity is predominantly human, and in most cases this referred to human A_1 type. The occurrence of sheep, horse, rabbit and mouse specificities implicates different carbohydrate specificities as binding targets on the erythrocyte membranes for different haemagglutinins. All the tested agglutinins are

TABLE 4 LIST OF METAZOAN INVERTEBRATE SPECIES WITH RECORDED AGGLUTINATING ACTIVITY.

PHYLUM MOLLUSCA
- CLASS GASTROPODA
  - SUBCLASS PULMONATA *
  - SUPERORDER STYLOMMATOPHORA
    - *Achatina fulica* (Sarkar, Bachhawat & Mandal, 1984)
    - *Achatina achatina* unpublished observations
    - *Arianta arbustorum* (Renwrantz & Berliner, 1978)
    - *Otala lactea* (Boyd and Brown, 1965)
    - *Helix aspersa* (Hammarström, Westöö & Björk, 1972)
    - *Helix pomatia* (Uhlenbruck & Prokop, 1966)
    - *Limax flavus* (Miller & Cannon, 1984)
  - SUPERORDER BASOMMATOPHORA
    - *Lymnaea stagnalis* (vd.Knaap,Doderer,Boerrigter-Barendsen & Sminia,1982)
    - *Biomphalaria glabrata* (Boswell & Bayne, 1984)
    - *Bulinus nasutus* (this thesis)
  - SUBCLASS OPISTHOBRANCHIA
  - ORDER APLYSIACEA
    - *Aplysia californica* (Pauley, Granger & Krassner, 1972)
- CLASS BIVALVIA
  - ORDER VENEROIDA
    - *Mercenaria mercenaria* (Arimoto & Tripp, 1977)
    - *Tridacna maxima* (Baldo, Sawyer, Stick & Uhlenbruck, 1978)
    - *Saxidomus giganteus* (Johnson, 1964)
  - ORDER MYTILOIDA
    - *Mytilus edulis* (Renwrantz & Stahmer, 1983)
    - *Velesunio ambigus* (Jenkin & Rowley, 1970)
    - *Crassostrea virginica* (McDade & Tripp, 1967)
    - *Crassostrea gigas* (Hardy, Fletcher & Olafsen, 1977)
    - Pinctada maxima (Flower, Wilcox & Pass, 1985)
- CLASS CEPHALOPODA
  - ORDER OCTOPODA
    - *Octopus vulgaris* (Rögener, Renwrantz & Uhelenbruck, 1985)

*
- *Planorbarius corneus* (Ottaviani & Tarugi, 1986)
- *Cepaea hortensis* (Wagner, 1982)
- *Australorbis spp.* (Gilbertson & Etges, 1967)

A review of gastropod species is given by Pemberton,(1974)

PHYLUM **ANNELIDIA**
CLASS POLYCHAETA
FAMILY TEREBELLIDAE
Neoamphitrite figulus (Dales, 1982)
Amphitrite ornata (Garte & Russell 1976)
FAMILY SABELLIDAE
Spirographis spallanzanii (Parinello, Rindone & Canicatti, 1981)
Sabella magnifica (Brown, Almodovar, Bhatia & Boyd, 1968)
FAMILY GLYCERIDAE
Glycera dibranchiata (Anderson, 1980)
FAMILY NEREIDAE
Nereis virens (Russell, Rodriguez & Lai, 1983)
FAMILY ARENICOLAE
Arenicola marina (Dales, 1982)
FAMILY CHAETOPTERIDAE
Chaetopterus variopedatus (Tyler, 1946)
CLASS OLIGOCHAETA
ORDER LUMBRICULIDA
Lumbricus terrestris (Cooper, Lemmi & Moore, 1974)
ORDER HAPLOTAXIDA
Eisenia fetida andrei (Lassegues, Roch & Val embois, 1989)

PHYLUM **ARTHROPODA**
SUBPHYLUM CHELICERATA
CLASS ARACHNIDA
ORDER SCORPIONES
Centruroides sculpturatus (Vasta & Cohen, 1982)
Paruroctonus mesaensis (Vasta & Cohen, 1983)
Hadrurus arizonensis (Vasta & Cohen, 1984b)
Vaejovis spiningerus (Vasta & Cohen, 1984c)
ORDER UROPYGI
Mastigoproctus giganteus (Vasta & Cohen, 1984a)

SUBPHYLUM CRUSTACEA
CLASS CIRREPEDIA
ORDER THORACICA
Megabalanus volcano (Kamiya, Muramoto & Goto 1987)
CLASS MEROSTOMATA
SUBCLASS XIPHOSURA
Limulus polyphemus (Marchalonis & Edelman, 1968)
CLASS MALACOSTRACA
ORDER DECAPODA
Macrobrachium rosenbergii (Vasta, Warr & Marchalonis, 1983)
Homarus americanus (Camus, 1899)

Homarus gammarus (Cantacuzene, 1912)
Eupagurus prideziixii (Cantacuzene, 1918)
Eupagurus pernhardus (Cantacuzene, 1918)
Panulirus interruptus (Tyler & Metz, 1943)
Panulirus argus (Weinheimer, 1971)
Cherax destructor (Tyson, McKay & Jenkin, 1974)
Parachaeraps bicarinatus (Tyson, McKay & Jenkin, 1974)
Birgus latro (Cohen, Rozenberg & Massero, 1974)
Maja squinado (Cantacuzene, 1918)
Libnia dubia (Smith, 1984)
Cancer antennarius (Cohen & Vasta, 1982)
Cancer productus (Cohen & Vasta, 1982)
Cancer anthonyi (Cohen & Vasta, 1982)
Cancer irroratus (Cornick & Stewart, 1968)
Callinectes danne (Brown, Almodovar, Bhatia & Boyd, 1968)
Callinectes sapidus (Pauley, 1974)
Macropipus puper (Ghidalia, Lambin & Fine, 1975)
Menippe mercenaria (Smith, 1984)
Carcinoscorpius rotundacauda (Bishayee & Dorai, 1980)

ORDER STOMATOPODA
Squilla mantis (Amirante & Basso, 1984)
Tachypleus tridentatus (Shimizu, Ito & Niwa, 1977)

SUBPHYLUM UNIRAMIA
CLASS INSECTA
ORDER DICTYOPTERA
Leucophea maderea (Amirante & Mazzalai, 1978)
Periplaneta americana (Scott, 1972)
Extatosoma tiaratum (Rowley, Ratcliffe, Leonard, Richards & Renwrantz, 1986)
Locusta migratoria (Brehelin, 1972)

ORDER ORTHOPTERA
Telogryllus commodus (Hapner & Jermyn, 1981)
Acrididae (Jurenka, Manfried & Hapner, 1982)

ORDER HEMIPTERA
Rhodnius prolixus (Pereira, Andrado & Ribeiro, 1981)
Melanoplus sanguinipes (Stebbins & Hapner, 1985)
Melanoplus differentialis (Stebbins & Hapner, 1985)
Schistocerca gregaria (Lackie, 1981)

ORDER DIPTERA
Glossina spp. (Ibrahim, Ingram & Molyneux, 1984)
Calliphora erythrocephala (Peters, Kolb & Kolb-Bachofen, 1983)
Calliphora vomitoria (McKenzie, 1988)
Sarcophaga bullata (Stynen, Vansteenwegen & DeLoof, 1985)
Sarcophaga peregrina (Komano, Mizuno & Natori, 1980)
Drosophila melanogaster (Ceri, 1984)

ORDER LEPIDOPTERA
Pieris brassicae (Mauchamp, 1982)

Manduca sexta (Minnick, Rupp & Spence, 1986)
Spodoptera exigua (Pendland & Boucias, 1986)
Antheria pernyi (Qu, Steiner, Engstrom, Bennich & Boman, 1982)
Bombyx mori (Suzuki & Natori, 1983)

ORDER COLEOPTERA
Allomyrina dichotoma (Umetsu, Kosaka & Suzuki, 1984)
Leptinotarsa decemlineate (Peferoen, Stynen & DeLoof, 1982)

ORDER HYMENOPTERA
Apis mellifera (Gilliam & Jeter, 1970)

PHYLUM **ECHINODERMATA**
CLASS HOLOTHUROIDEA
ORDER DENDROCHIROTA
Pentacta pygmea (Smith, 1984)
ORDER ASPIDOCHIROTIDA
Holothuria polii (Miglietta, Canicatti & Cooper, 1989)
CLASS ECHINOIDEA
ORDER CAMARADONATA
Echinometra oblongata (Smith, 1981)
Lytechinus variegatus (Smith, 1984)

PHYLUM **CHORDATA**
SUBPHYLUM UROCHORDATA
CLASS ASCIDIACEA
ORDER APLOUSOBRANCHIATA
Didemnum candidum (Vasta, Warr & Marchalonis, 1982)
Didenum patulum (Ey & Jenkin, 1982)
Diplosoma (Ey & Jenkin, 1982)
Aplidium australiensis (Vasta, Warr & Marchalonis, 1982)
Amaroucium stellatum (Vasta, Warr & Marchalonis, 1982)
Sycozoa tenuicaulis (Ey & Jenkin, 1982)
Clavelina picta (Vasta, Warr & Marchalonis, 1982)
Atapozoa fantasiana (Ey & Jenkin, 1982)
Podoclavella cylindrica (Ey & Jenkin, 1982)

ORDER PHLEBOBRANCHIATA
Phallusia depressiuscula (Ey & Jenkin, 1982)
Phallusia mammillata (Cantacuzene, 1923)
Ascidia nigra (Brown, Almodavar, Bhatia & Boyd, 1968)
Ascidia malaca (Parrinello & Patricolo, 1975)
Ascidia thompsoni (Ey & Jenkin, 1982)
Ecteinascidia turbinata (Brown, Almodavar, Bhatia & Boyd, 1968)
Axinella polyploides (Bretting, Kalthoff & Fehr, 1978)
Ciona intestinalis (Tyler, 1946)

ORDER STOLIDOBRANCHIATA
Halocynthia pyriformis (Anderson & Good, 1975)
Halocynthia roretzi (Yokosawa, Sawada, Abe, Numakunai & Ishii, 1982)
Halocynthia hispida (Ey & Jenkin, 1982)

Halocynthia papillosa (Bretting & Renwrantz, 1973)
Halocynthia hilgendorfi (Fuke & Sagai, 1972)
Microcosmus nichollsi (Ey & Jenkin, 1982)
Microcosmus sulcatus (Bretting & Renwrantz, 1973)
Pyura praeputiculis (Ey & Jenkin, 1982)
Pyura irregularis (Ey & Jenkin, 1982)
Herdmonia momus (Ey & Jenkin, 1982)
Boltenia ovifera (Vasta, Warr & Marchalonis, 1982)
Botrylloides leachii (Schluter, Ey, Keough & Jenkin, 1981)
Botrylloides magnicoeccus (Ey & Jenkin, 1982)
Botryllus schlosserii (Ey & Jenkin, 1982)
Cnemidocarpa etheridgii (Ey & Jenkin, 1982)
Polycarpa obtecta (Ey & Jenkin, 1982)
Polycarpa pedunculata (Ey & Jenkin, 1982)
Polycarpa papillata (Ey & Jenkin, 1982)
Styela plicata (Vasta, Warr & Marchalonis, 1982)
Styela partita (Vasta, Warr & Marchalonis,1982)
Styela montereyensis (Vasta, Warr & Marchalonis,1982)
Styela barnhati (Tyler, 1946)
Stolonica australis (Ey & Jenkin, 1982)

REFERENCES FOR TABLE 5

Bulinus nasutus 1214	Harris, (this report)
Achatina fulica	Sarkar, Bachhawat & Mandal, 1984; Mitra, Sarkar & Allen, 1987
	Mitra & Sarkar, 1988
Helix aspersa	Uhlenbruck & Prokop, 1966
Helix pomatia	Hammarström, Westöö & Björk, 1972
Arianta arbostorum	Renwrantz & Berliner, 1978
Lymnaea stagnalis	van der Knaap, Boerrigter-Barendsen, van der Hoven & Sminia, 1981
Biomphalaria glabrata	Boswell & Bayne, 1984
	Jeong, Sussman, Rosen, Lie & Heyneman, 1981
Aplysia californica	Pauley, Granger & Krassner, 1971
Mercenaria mercenaria	Arimoto & Tripp, 1977
Tridacna maxima	Baldo, Sawyer, Stick & Uhlenbruck, 1978
Mytilus edulis	Renwrantz & Stahmer, 1983
Crassostrea virginica	Li & Flemming, 1967; Acton, Bennett, Evans & Schroenloher, 1969
Octopus vulgaris	Rogener, Renwrantz & Uhlenbruck, 1985
Carcinoscorpius cauda	Bishayee & Dorai, 1980
Tachypleus tridentatus	Shimizu , Ito & Niwa, 1977
Limulus polyphemus	Marchalonis & Edelman, 1968; Roche & Monsigny, 1974; Cohen, Roberts, Nordling & Uhlenbruck, 1972; Nowak & Barondes, 1975, Pistole, 1979
Cancer antennarius	Ravindranath, Higa, Cooper & Paulson, 1985
Homarus americanus	Hall & Rowlands, 1974a; 1974b; Abel, Campbell, Vanderwall & Hartman, 1984
	Hall & Rowlands, 1974a;1974b
Sarcophaga peregrina	Komano, Mizuno & Natori, 1980; 1981;Komano, Nozana, Mizuno & Natori,1983
Spodoptera exigua	Pendland & Boucias, 1986
Teleogrylus commodus	Hapner & Jermyn, 1981
Melanoplus sanguinipes	Stebbins & Hapner, 1985
Periplaneta americana	Scott, 1971; 1972; Ingram, East & Molyneux, 1983;1984; Lackie, 1981
Caliphora vomitoria	McKenzie, 1988

TABLE 5 LECTINS CHARACTERISED FROM INVERTEBRATES

SPECIES	ERYTHROCYTE SPECIFICITY	LOCATION	THERMOLABILITY	CATION DEPENDENCY	INHIBITOR	M_r
Bulinus nasutus 1214	HUMAN	HL	+	-	porcine mucin	210
Achatina fulica	RABBIT	HL	+	-	2-deoxy-D-gal	210
	UMBILICAL CORD	AG	+	ND	methyl β-D-gal	220
Helix aspersa	HUMAN	AG	ND	ND	GalNAc, GlcNAc	79
Helix pomatia	HUMAN	AG	ND	ND	GalNAc, GlcNAc	79
Arianta arbostorum	RABBIT	AG	+	-	α-1-6 galactose sugars	ND
Lymnaea stagnalis	HUMAN	HL	+	ND	galactogen	600
Biomphalaria glabrata [a]	RABBIT	HL	+	+	GalNAc	1000
[b]	HUMAN	AG	+	ND	ND	55
Aplysia californica	CHICKEN	HL	+	+	ND	>150
Mercenaria mercenaria	ND	HL	+	+	GalNAc, GluNAc	21*
Tridacna maxima	HUMAN	HL	ND	+	GalNAc	470
Mytilus edulis	HUMAN	HL	ND	+	bovine mucin	ND
Crassostrea virginica	SHEEP	HL	+	+	ND	20*
Octopus vulgaris	MOUSE	HL	+	+	lactose	260
Carcinoscorpius cauda	RABBIT	HL	ND	+	sialic acid	420
Tachypleus tridentatus	HORSE	HL	ND	ND	ND	250
Limulus polyphemus	HORSE	HL	+	+	GalNAc, GlcNAc	335
Cancer antennarius	MOUSE	HL	ND	ND	bovine mucin	70
Homarus americanus LAg_1	HUMAN	HL	+	+	GlcNAc	700
LAg_2	MOUSE	HL	+	+	GlcNAc	700
Sarcophaga peregrina	SHEEP	HL	+	ND	D-galactose	190
Spodoptera exigua	HUMAN	HL	+	+	galactosides	30.5*
Teleogrylus commodus	HUMAN	HL	+	+	GalNAc, GluNAc	1000
Melanoplus sanguinipes	HUMAN	HL	+	+	galactosides	500
Periplaneta americana	HUMAN	HL	+	+	glucose, sucrose	ND
Calliphora vomitoria	HUMAN	HL	+	+	galactose	130

KEY: HAEMOLYMPH HL ; ALBUMIN GLAND AG ; POSITIVE + ; NEGATIVE - ; SUBUNITS *

seen to be thermolabile, suggesting the proteinaceous nature of the agglutinating molecules. Dependencies on cations are observed in the arthropod species, but not in the molluscs (*Biomphalaria glabrata* is an exception, Boswell & Bayne, 1984 is an exception). Sugar specificity is predominantly galactose or galactose-derivatives, with GalNAc and GluNAc being common inhibitory agents. Of those for which the relative molecular masses has been determined, the majority are large molecules in their native states, in excess of 200k.

Agglutinins in most invertebrates are expressed at a constant level and this cannot be increased by exposure to antigen, in contrast to the level of expression of vertebrate immunoglobulins. There are exceptions, including the fleshfly larvae *Sarcophaga peregrina* where agglutinin levels are increased after wounding (Komano, Mizuno and Natori, 1980); exposure of the Pacific Oyster *Crassostrea gigas* to bacteria (Hardy *et al*, 1977); and immunization of the earthworm *Lumbricus terrestris* to erythrocytes (Cooper, Stein and Wodjani,1984).

The serum of a number of mollusc species have been shown to have agglutinating activity. Agglutinins may be synthesized and secreted by snail haemocytes and albumen glands (organs that secrete fluids which coat oocytes). Agglutinins are not uniformally present in the gastropod family Planorbidae, and variations also arise with respect to the selection of sample, either haemolymph, albumen glands or egg masses. The haemagglutinating activity in many snails appears predominantly in the albumen gland, and may be absent or weak in the haemolymph. For example, agglutinins have been recorded in the haemolymph of six out of seventeen populations of *Biomphalaria glabrata* and in the egg masses of all seventeen populations (Michelson and Dubois, 1977). The albumen gland of *Otala*

lactea contains a powerful agglutinin that is specific for human A blood group antigen (Boyd and Brown, 1965; Boyd, Brown and Boyd, 1966). *Helix pomatia* has a stronger agglutinin in its albumen gland than in its haemolymph (Renwrantz and Mohr, 1978).

An animal may produce more than one agglutinin. Two agglutinins have been reported from the Giant African snail *Achatina fulica.* The first is a glycoprotein "cold agglutinin" of native M_r 220k, and comprises of three non-covalently bound subunits of M_r 84k, 74k and 62k respectively and is produced in the albumen glands (Mitra, Sarkar and Allen, 1987). The second is a galactose - specific agglutinin present in the haemolymph. It has a native M_r of 210kD and comprises of non - covalently bound subunits of M_r 15k (Mitra and Sarkar, 1988). In *Biomphalaria glabrata,* a ten subunit, non - covalently bound glycoprotein of native M_r 100k has been found to show agglutinating activity (Boswell and Bayne, 1984).

Amoebocytes of *Lymnaea stagnalis* produce a 600k protein which functions in defence recognition - in plasma it agglutinates and opsonises, and on the amoebocyte surface it is a receptor for foreign-ess. "Type 1" snails have a very reactive and highly opsonic agglutinin compared to that in "Type 2" snails (van der Knaap, Sminia, Schutte and Borrigter-Barendsen, 1983).

Snail haemolymph agglutinin titres fall when bacteria are experimentally-injected, and return to normal levels after 4-8 hours (temperature-dependent). Plasma agglutinins may have a number of roles in the molluscan defence system. They may cause aggregation of smaller particles such as bacteria, thus facilitating a more efficient phagocytic response, or as opsonins they may stimulate chemotactic responses of haemocytes towards foreign objects (Tripp, 1966; Stuart, 1968; Prowse and Tait, 1969; MacKay and Jenkin, 1970). They might act as receptors for

foreign ess on the surface of haemocytes, and as well as these "protective roles" haemagglutinins are also postulated to be involved in transport processes, especially in aquatic invertebrates (Bayne, Moore, Carefoot and Thompson, 1979). Plasma enzymes may serve to alter the surface properties of bacteria so that their apparent foreign ess is enhanced, with apparent opsonic effect. Opsonins can only enhance phagocytosis as long as the phagocytic system is not saturated. Recognition influences and regulates the outcome of numerous biological processes. The prerequisite for recognition of any non-self molecule, be it cell-bound or soluble, is its attachment to the cell surface via a receptor. Lectins on cell surfaces and in body fluids play an important role in recognition, binding and subsequent effector activity.

Lectins are ubiquitous glycoproteins found throughout the plant and animal kingdoms, and function in a variety of biological recognition processes at all phylogenetic levels. They are characterized by their ability to interact with specific carbohydrates, different lectins binding to different glycosyl residues. Lectins are capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrates, but do not have an enzymatic or immunological nature. These recognition functions include their involvement in interactions with cells or extracellular materials from the same organism (self-recognition, endogenous functions) and interactions with foreign particles or cells (non-self recognition). The ability of membrane lectin molecules and body fluid agglutinins to recognise and interact with specific carbohydrates found on the surface of prokaryotic and eukaryotic cells suggests lectins may represent the archetypical molecules by which cells acquire the capacity to recognize many different effector molecules. As such, lectins may function as specific recognition molecules (receptors) in defence phenomena.

Lectins have been implicated as the invertebrate receptor(s) for non-self (Wright and Cooper, 1981; Vasta, Sullivan, Cheng, Marchalonis and Warr, 1982; Renwrantz and Stahmer, 1983) and appear to be involved in diverse mammalian responses. Target cell recognition by natural killer cells (Stutman, Dien, Wilsun and Lattune, 1980), macrophage phagocytosis (Weir and Ogmundsdottir, 1977), secondary IgG responses (Tomaska and Parish, 1981) and recognition in T/B lymphocyte responses (Splitter and Everlith, 1982; Pallidino, Ranges, Scheid and Oettgen, 1983) can all be inhibited by specific carbohydrates. Many invertebrate soluble glycoproteins in body fluids are also lectins and include the naturally - occurring agglutinins (MacKay and Jenkin, 1970; Arimoto and Tripp, 1977), lysins (Dales, 1982) and possible bacteriocidins (Chain and Anderson, 1983) produced by leucocytes (Amirante and Mazzalai, 1978; Van der Knaap, Boerrigter-Barendsen, Van der Hoeven and Sminia, 1981; Wright & Cooper, 1981; Stein and Cooper, 1982) or specialized organs (Teichberg, Silman, Beitsch and Resheff, 1975). Due to striking similarities, a superfamily of related recognition molecules have been proposed and assumed to have evolved from a common ancestral gene which produced a diverse class of molecules i.e. humoral lectins, C-reactive protein, immunoglobulins, major histocompatability products, β_2- microglobulin, T/B lymphocyte antigen membrane receptors, and differentiation antigens such as Thy 1 (Warr, Vasta, Ledford and Marchalonis, 1983).

The discovery of serum and cell surface lectins in molluscan haemolymph has permitted studies aimed at understanding the molecular basis of several phases of recognition and subsequent response of phagocytes to foreign materials. Following lectin treatment *in vitro,* the morphological behaviour of haemocytes of *Biomphalaria glabrata* was shown to alter (Schoenberg & Cheng, 1981), and haemocytes of both

Biomphalaria glabrata and *Bulinus truncatus* formed pseudopods (Schoenberg & Cheng, 1980b). Each of these responses is thought to facilitate endocytosis.

The presence of haemocyte associated lectins was shown initially by Tyson, McKay and Jenkin, (1974), who demonstrated that haemocytes from the crayfish *Cherax destructor* could recognise bacteria through a membrane molecule probably related to haemolymph agglutinins. Antibacterial lectins in *Limulus polyphemus* have been identified - "Limulin" (Cohen, Roberts, Nordling and Uhlenbruck, 1972) binds to 2-keto-3-deoxyoctonate (KDA), a key sugar in polysaccharide cores of many Gram-negative bacteria (Rostam-Abadi and Pistole, 1982), and "Polyphenin" binds to the N-acetyl-D-glucosamine moiety of teichoic acids from certain Gram-positive bacteria (Brandin and Pistole, 1983). Limulin acts as a recognition molecule for foreign particles; a second humoral factor actually enables killing responses, but limulin will not independently. Lectins in the albumen glands of certain molluscs were termed "protectins" as they were postulated to protect oocytes from bacterial infection (Prokop, Uhlenbruck and Kohler, 1968). The precise roles of lectins in invertebrate systems are still unclear, although it appears that they do have the capacity to act as cell surface recognition molecules or receptors (Marchalonis, 1980; Simpson & Smithers, 1980; Weir, 1980).

Given the efficacy of the molluscan internal defence system (Bayne, 1983; Meuleman, Bayne and van der Knaap, 1986), a most important component of a compatible relationship is an ability on the part of the miracidium and both the primary and the secondary sporocysts to evade the defence defences of the host (Yoshino and Bayne, 1986). Other components include the ability to obtain nourishment from the host haemolymph (blood), to migrate to correct locations in the host, and to propogate the appropriate next larval stage (Bayne and Loker,

1988). By penetrating young, probably relatively defenceless immature individuals, it has been proposed that schistosomes may avoid the need for protective responses against host defences in the early stages of infection (Dikkeboom, Van der Knaap, Meuleman and Sminia, 1984). Later, the persistent parasite may adopt chronically effective active or passive strategies (Dikkeboom *et al*, 1985). Just as snails differ in their degree of resistance and susceptibility, so do trematodes vary intra- and interspecifically in their ability to evade (Lie, 1982).

Two possible strategies by which digenean larvae evade host defence responses have received the most attention - (1) passive evasion of recognition by way of molecular disguise, (2) active selective interference with intended defence functions, either "directly" when an individual sporocyst is aggressive to host haemocytes in its immediate vicinity, or "indirectly" when the parasite interferes systematically with the defence competence of the host (Van der Knaap *et al*, 1990). It is possible that both these strategies are employed simultaneously, or sequentially, or that the reliance of a given strategy varies both between different parasite species and different developmental stages of a species.

Molecular disguise may be achieved either by molecular mimicry, in which the parasite's genome encodes host-like determinants that are expressed at the tegumental surface (Damian, 1987), or by molecular masking, in which host determinants other than molecules absorbed for nutritional purposes are adsorbed onto the parasite's surface, as occurs with the adult stages within the definitive host, where protective host antigens are aqcuired on penetration (Clegg *et al*, 1971; Bout *et al*, 1974). Evidence consistent with both these hypotheses has been reported, using polyclonal and monoclonal antibody probes and a range of intramolluscan stages (Yoshino & Boswell, 1986).

If one assumes a role for lectins in snail non-self recognition, carbohydrates would be prime candidates for disguise of surface determinants. Shared carbohydrate epitopes of snail tissue and parasite larvae have been shown using labelled plant lectins (Yoshino *et al*, 1977; Zelck & Becker, 1990). Pretreatment of sporocysts with exogenous lectins (thereby modulating the tegumental surface), has been shown to provoke killing by normally benign haemocytes *in vitro* (Boswell & Bayne, 1985). It is unclear whether this exogenous lectin modulation served to obscure normally disguising carbohydrate epitopes, or to mark sites for endogenous recognition markers (e.g. snail haemolymph lectins). The precise nature of such defence evasion strategies still requires elucidation.

The interference hypothesis states that "parasites survive in invertebrate hosts by interfering with the host's defence system" (Lie, 1982). Suppression of anti-parasite responses is well-documented - *Trichobilharzia brevis*- treated *Lymnaea rubiginosa* gave higher infections of *Echinostoma hystricosum* challenges (Van der Knaap *et al*, 1987); *E. paraensi*-infected *Biomphalaria glabrata* cannot encapsulate *S. mansoni* as usual, even though other foreign materials are not "protected" (Lie, Heyneman and Richards, 1977); *E. paraensi* elicits a higher degree of resistance to *E. lindolense* than to itself, implying that a "fixed specificity" is stimulated nonspecifically by either species (Lie *et al*, 1982). This defence subversion may be due to functional alterations of haemocytes by the parasites, in humoral defence factors related to their functioning, or both these. Haemocytes from snails with echinostome infections are less likely to kill trematode larvae in *in vitro* cytotoxicity assays (Loker, Bayne & Yui, 1986), which is evidence to support the hypothesis that the haemocytes are targets of interference. *In vivo* studies suggest that the suppressive effect is strong near the trematode larva and weaker at a distance, possibly implying that haemocyte activity

is subverted by substances produced by the larvae, which follow a concentration gradient of effectiveness (Lie, 1982). Haemocytes of a susceptible *Biomphalaria glabrata* strain show reduced phagocytic activity when incubated in whole haemolymph with *S. mansoni* sporocysts, while haemocytes from a resistant strain show increased phagocytosis under identical conditions (Fryer & Bayne, 1989). These observations also allow for depression of haemocyte activity in susceptible hosts by snails from parasite-exposed haemocytes, as well as direct effects of parasite on haemocytes. Figure 6 summarises these concepts. Dual infections of snails occurs naturally, but not all result in suppression of host defence responses. Indeed, in *Oncomelania formosana*, resistance to normally compatible strains of *S. japonicum* is seen after primary exposure to incompatible strains (Lin, Fang, Huang and Chen, 1974). Similarly, the defence system of *Biomphalaria glabrata* is aided by an infection of *Nucleria*, amoebae which kill sporocyst stages of *S. mansoni* without killing the snails themselves, although their fecundity may be altered (Stibbs, Owczarzak, Bayne and DeWan, 1979).

Protective mechanisms of schistosomes also include their ability to heal wounds (Popiel, Irving and Basch, 1985). In the dynamic interplay between host and parasite this may be important in determining the fate of individual schistosomes which suffer minor damage at any stage of the life cycle.

Immunological memory has been defined as the alteration of the immune response resulting from previous exposure to an antigen (Bayne, 1980). It is well demonstrated in the vertebrate immune system by the proliferation of B lymphocyte "memory cells", which enable a rapid clonal expansion of antibody - producing lymphocytes when a particular antigen is re-encountered. An equivalent system does not occur in invertebrates, although evidence has been proposed for adaptive

FIGURE 6 . POTENTIAL STRATEGIES OF DEFENCE EVASION BY LARVAL SCHISTOSOMES.
(a) compatability depends on exploitation of a "blind spot" in the host's recognition system. The parasite takes advantage of the host's lack of appropriate recognition molecules.
(b) Molecular Masking - compatible parasites acquire disguising host molecules either by passive uptake or by specific receptor-mediated processes.
(c) Molecular Mimicry - compatible parasites synthesize and display molecules on their surfaces which successfully mimic self and prevent host responses. Incompatible parasites synthesize inappropriate molecules that stimulate a response.
(d) Interference Hypothesis - secretory/excretory products of larval schistosomes actively interfere with host defence responses.

Key
H = haemocyte
I = incompatible parasite
C = compatible parasite
S = self hapten
N = nonself hapten

6a. HOST LACK OF RECOGNITION MOLECULES

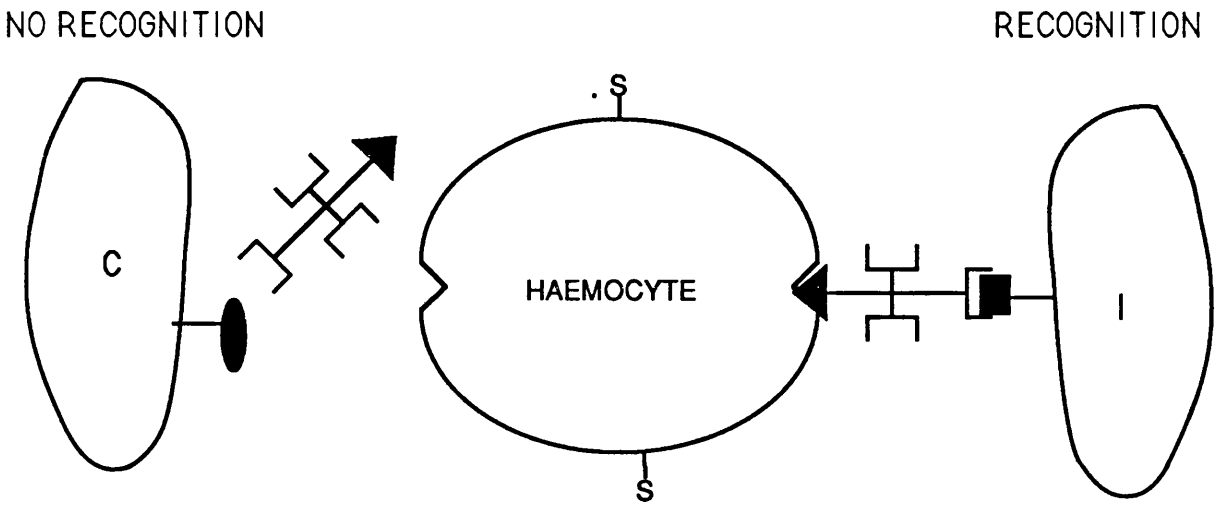
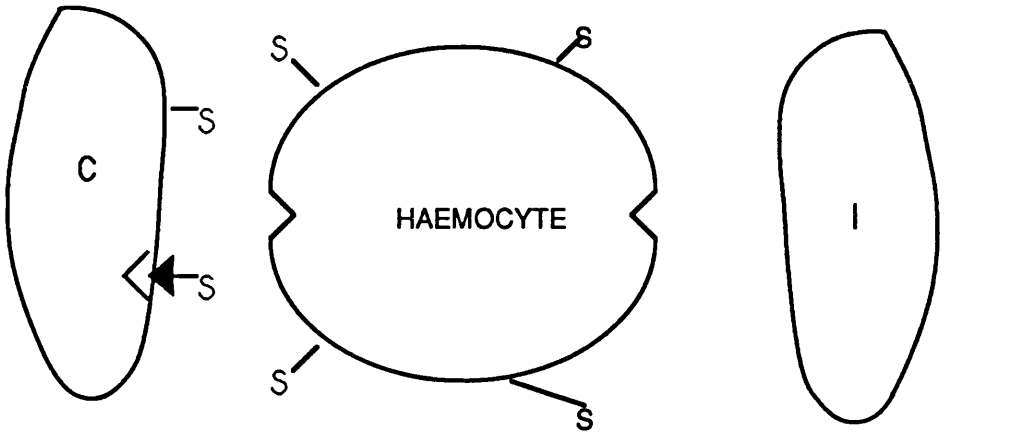


FIGURE 6b. MOLECULAR MASKING



RECEPTOR MEDIATED
AQUISITION OF SELF

NO AQUISITION
OF SELF

FIGURE 6c. MOLECULAR MIMICRY

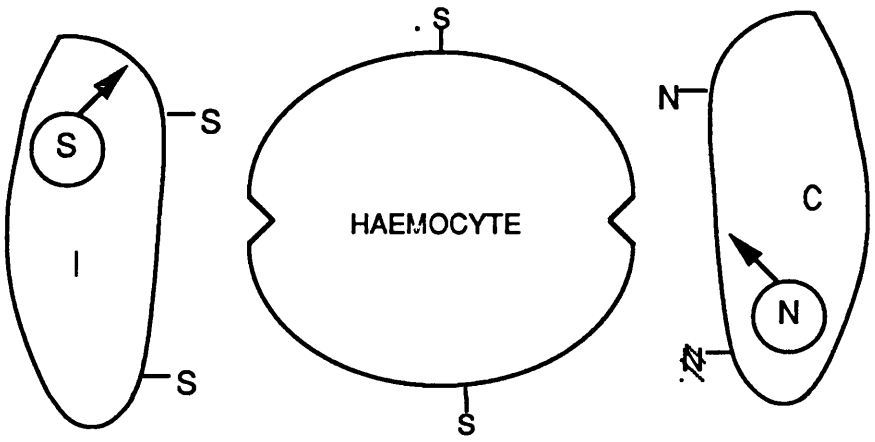
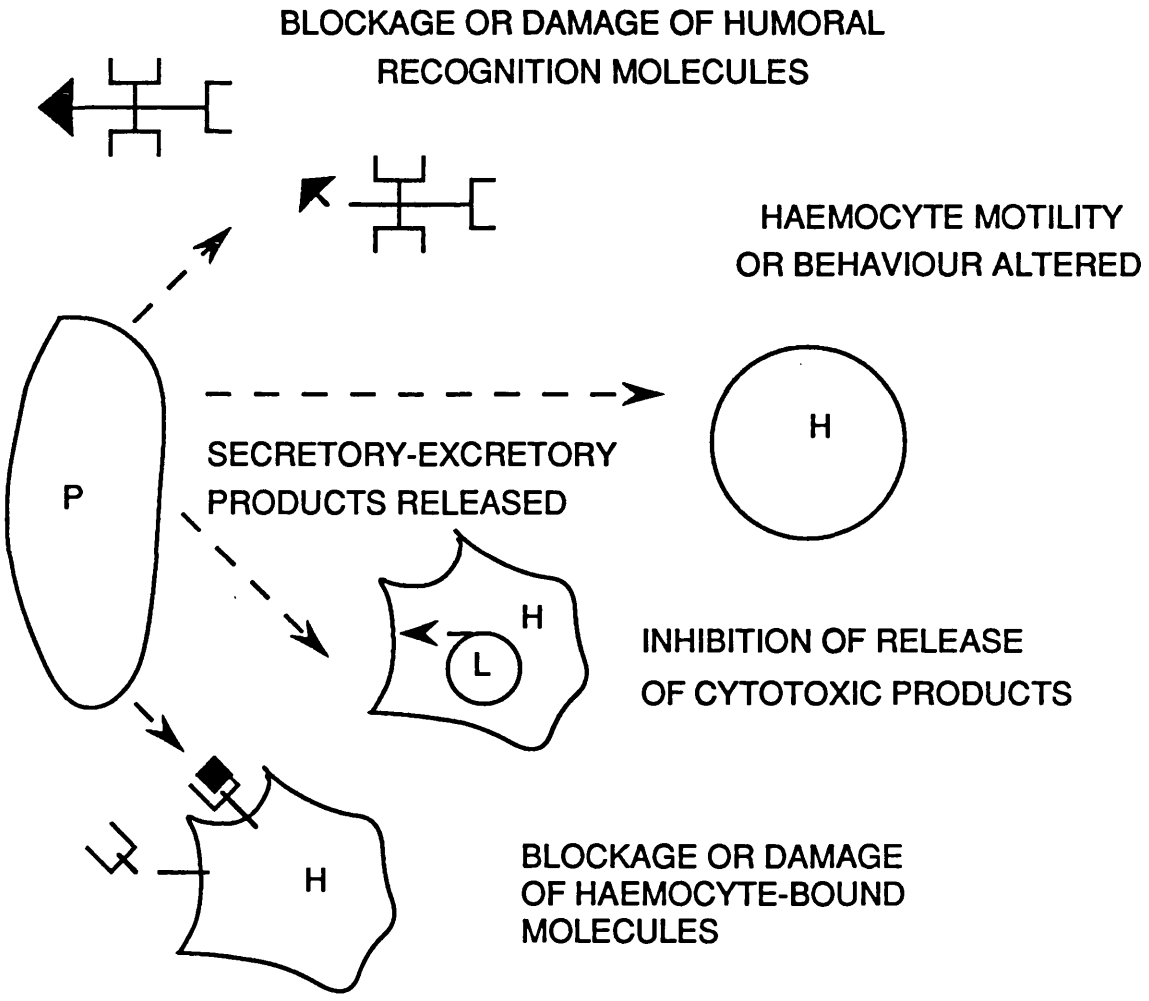


FIGURE 6d. INTERFERENCE HYPOTHESIS



mechanisms in some molluscs, there being a faster secondary reaction. In *L. stagnalis,* an adapted response to *Escherichia coli* infection and a dose-dependent response to *Staphylococcus saprophyticus* infection have been demonstrated, showing that the internal defence system is specifically activated. However, the secondary response is probably non-specific, and thus it is better not to use the term "immunological memory", but descriptions such as "duration of the induced activation" (Sminia and Van der Knaap, 1986). As yet, adapted responses directed against invading schistosome larvae have not been demonstrated.

It is clear that many aspects of the functioning of invertebrate defence responses still need to be clarified. In particular, much evidence has been proposed for the roles of humoral factors in molluscan defence responses, but the precise activity of these molecules is still unclear.

This project encompasses a study of the humoral defence responses in gastropod molluscs. Two species of *Bulinus* have been selected, both of which act as intermediate hosts in the transmission of schistosomiasis. A scheme of classification for the snail species used in this study is given in Figure 7. In the **africanus** group, morphological intermediates among *B. africanus, B. globosus* and *B. nasutus* in the East African coastal belt create difficulties in identification. The distribution of *B. nasutus* is restricted to East Africa (Mandahl-Barth, 1965). It serves as the main intermediate host of *S. haematobium* in north-western Tanzania (Webbe, 1962), and naturally-infected snails have been found in Uganda and West Kenya (Cridland, 1955; Kinoti, 1971). Its habitat is associated with seasonal waterbodies which may dry out for periods of about 5 months (Webbe and Msangi, 1958; Pringle, Otieno and Chimtawi, 1971)

FIGURE 7. CLASSIFICATION OF SNAIL SPECIES .

PHYLUM - MOLLUSCA

CLASS - GASTROPODA

SUB CLASS - PULMONATA

FAMILY - PLANORBIDAE

SUB FAMILY - BULININAE

Bulinus nasutus (Martens, 1879) (**africanus** group)

Bulinus natalensis (Kuster, 1841) (**truncatus\tropicus** group)

particular it occurs in isolated pools formed by seepage (as in NW Tanzania). Sexual maturity is reached about 8 weeks after hatching (shell height 8mm). The life expectancy of adults is 30 days (Webbe, 1965), in which time infected snails may shed up to 2000 cercariae in a day (McClelland, 1967). The habitats of *B. natalensis* encompass a wide variety of waterbodies including small pools, slowly-flowing rivers and also some lakes. Present in Eastern Africa from Ethiopia to South Africa, it does not serve as an intermediate host for *S. haematobium,* but has shown low infectivity in experimental infections (Lo, Burch and Schutte, 1970). *S.margrebowiei Le* Roux, 1933 is a parasite of cattle, goat and wild artiodactyls. It is transmitted naturally by snail species belonging to the **B.natalensis/tropicus** complex (Pitchford, 1976) and the **B. forskalii** group (Wright, Southgate and Howard, 1979). It is known from an area which includes the Chobe Game Reserve in Botswana, the Eastern Caprivi Strip, Southern Zambia and Katanga (Pitchford, 1976) and Chad (Graber, 1978). *S. margrebowiei* has been shown to be compatible under experimental conditions with the following snail species - *B. tropicus* (Pitchford, 1976; Southgate and Knowles, 1977), *B. octoploidus, B. truncatus, B. beccarii, B. bavayi* and *B. wrighti* (Southgate and Knowles, 1977).

A powerful agglutinin of human erythrocytes has been discovered in *B. nasutus. This* species is resistant to infection by *S. margrebowiei,* and it is postulated that the agglutinin may contribute to this resistance. By the same token, the lack of such an agglutinin in *B. natalensis* might contribute to its susceptibility to *S.margrebowiei.* The isolation and characterization of the agglutinin in *B. nasutus* was undertaken, in order that the the role(s), if any, of this molecule in defence responses of these snails invoked by parasitism and other stimuli could be investigated.

or
invoked

1.0 ANIMALS.

1.1 Origin and Maintenance of Snails.

The population of *Bulinus nasutus* used in this study originates from a collection made in Morogoro, Tanzania in 1974. These snails and their progeny have been kept in culture for 16 years at the Experimental Taxonomy Unit of The Natural History Museum, London. Each isolate is designated a collection number, which in this text will appear after the species name; hence, *B. nasutus* 1214.

The population of *B. natalensis* 272 originates from Lake Sibayi, South Africa, and has been kept in culture at The Natural History Museum for 17 years.

Snails were maintained in a constant temperature room at 27°C in plastic trays (35 x 25 x 5cm) covered with a sheet of glass (to maintain humidity) in "snail conditioned water." This was Bloomsbury tapwater in which fish (*Poecilia spp.*) had been kept for at least a fortnight, to remove chlorine. Fresh water was supplied at 14 day intervals. Snails were fed fresh lettuce *ad libitum*. Egg masses were collected on polyethylene sheets, and were transferred to new trays, in order that juveniles could hatch unmolested by adults.

1.2 Origin and Maintenance of Parasite.

S. margrebowiei, (a schistosome occuring naturally in the lechwe, *Kobus leche*), used in this study was isolated from the Kafue Flats, Lochinvar National Park, Zambia. This trematode was maintained in the laboratory using Tyllers' Original Swiss mice as the definitive host. Mice were routinely infected by exposure to about 200 cercariae using

the partial immersion technique of Watson and Azim (1949). Mice are allowed to paddle in a suspension of cercariae in snail conditioned water within the confines of a beaker, thereby facilitating contact between parasite and animal. Livers from infected mice were removed at 45 days post-infection by dissection using aseptic techniques, and were homogenised in a Waring blender with 100ml 0.85% saline. The resultant tissue suspension was passed through a series of sieves (mesh sizes 212-106μm). Coarse liver tissue was retained on the meshes, and the eggs were allowed to sediment under gravity for 30 minutes. Eggs were then washed in 0.85% saline several times by low speed centrifugation in a Beckman bench centrifuge, discarding the supernatants each time.

Hundreds of eggs were obtained in this way, and these were hatched by incubating in snail conditioned water at 20°C for 30 minutes. The miracidia could be collected by use of a side-arm flask that exploits the negative geotaxis and positive phototaxis of the larvae (See Smyth & Halton, 1983).

Snails were infected by placing individuals into small pots containing 20 miracidia in snail conditioned water. This number was selected as infection using fewer could not be assured, and using a greater number usually resulted in snail death. The snails were left overnight with miracidia, and then transferred to a plastic tray with fresh water and incubated at 27°C.

Cercarial shedding usually began about 30 days post- infection, and continued for 10 days. Infected snails were kept in the dark until cercariae were required, as they are shed in the early morning in the wild (Southgate & Knowles, 1977). Exposure to light stimulated shedding in the laboratory, and the resulting cercariae could be collected from the water surface where they accumulated, by direct observation using dark ground illumination in conjunction with a stereo microscope.

1.3 Haemolymph Collection.

Snails were swabbed with 70% alcohol to remove surface deposits and mucus, immediately before haemolymph was withdrawn. The foot of each snail was forced by gentle pressure with a finely-drawn Pasteur pipette, to retract into the shell. Foot retraction was accompanied by exudation of red haemolymph (haemoglobin is the respiratory pigment of these snails), which could be aspirated off. Approximately 10-15μl could be obtained from each snail using this non-damaging method. Following a period of 30 days recovery, snails could be bled again. The cells (haemocytes) were separated from the serum (cell-free haemolymph) by centrifugation at 10000 x g for five minutes. Haemolymph serum not used immediately was stored at -20°C in 20μl aliquots, in order that future use of haemolymph would result in minimal wastage (as repeated freezing and thawing is detrimental to many proteins).

2.0 INVESTIGATION OF PLASMA COMPONENTS.

2.1 Erythrocyte Preparation.

Human blood (groups A_1, A_2, B and O) was obtained when required from University College London Blood Transfusion Unit. Heparinized blood was collected from rabbits and mice (Balb C, A-Thy-1, CBA and Brown strains). Sheep erythrocytes, stored in Alsever's solution, were obtained from the ICRF Tumour Immunology Unit, UCL. Before use in agglutination assays, all erythrocyte types were washed five times in phosphate buffered saline (PBSA), pH 7.4. The cells were resuspended in the same buffer to give a final cell suspension of 2% (packed volume), approximately 10^7 cells.ml^{-1}.

2.2 Agglutination Assays.

The haemolymph of a number of bulinid and other snails were as asssayed for agglutinating activities. These included *B. nasutus* 1214, *B. ugandae* 1250, *B. africanus* 1261, *B. umbilicatus* 405, *B. obstussipira* 1476, *B. globosus* 1381 (all **africanus** group snails), *B. beccarii* 1416, *B. forskalii* 1162, (**forskalii** group snails), *B. guernei* 1126, *B. rohlfsi* 1326, *B. tropicus* 28, *B. truncatus* 1521, *B. coulboisi* 1087, *B. permembranaceus* 1256, *B. octoploidus* 1077, *B. trigonis* 755 (all **truncatus/tropicus** complex snails), *Indoplanorbis exustus* 1275, *Biomphalaria glabrata* 1144, *Biomphalaria pfefferi* 978, *Lymnaea stagnalis*, *Lymnaea peregra*, *Achatina achatina*, *Achatina fulica*, *Helix aspersa* and *Planorbis contortus*. The geographical origins of the bulinid snails are shown in Figure 8. Assays were performed in U-bottomed well microtitre plates (Falcon). Haemagglutinating activity was determined using serial two-fold dilutions of haemolymph in PBSA, pH 7.4. Each well contained 20μl of cell-free haemolymph of known dilution, and 20μl of 2% erythrocyte suspension. Control wells containing no haemolymph were always included with each test. The plates were vigorously shaken for 30 seconds (to ensure even distribution of erythrocytes), and left to stand for 2 hours at room temperature before any agglutinating activity was recorded. The haemagglutinating titres were expressed as the reciprocal of the dilution at the end point (the last well in which there was complete agglutination of the erythrocytes).

2.3 Heat Stability.

In order to determine the thermal stability of the haemagglutinins from a given snail isolate, 20μl samples of cell-free haemolymph were incubated in pre-heated water baths at various temperatures (22, 37, 42, 50, 60 and 70°C) for 30 minutes. Samples were

subsequently centrifuged at 10000 x g for five minutes, in order to sediment any formed precipitate. The agglutinating activity of the supernatant was then assayed with human A_2 type erythrocytes, using unheated cell free haemolymph from the same snail as the positive control.

2.4 pH Dependency.

In order to determine the optimum pH for agglutinating activity, agglutination assays were conducted using cell-free haemolymph in PBSA of varying pH, over the range 3-11. Stock PBSA pH 7.4 was adjusted to the appropriate pH with 1M NaOH or 1M HCl as required. Haemolymph was incubated in the PBSA solution for 30 minutes before the test human B type erythrocytes were added.

2.5 Inhibition by Proteolytic Enzymes.

The proteolytic enzymes pepsin, trypsin and pronase were used to determine whether agglutinating activity was dependent on protein molecules. The enzymes were made up in PBSA, pH 3 and added to cell-free haemolymph to give a final concentration of $1mg.ml^{-1}$. Control samples containing no enzymes were also set up. All test samples were incubated at room temperature, and agglutination assays were performed using human A_2 type blood erythrocytes.

2.6 Divalent Cations.

In a number of invertebrate species studied, the presence of free divalent cations in haemolymph is necessary for agglutinating activity. To investigate whether this is a

requirement of *B. nasutus* 1214 agglutinins, the chelating agents EGTA and EDTA were used in agglutination assays, in order to remove free calcium and magnesium ions respectively. Final concentrations of 1mM and 5mM in PBSA, pH 7.4, were used.

2.7 Inhibition Tests.

In order to investigate whether agglutination was due to a simple sugar-specific lectin, a variety of sugars and glycoproteins was tested to determine whether any would act as a competitive inhibitor of the *B. nasutus* 1214 haemagglutination activity. Stock solutions of carbohydrates and glycoproteins were made up in PBSA, pH 7.4. Sugar solutions were added to the agglutination assays, prior to the addition of erythrocytes, to give final concentrations of 500mM. The sugars tested are listed below.

AGENTS EMPLOYED TO INHIBIT AGGLUTINATION.

MONOSACCHARIDES	D+glucose
	D-glucose
	D+galactose
	D-mannose
OLIGOSACCHARIDES	D-ribose
	D-maltose
	D-sucrose
	α-lactose
GLUCOSIDES	α-D-galactopyranoside
	methyl ß-D-galactopyranoside
	methyl α-D-mannopyranoside
	α-methyl-D-mannose
	methyl-D-mannoside
OTHER	fetuin
	porcine mucin
	bovine mucin

2.8 Adsorption.

In vivo, if the *B. nasutus* 1214 agglutinin is mediating defence reactions by recognition of nonself, then in a schistosome infection of this snail one would expect a specific interaction to occur between the agglutinin and the parasite. As it is highly likely that it is the miracidium, and not the subsequent sporocyst which initiates defence reactions on penetration of snail tissues, this larval stage was chosen for test.

500μl cell-free 1/64 diluted haemolymph was reacted under constant rotation for 60 minutes with a pellet of live *S.margrebowiei* miracidia. Following centrifugation at 11600 x g, the supernatant was assayed for agglutinating activity with 2% human A_2 erythrocytes as test cells. As the agglutinin is reactive towards human erythrocytes, then it must bind to their surfaces. If an excess of surface membrane is provided to the agglutinin (i.e. sub-agglutinating concentration of agglutinin) then the protein should effectively be removed from haemolymph.

10μl of cell-free haemolymph was rotated for 60 minutes with 0.5ml 2% human A_2 erythrocyte cell suspension in PBSA, pH 7.2. Following centrifugation at 11600 x g for 3 minutes, the supernatant was tested for agglutinating activity with A_2 erythrocytes. An aliquot of this supernatant was also applied to a Superose 12 gel filtration column (see section 3.7).

2.9 Egg Masses.

In order to investigate whether snail egg masses have agglutinating activity, egg masses were removed from sheets of plastic film which had been placed previously in stock aquaria. No effort was made to separate the masses with respect to age of

development. The masses were rinsed in distilled water, blotted on filter paper and weighed on a micro-balance. They were then homogenised by hand in a pestle with 5μl PBSA, pH 7.4 added for each mg of egg-mass. The homogenate was then centrifuged for 5 minutes at 11600 x g, and the supernate used as the test sample. Agglutination was assayed using human B type erythrocytes.

Egg masses were selected that were in an early stage of development (embryo small and transparent). Fluid was released from the embryonic sac by puncture of the underside of the egg-mass with a fine needle, and about 5μl could be aspirated off with a Pasteur pipette. A sample was tested for agglutinating activity with human A_1 cells. The remaining portion of the egg masses was homogenised and prepared for assay as before.

2.10 Antibacterial Activity.

In order to test for antibacterial activity (as in Insecta) an Inhibition Zone Assay (Faye & Wyatt, 1980) was employed. Four wells of 5mm diameter were cut into a thin-layer 2% nutrient agar plate. 5μl aliquots of PBSA, pH 7.4 were pipetted into two of these wells, and 5μl aliquots of *B. nasutus* 1214 cell-free haemolymph were pipetted into the remaining wells. Approximately 0.5ml of a bacterial suspension of *Escherichia coli* 10^6 cells.ml^{-1} had been spread over the whole plate. The plate was incubated at 37°C, and viewed after 24 hours.

3.0 PROTEIN CHEMISTRY.

3.1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

To investigate the protein compositions of haemocytes and haemolymph, SDS-PAGE was conducted using a discontinuous buffer system based on the method of Laemmli (1970). A vertical gel electrophoresis system was used (model V16, Bethseda Research Laboratories or Mini Protean II dual slab cell, Bio-Rad Laboratories). Gels were constructed using glass plates which had been thoroughly acetone and alcohol-cleaned, using 0.75mm spacers. Gel compositions are given in Table 6.

Electrophoresis was carried out at under conditions of constant voltage; 50V to carry the samples through the stacking gel and then at 200V through the resolving gel until completion.

3.2 Sample Preparation.

Sample buffer [2.3% SDS, 0.0625M Tris-base (pH6.8), 10% glycerol, 0.15% dithioerythritol (DTE) and 0.002% bromophenol blue] was boiled for 5 minutes before being added to the protein sample to be electrophoresed. The sample was then boiled for 15 minutes and centrifuged for 3 minutes at 11600 x g before being loaded into wells using a 50μl Hamilton syringe. Determination of the relative molecular masses (M_r) of sample proteins was made by reference to the mobilities of polypeptides of known relative molecular mass (M_r) run under the same electrophoretic conditions.

TABLE 6 COMPOSITION OF SDS POLYACRYLAMIDE GELS

STOCK SOLUTIONS	STACKING GEL	RESOLVING GEL			
	3%	15 %	12.5%	10%	7.5%
30% acrylamide	1.5	10.0	8.34	6.67	5.0
30% acrylogel	1.5	10.0	8.34	6.67	5.0
UGB 5X [a]	4.0	-	-	-	-
LGB 5X [b]	-	8.0	8.0	8.0	8.0
Temed [c]	0.025	0.05	0.05	0.05	0.05
distilled water	12.825	11.65	14.97	18.31	21.65
$(NH_4)_2SO_4$	0.15	0.15	0.3	0.3	0.3

All volumes are in ml

[a] UGB Stacking gel buffer 0.5M Tris-glycine, pH 6.8

[b] LGB Resolving gel buffer 1.87M Tris-glycine buffer, pH 8.8

[c] NNN'N'-Tetramethyl-1,2-diaminoethane

M_r Standards (Pharmacia) -

thyroglobulin	669k
ferritin	440k
catalase	232k
lactate dehydrogenase	140k
phosphorylase b	94k
bovine serum albumin	67k
ovalbumin	43k
carbonic anhydrase	30k
soybean trypsin inhibitor	20.1k
α - lactalbumin	14.4k

3.3 Staining.

A number of staining techniques were employed for the visualization of proteins in polyacrylamide gels following electrophoresis.

(1) Coomassie Brilliant Blue (CBB).

Gels were incubated in CBB solution [35% v/v methanol, 7.5% v/v glacial acetic acid, 0.05% w/v CBB (R-250, Pharmacia) in distilled water] for two hours or longer. Excess dye was removed by incubation of gels in destain solution (25% methanol, 7.5% glacial acetic acid in distilled water) until a clear background was obtained. This stain enabled protein bands of as little as 0.2-0.5 μg to be detected in a quantitative manner.

(2) Sensitive Silver Staining.

Protein concentrations as low as 40ng (depending on the polypeptide in question, as some are less argentophilic than others) could be detected with highly sensitive staining methods using silver nitrate. These techniques were employed either directly after electrophoresis had been completed, or more frequently subsequent to staining with CBB.

The following protocols were adopted -

(A). The first method is one published by Morrissey (1981), with a modified step.

Experience showed that an additional incubation in 10% w/v glutaraldehyde prior to staining did not reduce background staining sufficiently to warrant use of this chemical. Thus the revised methodology was as follows-

Following fixation of gels in destain (as for CBB staining), the gels were drained and washed thoroughly in distilled water in order to equilibrate the pH of the gel with that of the water. Gels were then incubated in DTE ($5\mu g.ml^{-1}$) for 30 minutes. After draining, gels were incubated in fresh 0.1% silver nitrate solution in complete darkness for 30 minutes. Following drainage, the gels were rapidly rinsed in distilled water. Incubation in 3% sodium carbonate (anhydrous) was then conducted for 15 seconds, in order to develop the stain. This stage was repeated to ensure full development, and 100μl 40% v/v formaldehyde added to regulate the process. Gels were agitated gently over a light box until bands could be seen satisfactorily (usually less than five minutes). The reaction was stopped by addition of 2.3M citric acid to a final concentration of 0.1M.

(B). Sammons, Adams and Nishizawa (1981).

Gels were fixed in destain solution (as for CBB staining) for two hours or more, and were then washed in successively diluter destain solutions (25% v/v methanol, then 10% v/v methanol, both 10% v/v glacial acetic acid) for 30 minutes each. Gels were then equilibrated in 0.019% silver nitrate solution for two hours. Following drainage the gels were briefly rinsed in distilled water for 20 seconds to remove surface silver deposits, and were then immersed in a reducing environment for 10 minutes. The reducing solution was 8.75% w/v sodium borohydride, 0.75% v/v formaldehyde in 0.75N sodium hydroxide. Technicolour formation was enhanced by two washes in 0.075% w/v sodium

carbonate for one hour each. The colour of each polypeptide - silver complex is clearly visible above the light background of the stained polyacrylamide gel, and blue, green, yellow and red colours can be obtained.

3.4 Glycoprotein Staining.

As any molecule within snail plasma that has agglutinating activity is likely to be a glycoprotein, for the detection of sugars in glycoconjugates, a Glycan Detection Kit (Boehringer) was employed (O'Shannessy, Voorstad and Quales, 1987). Adjacent hydroxyl groups in sugars of glycoconjugates were oxidised to aldehyde groups by mild periodate treatment. The spacer linked steroid hapten digoxigenin was then covalently attached to these aldehydes via a hydrazide group. Digoxigenin labelled glycoconjugates were subsequently detected in an enzyme immunoassay using an antibody alkaline phosphatase glycoconjugate.

3.5 Flat Bed Isoelectric Focusing.

In order to investigate the pI characteristics of proteins present in cell-free haemolymph, a flat-bed isoelectric focusing technique was employed. Gels were cast on glass plates at room temperature, with a thickness of 5mm. The composition of gels is given in Table 7.

Electrolyte strips were soaked in 0.1M phosphoric acid, (anode) and 1M sodium hydroxide (cathode), and placed on the gel surface. Samples were loaded directly onto the gel surface. Haemoglobin standards were loaded at both top and bottom of the gel as references of mobility, as the red bands were easily visible, and a major band is

TABLE 7 COMPOSITION OF ISOELECTRIC FOCUSING GELS

	NUMBER OF PLATES					
	2	**3**	**4**	**6/7**	**8/9**	
Distilled water	72	108	144	226	228	ml
sucrose	15	22.5	30	47	60	g
29.1 % acrylamide	20	30	40	63	80	ml
0.9 % bis-acrylamide	20	30	40	63	80	ml
ampholines+	8	12	16	25	32	ml
0.005% riboflavin 0.8	1.2	1.6	2.5	3.2	ml	

+ pH 3.5 - 9.5 Stock.	Pre-blended LKB 1818-101
pH 4.0 - 7.5 Stock.	4-6 11 vols. LKB 1809-116
	5-7 5 vols. LKB 1809-121
	5-8 20 vols. LKB 1809-126

known to have a pI of 7.25.

Electrofocusing was conducted on a 2117 Multiphor cooling system (LKB) with a 2103 D.C. Power Supply (LKB). Running conditions depended on the pH range of the gel -

pH 3.5 - 9.5 = 1200V 30W 50mA

pH 4.0 - 7.5 = 1200V 30W 30mA

All gels were run for a two hours, after which time electrofocusing was completed. When the run was completed, a surface pH electrode was placed sequentially along the gel at 10mm intervals, to give a pH profile of the gel composition. This was cross-referenced with a major haemoglobin band of known isoelectric point (pI) 7.25.

3.6 Staining.

Gels were fixed in 3.5% sulphosalicylic acid, 11.5% trichloroacetic acid in distilled water for 1 hour, in order to precipitate the proteins and wash out most of the ampholines (which give background staining). After draining, gels were washed in three changes of destain solution (25% ethanol, 8% glacial acetic acid in distilled water), for 10 minutes each wash. Several changes were necessary to adjust the pH of the gel to that of the staining solution. The gels were then incubated in staining solution [0.1% Coomassie Brilliant Blue (R-250 Pharmacia) in destain] for 30 minutes. Gels were subsequently destained until the backgrounds were clear, and were then incubated in a preserving solution (0.003% glycerol in destain) for 1 hour.

3.7 Fast Protein Liquid Chromatography (FPLC).

When attempting a protein purification such as in this study, one requires maximum recovery to achieve high purity when working with low initial concentrations and extremely small volumes (e.g.50μl), and also high selectivity when working with a diverse range of biomolecules.

FPLC is the only high performance chromatography system that allows one to run analytical or preparative, manual or automated applications, and to combine multi-step purification schemes, all with full biocompatibility and high chemical resistance.

Columns are connected into the system via a manual selection valve, and samples injected onto the columns from standard sample loops. Automated isocratic or gradient elution techniques are incorporated into programmed separation methods. The high resolution generated in the column is preserved throughout by use of low dead-volume components. The biocompatibility of media and wetted surfaces of instruments ensures maximum recovery of biological activity and protects samples and columns from contamination. The high chemical resistance of the FPLC system also means that all wetted surfaces can withstand the extremes of pH or salt concentration needed for optimal biomolecule separation.

MonoBeads are monodisperse polymer particles to which a hydrophilic layer and ligands have been bonded. MONO Q is a "strong" anion exchanger, in that it has no buffering capacity in the pH range 2-12. For size fractionation, Superose enables fast gel filtration, the highly cross-linked agarose beads making the matrix rigid and pH stable.

In order to separate the protein constituents of cell-free haemolymph, chromatography was conducted using Mono Q HR 5/5 (Pharmacia) cation exchange,

Superose 12 10/30 (M_r range $1x10^3$- $3x10^5$) and Superose 6 HR 10/30 (M_r range $5x10^3$-$5x10^6$) (all Pharmacia) gel filtration columns, using an FPLC system (Pharmacia). Details of gradients and flow rates are given for each separation in the Results section.

3.8 Optimization of Fast Protein Liquid Chromatography (FPLC).

In order to maximise the efficiency of FPLC, the pH and ionic strength of buffers used has to be selected to match the given protein whose purification is sought.

pH - as the pI of the agglutinin was unknown, a simple assay was performed to determine the starting pH to be used in ion exchange chromatography. A series of test-tubes were set up with 1.5ml of ion exchanger, sepharose Q-FF (Pharmacia). The gel in each tube was equilibrated to a different pH by washing 10 times with 10ml 0.5M Tris-HCl of the pH range 5-9. Gels were then equilibrated with 0.01M Tris-HCl of the same pH 10 times. The buffer volume was reduced to 2ml, and 50μl of cell-free haemolymph applied to the slurry. The gel was mixed for 10 minutes, and then allowed to settle for 30 minutes. The supernatants were assayed for agglutinating activity with human type B blood cells.

Ionic Strength - in order to select the correct ionic strength of starting buffer, a series of tubes with ion exchanger were set up as before. The gels were equilibrated with ten 10ml washes of 0.5M Tris-HCl, pH7. The gel in each tube was then equilibrated to a different ionic strength using a range from 0.01-0.3M sodium chloride. 50μl of cell-free haemolymph was added and the whole was shaken for 10 minutes. After settling, the supernatants were assayed for agglutinating activity with human A_2 type blood cells.

3.9 Protein Concentrations.

Protein concentrations from all the purification methods were assayed using the modified Lowry assay (Markwell, Haas, Bieber & Tolbert, 1978).

3.10 Polyclonal antibody production.

Due to the dilution factors involved with the adopted purification protocol, only very low concentrations of protein would be yielded. Due to a lack of available snail material, protein bands of M_r 135k were prepared in SDS PAGE gels as outlined above. The gel slices were carefully dissected from the gels, and extensively washed in distilled water. Excess water was removed, and the gel slices frozen at -20°C until immunization. Gel slices were homogenized by hand in 200μl PBSA, pH 7.4, and injected subcutaneously into Balb C strain mice. The acrylamide acts as an adjuvant, compensating for the low concentration of protein in the samples. Individual mice received 3 immunizations at 14 day intervals. Antisera were then collected using the tail bleeding method.

3.11 Western Blotting.

SDS-PAGE gels were set up to include tracks for positive and negative controls, and tracks to stain with CBB, with molecular weight markers and amido black.

Transfer of proteins from the gel to nitrocellulose paper (Schleicher and Scheull) was facilitated with a Miniblot system (BioRad) using transfer buffer [25mM Tris-base,(pH8.3), 192mM glycine, 20% (v/v) methanol]. Air was carefully excluded from between the gel, nitrocellulose filter, filter paper and spacer pads. The apparatus was

run at 100V for 2 hours. Blots were stained with Ponceau Red to allow a direct record of transfer and position of polypeptides, prior to immunoblotting.

3.12 Cleveland mapping (Cleveland, Fischer, Kirschiner & Laemmli, 1977).

7.5% SDS PAGE gels were run with the appropriate samples. Bands of interest were cut out of the gel, and placed in separate wells of a second gel (15% resolving). 20-30μl of Cleveland's buffer (0.0625M Tris pH 6.8, 50mM DTT, 105 glycerol, 0.2% SDS) containing 20μg.ml^{-1} of Staphylococcal V8 protease was added to each well. An overlay of running buffer was added, and the whole allowed to sit at room temperature (24°C) for 10 minutes. The dye front was run quickly into the stacking gel (50mA) and then the current was reduced to 15mA. When the dye front was well into the stacking gel, the current was stopped for 30 minutes. It was then reapplied at 20mA until completion of the run.

4.0 PURIFICATION OF *B. nasutus* 1214 AGGLUTININ.

Attempts at purification of the agglutinating agent(s) present in the haemolymph of *B. nasutus* 1214 have been problematical. Application of whole haemolymph to FPLC columns has resulted in poor resolution of component proteins. This is largely due to haemoglobin, which accounts for the majority of haemolymph proteins (97% in *Biomphalaria glabrata*, Figueiredo, Gomez, Heneine, Santos & Hargreaves, 1973). Unlike vertebrate haemoglobins, invertebrate haemoglobins exist as a protein complex of the order of 1.6-3.2 x 10^6 k in size (Figueiredo *et al.*, 1973), and consisting of numerous components of different sizes. Granath, Spray & Judd (1987) reported

ultracentrifugation of *Biomphalaria glabrata* (PR albino M-line) to yield very few major proteins in the resultant supernatant, once the haemoglobin had been pelleted. This is in contrast to an earlier study (Yoshino and Bayne, 1983) in which ultracentrifugation was employed as an initial step for isolation of specific proteins from *Biomphalaria glabrata* (PR albino M-line) haemolymph.

4.1 Ultracentrifugation.

Haemolymph samples were centrifuged in an Airfuge Ultracentrifuge(Beckman) in an attempt to pellet out haemoglobin, as described by Yoshino and Bayne (1983). Samples were spun for 30 minutes at 170000 xg at a pressure of 207 kPa. The resultant supernatant and pellet fractions were then assayed for agglutinating activity as described previously, using human type A_2 erythrocytes.

4.2 Adsorption onto Concanavalin A-Sepharose 4B beads.

Invertebrate haemoglobins differ fundamentally from their vertebrate counterparts in being glycosylated. Afonso, Arrieta & Neves (1976) report a 3% sugar composition of *Biomphalaria glabrata* (PR albino M-line) haemoglobin, with mannose, galactose and fucose being the three most-represented sugar residues. α-D mannopyranoside is a specific hapten for the lectin Concanavalin A. Were the mannose residues of molluscan haemoglobins available for interaction with Con A, and not inhibited by steric factors, then in principle this lectin coupled to a solid matrix might provide a means to remove haemoglobins from haemolymph by affinity binding. Since the mannose hapten did not abrogate agglutination of A_2 cells it might be expected that the agglutinin should remain

in solution, and then could be purified by FPLC without the interference of haemoglobins experienced with whole haemolymph. With this in mind, concanavalin A linked to sepharose 4B beads (Pharmacia) was used in an attempt to adsorb out haemoglobins from *B. nasutus* 1214 haemolymph.

100μl Con A sepharose 4B bead suspension in PBSA, pH 7.4 was rotated for 60 minutes with 200μl 1/64 diluted *B. nasutus* 1214 cell-free haemolymph. Following centrifugation at 11 600 x g, the supernatant was further rotated with 100μl Con A sepharose 4B beads. An aliquot of this second supernatant was assayed for agglutinating activity with 2% human A_2 erythrocytes. Sepharose 4B beads were used in control experiments.

200μl of this second supernatant from the Con A sepharose treatment was applied to a Superose 12 gel filtration column (FPLC, Pharmacia), sensitivity 0.05, flow rate 0.5ml.min^{-1}, 0.05M phosphate buffer, pH 7.0 with 0.15M NaCl. 1ml fractions were collected, and 20μl aliquots were tested for agglutinating activity with 2% human A_2 cells as before. Aliquots of three fractions, and of the original second Con A bead supernatant were processed for SDS-PAGE.

4.3 Analysis of *B. nasutus* 1214 agglutinin.

In order to investigate the native M_r of the agglutinin, it was necessary to run the purified protein under non-reducing conditions. The FPLC gel filtration studies indicated that the M_r would be very large (in excess of 200k); thus SDS-PAGE was performed using a 5-15% gradient gel. Three samples were used; 1/4 diluted

B. nasutus 1214 cell-free haemolymph, 1/2 diluted Con A bead supernatant and FPLC purified fraction 9. Duplicate sets of these samples were prepared, one of which had been exposed to the reducing agent DTE. 20μl aliquots of all samples were loaded into individual wells of the gel, the reduced samples being distinctly spatially separated from the non-reduced in order to prevent lateral diffusion of the DTE (which could cause alteration of the non- reduced samples while they migrate through the gel).

4.4 Comparison of *B. nasutus 1214* agglutinin with *B. natalensis* 272.

To investigate whether the other test bulinid, *B. natalensis* 272 possessed the same 135k band, although it lacked agglutinating activity against human A2 red blood cells an identical experimental procedure was adopted using haemolymph from this species as well as *B. nasutus* 1214. 25μl of *B. natalensis* 272 and *B. nasutus* 1214 cell-free haemolymphs were diluted with 75μl PBSA, pH 7.4, and 200μl of a Con A sepharose 4B bead suspension was added. Each mixture was rotated for 30 minutes, and following centrifugation at 11600 x g the procedure was repeated with the resultant supernatants. These preparations were subjected to SDS-PAGE.

4.5 Analysis of bulinid haemolymphs.

Although a humoral agglutinin has only been detected in one of the bulinid species tested (*B. nasutus* 1214), one hypothesis is that this protein is not unique to this single species. Therefore, seven other species of *Bulinus* and one species of *Biomphalaria* were investigated, employing the same purification procedures as was adopted for *B. nasutus*

1214 and *B. natalensis* 272. Haemolymph was collected from *B. truncatus* 1521, *B. ugandae* 1250, *B. africanus* 1227, *B. tropicus* 28, *B. umbilicatus* 478, *B. obtusispira* 1476, *B. globosus* 1381 and *Biomphalaria glabrata* 1144.

For each species, 25μl of cell-free haemolymph was diluted with 75μl PBSA pH 7.4 (i.e. 1/4 dilution), and three sample preparations were made -

(1) 10μl of this 1/4 diluted haemolymph was diluted with 20μl sample buffer, and 15μl of this was loaded into individual wells of a 7.5% SDS-PAGE gel.

(2) 200μl of Con A sepharose 4B bead suspension was added to the remaining 90μl 1/4 diluted haemolymph, and the whole was rotated for 30 minutes. Following centrifugation at 11600 x g, a further adsorption to Con A beads was conducted as before with the supernatant. 20μl of this second supernatant was diluted with 20μl sample buffer, and 15μl of this was loaded into individual wells of a 7.5% SDS-PAGE gel.

(3) 20μl of the Con A bead suspension with adsorbed proteins (i.e. haemoglobins) were diluted with 20μl sample buffer, and following centrifugation at 11600 x g, 10μl aliquots were loaded into individual wells of a 7.5% SDS-PAGE gel.

5.0 INDUCED RESPONSES.

5.1 Injection of Snails.

Stimulation of snail defence reactions occurs naturally by the invasion of microorganisms and metazoan parasites such as schistosomes. Experimental stimulation in the laboratory was achieved by the injection of biological or inert materials, using the protocol of Michelson (1958).

As large snails as possible were selected for injection with test substances. Snails were incubated individually in freshly prepared anaesthetic (0.16% sodium

pentobarbitone in snail conditioned water) for 2 hours. Once fully anaesthetized, the majority of the snail body protruded from the shell. By means of a binocular microscope, the male genital orifice positioned posterior to the right antenna could be identified, and a hooked capillary tube was inserted therein. The tube was anchored, and the snail shell restrained so as to create a tension which kept the snail still. Approximately 0.05-0.1 ml of the test substance could then be injected via a 30 gauge needle slowly (10-20 seconds) into the cephalopedal sinus. Following injection, snails were allowed to recover in frequent changes of snail water. Haemolymph was then collected for assay.

In a separate study, a second wounding experiment was conducted with *B. africanus* 1583, without the use of anaesthetic. This study was carried out in parallel to an infection study (see 5.2) in an attempt to discern any difference in activation of snail defence systems by invading schistosome miracidia due to (a) actual damage of the snail body wall during penetration, or (b) the subsequent presence of the "nonself" parasite within snail tissues. In order to mimic the penetration event, specimens were pierced with a 30 gauge needle three times, and haemolymph was subsequently collected from batches of seven snails at 3, 24, 48, 72 and 96 hours post-wounding. Absorbance measurements were made at 280nm (total protein) and 410nm (haemoglobin).

5.2 Infection with Parasites.

Specimens of *B. natalensis* were placed in individual vessels and exposed to miracidia of *Echinostoma togoensis* originating from Liberia. Five days post-infection haemolymph was recovered from some specimens.

In order to investigate the effects of progressive infection on protein levels in cell-free haemolymph, approximately 10mm diameter specimens of *B. natalensis* 272 (susceptible) and *B. umbilicatus* 478 (non-susceptible) were exposed to freshly-hatched miracidia of *S. margrebowiei*. Batches of five snails were isolated in separate crystallizing dishes, and 25 miracidia per snail were added. After 60 minutes incubation at room temperature, the dishes were scrutinised for the presence of free-swimming miracidia which had not attached to or penetrated snails. Specimens were then returned to plastic trays for the duration of the experiment.

Haemolymph was subsequently collected from batches of ten snails, at time intervals of 3 hours, 21 days and 30 days post-infection. These collection times were chosen to coincide with the expected stages of infection, namely (1) the penetration of snail tissues and transformation of miracidium to sporocyst which is initiated during the first three hours post-infection, (2) the multiplication and development of the two sporocyst generations by 21 days post-infection, and (3) the emergence of cercariae which began at 30 days post-infection. Haemolymph was also collected from unchallenged specimens of *B. natalensis* 272 and *B. nasutus* 1214 by way of controls (i.e. resting levels of haemolymph proteins).

Protein concentrations were determined by measurement of absorbance with a Cecil CE 72 linear readout spectrophotometer, using bovine serum albumin standards. I n a separate study, batches of five specimens of *B. natalensis* 272 and *B. umbilicatus* 1555 were exposed to *S. margrebowiei* miracidia as described above. Haemolymph was sampled at 3, 24, 48 and 72 hours post-infection from separate batches of snails. As well as measurements of total protein concentration, an estimation of haemolymph

haemoglobin content was also made by measuring absorption at 410nm. Specimens of *B. natalensis* 272 were fixed in 3% paraformaldehyde overnight. The shells were removed, and the bodies were placed into 1M sucrose solution for 3 hours. Specimens were then carefully blotted to remove excess sucrose solution, and frozen in liquid nitrogen in blocks of embedding medium (Tissue-Tek O.C.T. compound). 20μm sections were cut using a Bright Instruments Cryostat. Sections were air-dried, and aliquots of *B. truncatus* 1521 antiserum (1/100 dilution) in MEM with 10% FCS were applied to the sections. Following incubation overnight at 4°C, the slides were washed for 1 hour with PBSA pH 7.4, and the second layer, a goat α-mouse-FITC conjugate (1/100 dilution) (Cappell) applied for 1 hour at room temperature (24°C). Following further washing with PBSA, slides were mounted in glycerol containing 1,4-diazobicyclo-(2,2,2)octane (DABCO) to inhibit fading of fluorescence, and viewed with a Zeiss Universal microscope.

6.0 HAEMOCYTES.

6.1 Cell Counts.

A known volume of cell-free haemolymph collected from a known number of snails was diluted in a known volume of sterile snail saline (SSS). 10μl of this cell suspension was pipetted onto an Improved Neubauer haemocytometer (Hawksley Cristalite) and the cell number was counted. The mean number of cells.ml^{-1} and cells.snail^{-1} could then be calculated.

This methodology allows only for the collection of freely circulating cell types. Other cell types are fixed in intercellular spaces in various tissues, and duly would not be readily available under the employed conditions. Also, a slow circulation of haemolymph

would allow a random distribution of cells, thus the time of collection could also influence the number of cells available for collection.

6.2 Haemocyte preparation.

Experience has shown that snail haemocytes will readily aggregate once haemolymph had been collected from an animal. In order to conduct *in vitro* studies with cells, it was necessary to recover haemocytes from snails without them aggregating. For this purpose, an anti-clumping buffer ("Anti-C") was used (30mM NaCl, 20mM glucose, 6mM tri-sodium citrate, 5mM citric acid and 2mM EDTA). This buffer has been specifically devised for haemocytes of *Lymnaea stagnalis*. Haemolymph was diluted 1/5 in this buffer during collection. Centrifugation at 1000 rpm. at 4°C for 3 minutes resulted in a pellet of haemocytes. The pellet was washed twice with PBSA, pH 7.4, and finally resuspended in 200µl volume.

6.3 Monoclonal antibody production.

Haemocytes were collected in "Anti-C" buffer as described earlier. Pellets were resuspended in 200µl PBSA, pH 7.4 and injected intraperitoneally into Balb C mice. After 4 successive injections at 14 day intervals, spleens cells were extracted for fusion. A NS1 myeloma cell line was used for the fusions, and these were conducted in accordance with Kohler & Milstein, 1975.

Three related culture media are involved in monoclonal production:

(1) Tissue culture medium (TCM):-

RPMI with HEPES	(Gibco 041-2400-M)	500ml
Glutamine 200mM	(Gibco 043-5030-H)	5ml
Pyruvate	(Gibco 043-1369-H)	5ml
Penicillin-Streptomycin	(Gibco 043-5140-H)	5ml
Foetal Calf Serum	(Gibco 011-6180-H)	100ml

(2) HAT medium:-

Tissue culture medium		600ml
HAT (50 X concentrate)	(Gibco 043-1060-H)	12ml

(3) HT medium:-

Tissue culture medium		600ml
HT (50 X concentrate)	(Gibco 043-1065-H)	12ml

Spleens were transferred to a 35mm petri dish containing 1ml medium. Spleens were teased apart with two 25 gauge needles to make a cell suspension. Cells were transferred to a 15ml conical tube and left standing for 5 minutes to allow clumps to settle. The supernatant was transferred to a fresh tube. The cell suspension was centrifuged for 5 minutes at 44 xg and resuspended in serum-free RPMI. An aliquot was counted with Trypan Blue for viability. 2 x 10^7 NS1 myeloma cells in logarithmic growth were centrifuged for 5 minutes at 400 x g. The cell pellet was mixed with the spleen cell suspension and co-pelleted in a 50ml conical tube. The pellet was resuspended and warmed in a 37°C waterbath for 1 minute. Prewarmed polyethyleneglycol (PEG 1500) (Boehringer Mannheim 779 512) was added dropwise over 1 minute, mixing continually. In the same way, 1ml of serum-free medium was immediately added. 4ml of serum-free medium was then added over 4 minutes without mixing and followed by gradual addition of 20ml of the same medium. Then 20ml of medium containing 20% FCS was added. The tube was inverted gently to mix and left standing at 37°C for at least 1 hour. Cells

were spun down and resuspended in 100ml 2xHAT medium for distribution into 96-well plates (100μl per well). Plates were placed in a 37°C incubator until feeding was required. At day 5, 100μl of culture medium was removed from each well and replaced with fresh HAT medium. Wells were examined for growing clones each day. The same procedure was repeated at day 9. Cells were fed with HAT medium until day 14, and from then onwards HT medium was used. Hybridomas were fed up to day 28, thence with TCM.

6.4 Monoclonal antibody staining of Western blots.

Western blots were washed twice for 5 minutes in TBS (0.01M Tris, pH 7.4 0.15M NaCl). They were then blocked for 60 minutes in 5% w/v BSA in TBS, to prevent non-specific binding of antibody. Following two 5 minute washes in TBS the blots were incubated with the relevant monoclonal antibody (culture supernatants) or polyclonal antiserum diluted in 5% w/v BSA for 2 hours. Blots were then rinsed in 0.05% v/v Nonidet P40 in TBS and then twice in TBS, 5 minutes each in order to remove excess antibody. The second layer was then added (biotinlylated anti-mouse goat serum) in blocking buffer, and incubated at room temperature for 60 minutes. The washes in NP40 and TBS were repeated, to remove excess antibody as before, and then the blots were incubated in the third layer, an avidin-peroxidase conjugate, for 60 minutes. Following three washes in PBSA, pH 7.4 with 0.1% v/v Tween 20, and three subsequent washes in PBSA, colour was developed by addition of the substrate, which was prepared as follows. 60mg of 4-chloronapthanol were dissolved in 20ml ice-cold methanol, and 90ml PBSA, pH 7.4 was added. This was thoroughly mixed before the addition of 100μl H_2O_2. This solution was then applied to the nitrocellulose sheet and rocked to ensure

even distribution. When sufficient colour had developed, the reaction was stopped by quenching in distilled water.

6.5 Lectin staining.

Fluorescent lectins were utilised in an attempt to distinguish different subpopulations of haemocytes by the nature of the sugar moieties at their cell surfaces. The following lectins conjugated to FITC were used in this study (Sigma)-

LECTIN		SUGAR SPECIFICITY
concanavalin A (*Canavalia ensiformis*)	CON A	α-D-mannose
wheat germ agglutinin (*Triticum vulgaris*)	WGA	D-GlcNAc
soybean agglutinin (*Glycine max*)	SBA	D-GalNAc D-galactose
peanut agglutinin (*Arachis hypogea*)	PNA	ß-D-gal(1-3)- D-GalNAc
red kidney bean (*Phaseolus vulgaris*)	RKB	oligosacc.

Lectins were made up in PBSA at 1mg.ml^{-1}. 36μl of haemolymph was added to 4μl of labelled lectin, and this was incubated in the dark for one hour at room temperature (24°C). Monolayers were washed three times for 5 minutes, and then 40μl SSS:glycerol (50:50 v/v) was added to each well. Cells were examined using a Leitz Orthoplan fluorescence microscope, and intensity of staining was scored.

7.0 PRODUCTION OF REACTIVE OXYGEN INTERMEDIATES BY HAEMOCYTES.

7.1 Haemocyte monolayer preparation.

In order to prepare haemocyte monolayers, it was necessary to maintain haemocytes for several hours. Previous work with haemocytes from bulinid snails was conducted with Chernin's balanced salt saline. This proved to be disruptive for the haemocytes. Alternative media in which to prepare haemocytes were sought.

Pooled whole haemolymph from snails was collected in ice- cold sterile snail saline SSS (5mM Hepes, 3.7mM NaOH, 36mM NaCl, 2mM KCL, 2mM $MgCl_2.H_2O$, 4mM $CaCl_2.2H_2O$, pH7.8, sterilized through a 0.45μm Millipore filter), at a dilution of 1:5 (haemolymph:SSS). 40μl aliquots were applied to individual wells of Multispot microscope slides (Hendley and Essex), and allowed to settle in a humid chamber for 60 minutes.

7.2 Morphology.

The morphology of haemocytes attached to glass was studied by light microscopy.

7.3 Endogenous peroxidase staining.

Endogenous peroxidase activity was demonstrated by reduction of a substrate to yield a visible product. Haemocyte monolayers were rinsed in SSS. 40μl SSS was added to each well, and cell morphologies were examined with a light microscope. 40μl 0.5ng.ml^{-1} 3,3'diaminobenzidine-tetra-HCl (DAB) /0.01% H_2O_2 in SSS was added to each well, and slides were incubated in a humid chamber for 10 minutes. Additional wells also contained KCN in the incubation mixture as enzyme inhibitor. Slides were

then washed twice with SSS, and left to fix overnight in 3% paraformaldehyde in SSS.

7.4 Phagocytosis.

Haemocyte monolayers were prepared as before, and following sedimentation were rinsed with SSS. A suspension of yeast cell walls using zymosan A was prepared in accordance with Dikkeboom, Tijnagel, Mulder and van der Knaap (1986). 40mg of zymosan A was added to 10ml SSS and heated at 100°C for 30 minutes. The yeast cells were spun down (11600 x g for 3 minutes), washed twice in SSS, and resuspended to a final concentration of 1.5 X 10^8.ml^{-1}. 40μl of a suspension of zymosan in SSS (1 stock:8 SSS) was applied to each well, and allowed to sediment for 60 minutes. Excess zymosan was rinsed away with SSS, and then haemocytes were fixed with 3% paraformaldehyde in SSS. Slides were then passed through a series of graded alcohols and xylene, and mounted in Dabco. Cells were scored as having successfully phagocytosed if more than one particle was apparent within the cytoplasm. To distinguish between yeasts present on the surface of a cell and those actually internal, the field of view was carefully focussed in and out of the plane of the preparation.

7.5 Production of superoxides.

The kinetics of oxygen metabolite generation in snail haemocytes, in relation to the kinetics of phagocytic activity was studied. Haemocyte monolayers were prepared on slides as described above, and were kept on ice.

While still on ice, the SSS supernatant was replaced by a suspension of zymosan (using a ratio of 6 zymosan particles for every haemocyte) and 0.2% nitro blue tetrazolium (NBT), with or without the presence of 0.5mg.ml^{-1} superoxide dismutase (SOD). The SOD is a specific inhibitor of NBT reduction by superoxide. The suspensions were allowed to sediment onto the monolayers by centrifugation as before, thus establishing contact between haemocytes and zymosan. Slides were incubated for 60 minutes at room temperature. They were then rinsed in SSS, fixed in 3% paraformaldehyde and mounted as before.

7.6 Luminol-dependent chemiluminescence.

Haemocyte monolayers were prepared in chemiluminescence tubes (lumacuvette P, Lumac, The Netherlands) by centrifuging 750μl of 2/3 diluted haemolymph in SSS at 1000 rpm. for 5 minutes at 0°C (Kontron centrifuge). The chemiluminescence tube was kept horizontally during this procedure in order to produce a confluent monolayer along the whole length of the tube. The tube was kept on ice.

The haemocyte layers were stimulated by zymosan (6 particles per haemocyte) in SSS containing 3.3% Lumanol-100 (10^{-3} M 5'- amino-2,3-dihydro-1,4-phthalazinedione in NaOH, pH 12; Lumac). Phagocytosis was initiated (t=0) by placing the tubes in a waterbath at room temperature. Immediately after initiation of phagocytosis, luminol-dependent chemiluminescence by zymosan-stimulated haemocytes was detected using a Lumacounter 2080 (Lumac). Every 4 minutes, the mean of five measurements of 2 seconds each was recorded with a Hewlett-Packard (USA) HP 97S microcomputer. Controls consisted of incubations of haemocytes without zymosan, and of zymosan

stimulated haemocytes with SOD (final concentration 1mg.ml^{-1}) to assay for oxygen radical involvement in chemiluminescence.

RESULTS

ANIMALS

A major limitation of this study has been the availability of snail material. The main test species, *Bulinus nasutus* 1214, has proved very difficult to culture. This species, above all other bulinid species kept in culture, appears to be the most sensitive to the imposed laboratory environment. Individual *B. nasutus* 1214 snails were also more likely to die following haemolymph collection than any other species used. Despite careful maintenance in the laboratory, snails never attain the same size as their wild counterparts (e.g. *B. nasutus* sent over from Tanzania was twice the size of the laboratory line 1214). With an average of 10μl haemolymph collected from a single snail, and the minimum time interval between successive bleedings (in order to obtain a further 10μl) of 30 days, the total amount of readily available haemolymph has been severely limited. A limited store of frozen haemolymph was available, and was utilised in many of the early experiments. However, the titre of the frozen aliquots was so variable that reproduction of results proved difficult. Therefore, the frozen haemolymph samples were not used further.

Nonetheless, the extensive range of *Bulinus spp.* made available has enabled unique comparative investigations to be undertaken, and following purification of similar proteins from all species of *Bulinus*, the dependence on stocks of *B. nasutus* 1214 was relieved.

PRESENCE OF AN AGGLUTININ IN *Bulinus* HAEMOLYMPH.

Despite employing a variety of human and other vertebrate erythrocyte types as test cells in agglutination assays, agglutination could only be recorded in a single bulinid species, *B. nasutus* 1214. Fortunately, the haemolymph of this species showed

strong agglutination of human erythrocytes, with a maximum titre (1:65536) directed against human A_2 cells (Table 8).

Agglutinating activity of *B. nasutus* 1214 haemolymph was thermolabile, with a reduced titre on heating, and total loss of activity when heated to 50°C or above (Table 9). The effect of pH on this agglutinating titre shows a clear pH optimum at neutrality (Table 10). This would be expected as the pH of the haemolymph of bulinid snails is neutral. Incubation of haemolymph with the proteolytic enzymes pepsin and trypsin gave a reduction in agglutinating titre, indicating the proteinaceous structure of the lectin. With pepsin, which cleaves specifically at phenylalanine, tryptophan and tyrosine, agglutinating activity was totally lost. Trypsin is a more specific protease, cleaving at lysine and arginine residues only. Following trypsin treatment, the agglutinating titre was reduced by 75% (Table 11). Agglutinating activity was independent of the presence of divalent cations, as treatment with 5mM EDTA and EGTA had no effect on titre.

Despite employment of a wide range of inhibitory substances, the only effective inhibitors of agglutination were bovine and porcine mucins, the latter giving total inhibition at a lower concentration than the former (Table 12). Combinations of simple sugars were also used in inhibition tests, as mucins are O-glycosylated glycoproteins with multiple carbohydrate complexes. However, no inhibitory effect was observed using such combinations, indicating either that a vital carbohydrate specificity was absent, or that the spatial arrangement of the multiple carbohydrate specificities used is an important consideration for effective inhibition to occur.

TABLE 8 AGGLUTINATION OF HUMAN ERYTHROCYTES BY *B. nasutus* 1214 HAEMOLYMPH

CELL TYPE	TITRE
A_1	131 072
A_2	262 144
B	131 072
O	65 536

Titres are expressed as the reci procal of the last dilution giving total agglutination

TABLE 9 EFFECT OF TEMPERATURE ON HAEMAGGLUTININ TITRE

TEMPERATURE °C	TITRE
22	458 752
37	229 376
42	229 376
50	0
55	0
60	0
70	0
control	458 752

Titers are expressed as the reciprocal of the last dilution giving total agglutination

TABLE 10 EFFECT OF pH ON HAEMAGGLUTININ TITRE

pH	TITRE
3	6 400
5	25 600
7	409 600
9	102 400
11	800
control	409 600

Titres are expressed as the reciprocal of the end dilution giving total agglutination

TABLE 11 EFFECT OF ENZYME TREATMENT ON HAEMAGGLUTININ TITRE

ENZYME	TITRE
trypsin	28 672
pepsin	0
control	57 344

Enzymes at 1 $mg.ml^{-1}$ concentration in PBSA

Titres are expressed as the reciprocal of the end dilution giving total agglutination

TABLE 12 INHIBITION OF HAEMAGGLUTINATING ACTIVITY BY COMPLEX POLYSACCHARIDES

TEST		TITRE
Bovine mucin	1 $mg.ml^{-1}$	0
	0.1 $mg.ml^{-1}$	0
	0.01 $mg.ml^{-1}$	512
	0.001 $mg.ml^{-1}$	512
Porcine mucin	1 $mg.ml^{-1}$	0
	0.1 $mg.ml^{-1}$	0
	0.01 $mg.ml^{-1}$	0
	0.001 $mg.ml^{-1}$	512
control		512

Titres are expressed as the reciprocal of the end dilution giving total agglutination

THE AGGLUTININ CAN BE ABSORBED OUT OF THE HAEMOLYMPH.

Following incubation of *B. nasutus* 1214 haemolymph with a pellet of miracidia of *S. margrebowiei*, no agglutinating activity was recorded in the supernatant following re-pelleting of the miracidia.(Table 13).

Either all the molecules had bound to the miracidial surface, or a sub-agglutinating concentration of the agglutinin remained in the supernatant. SDS PAGE analysis of miracidia pre- and post-incubation is shown in Figure 8. A greater number of protein bands are evident in the post-incubation sample (lane 1), presumably reflecting snail components adsorbed to the surface of the miracidia. In particular, a strongly staining extra band at an apparent Mr of 135k is noticeable in the profile of this sample.

Agglutinating activity could also be adsorbed out of the haemolymph by preincubation with human erythrocytes (Table 13).

Four of human ABO blood groups erythrocyte types were used in a series of adsorption experiments, preadsorbing with one cell type, and testing the supernatant with each of the remaining types. However, all agglutinating activity was always adsorbed out with the preincubation cell type, indicating the presence of a common haemagglutinin (or family of agglutinins) present in the haemolymph. When post-adsorbed samples were applied to a Superose 12 gel filtration column, the void agglutinating peak was seen to be absent from the trace (Figure 9). A large peak was evident at an approximate M_r of 18k. This peak was expected to be vertebrate haemoglobin. A preparation of vertebrate haemoglobin was prepared by lysing A_2 erythrocytes, and when this was applied to the column, the same 18k peak was evident (Figure 10). Thus the absence of the void agglutinating peak in the initial experiment indicates that the agglutinating molecules had adsorbed to the surfaces of the erythrocytes.

TABLE 13 ADSORPTION OF HAEMAGGLUTININ ACTIVITY

Cell-free haemolymph of *B. nasutus* 1214 was incubated with the test material for 30 mins. Following sedimentation of the test material, the supernatant was assayed for activity with human A1 cells.

TEST	TITRE
excess A2 erythrocytes	0
S. margrebowiei miracidia	0
CONTROL	32768

FIGURE 8. SDS PAGE 7.5% Adsorption of haemolymph proteins by miracidia. Newly-hatched miracidia of *S. margrebowiei* were incubated with *B. nasutus* 1214 cell-free haemolymph for 1 hour. The miracidia were sedimented, and the pellet prepared for SDS PAGE (lane 1). Extracts of control miracidia with no exposure to haemolymph proteins are shown in lane 2. Gel silver stained.

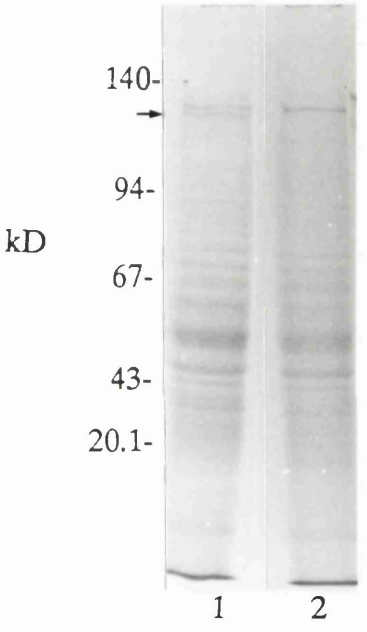
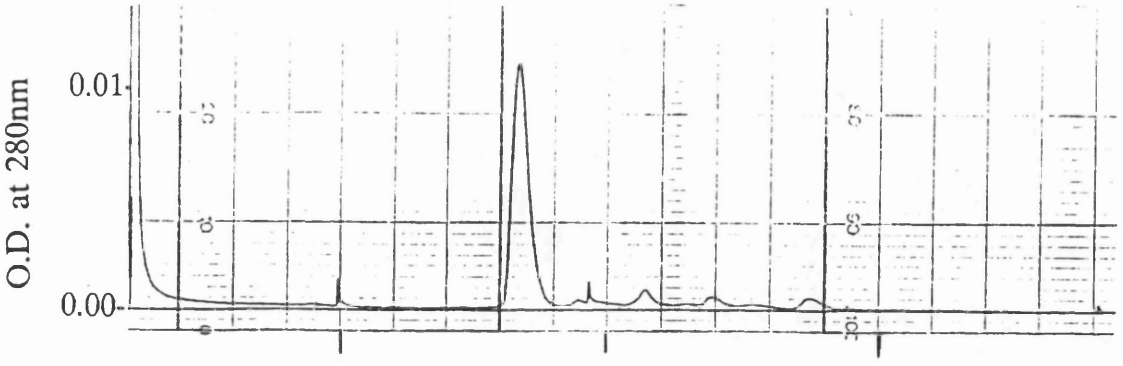

140-
94-
kD
67-
43-
20.1-
1
2

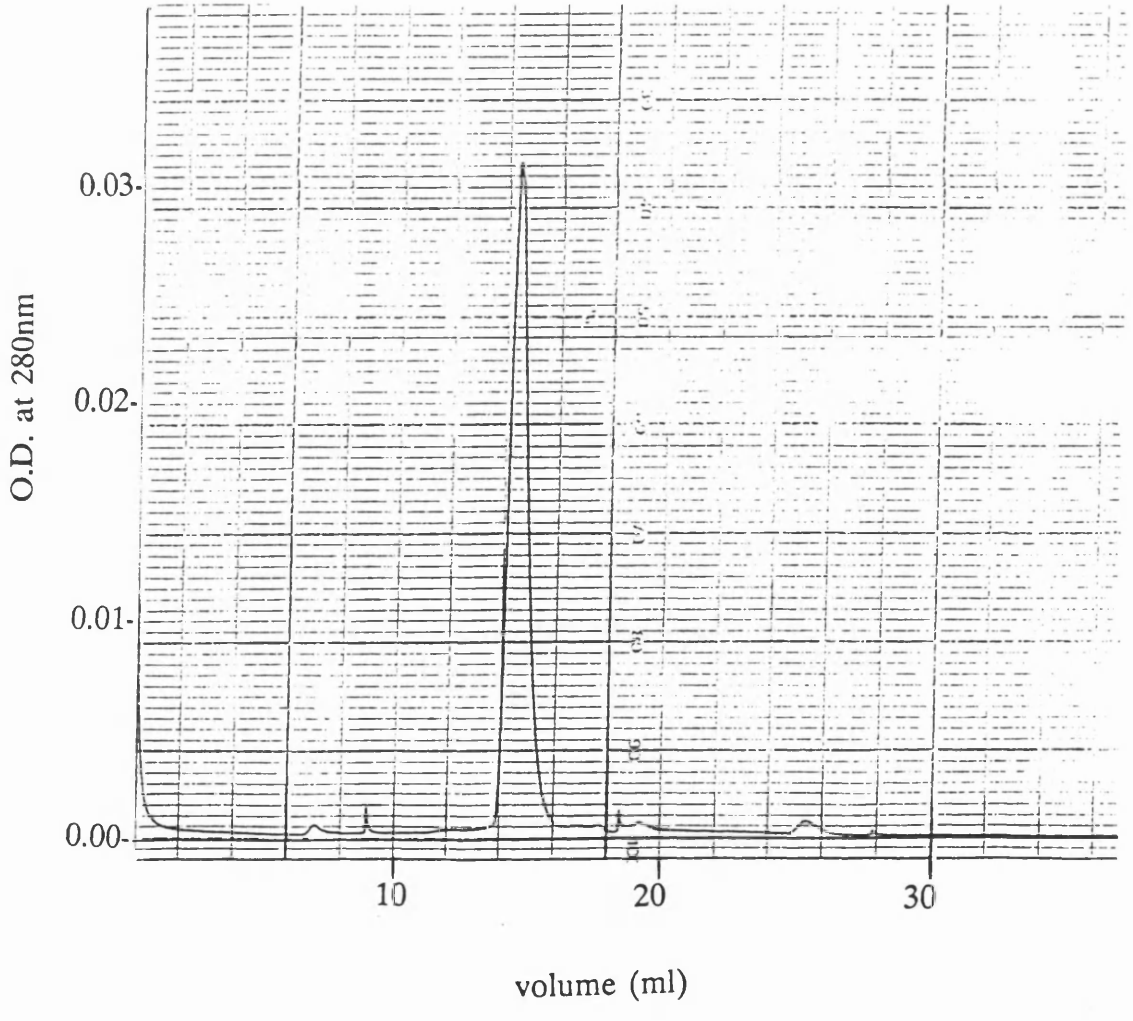
FIGURE 9

SAMPLE:	*B. nasutus* 1214 200μl 1/64 diluted preadsorbed with human A_2 cells
COLUMN:	SUPEROSE 12 HR10/30 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE:	0.5ml.min^{-1}
SENSITIVITY:	0.05

FIGURE 10

SAMPLE:	lysed human A_2 erythrocytes 200μl
COLUMN:	SUPEROSE 12 HR10/30 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE:	0.5ml.min^{-1}
SENSITIVITY:	0.05


O.D. at 280nm
0.01
0.00


O.D. at 280nm
0.03
0.02
0.01
0.00
10
20
30
volume (ml)

AGGLUTINATING ACTIVITY IS CONFINED TO THE HAEMOLYMPH.

Agglutinating activity was recorded only from the haemolymph of *B. nasutus* 1214. Extracts made from the egg masses would not agglutinate human, mouse, rabbit or sheep erythrocytes.

UNCHALLENGED HAEMOLYMPH DOES NOT EXHIBIT ANTIBACTERIAL ACTIVITY.

A confluent layer of bacterial growth was observed over the whole of the agar plate. There was no observed antibacterial activity (i.e. clearance rings) around the test wells. Thus haemolymph from unchallenged snails did not contain active levels of antibacterial substances.

FAST PROTEIN LIQUID CHROMATOGRAPHY.

As a first step in purification of plasma components using the FPLC, a MONO Q anion exchange column was the preferred choice, as most proteins will bind to this column and can be eluted off by a concentration gradient of anion e.g.Cl^-. Cell-free haemolymph of *Bulinus spp.* was known to contain numerous proteins, as SDS PAGE analysis shows (Figure 11). When cell-free haemolymph collected from *Biomphalaria glabrata* 1144 was applied to the MONO Q column, there was very little resolution of plasma components on elution with a linear gradient (0-250mM NaCl over 25 minutes) (Figure 12). In fact, a single major peak eluting at 330mM salt was found to comprise the majority of haemolymph proteins following assay by SDS PAGE. Similarly, when cell-free haemolymph from *B. nasutus* 1214 was applied to the MONO Q column and the identical linear salt gradient run, the great majority of proteins eluted in a single peak at 350mM salt (Figure 13). It was therefore

FIGURE 11. SDS PAGE 7.5% Comparison of cell-free haemolymph of *Bulinus spp.* Cell-free haemolymph of bulinid species were applied at equivalent dilution. *B. truncatus* 1521 (lane 1), *B. ugandae* 1250 (lane 2), *B. africanus* 1227 (lane 3), *B. tropicus* 28 (lane 4), *B. umbilicatus* 478 (lane 5), *B. obtussispira* 1476 (lane 6), *B. globosus* 1381 (lane 7). Gel stained with Coomassie Brilliant Blue.

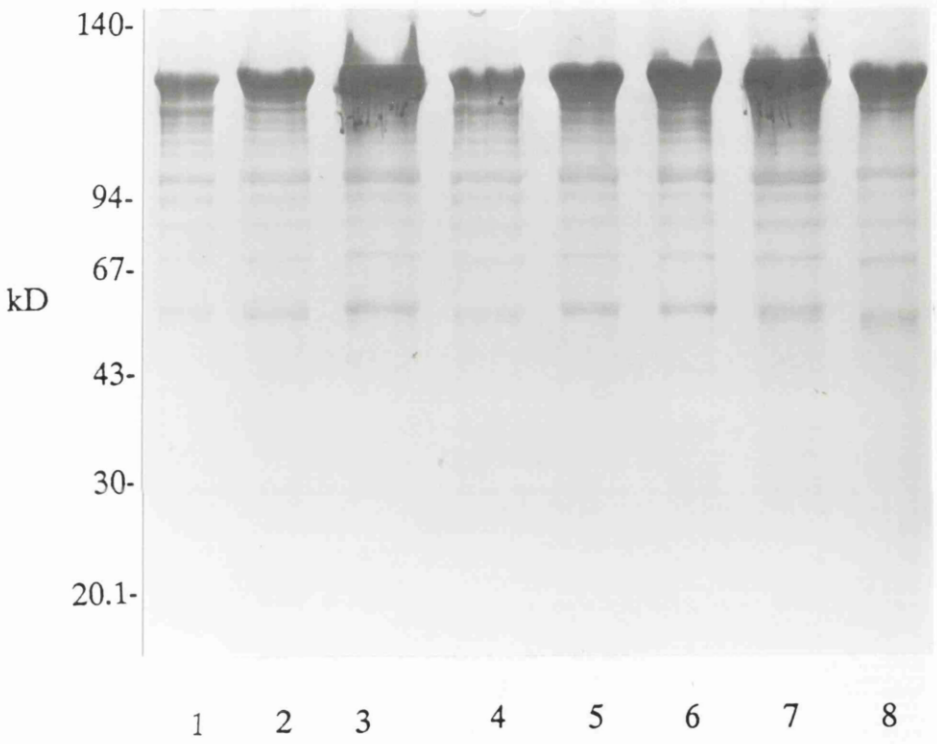
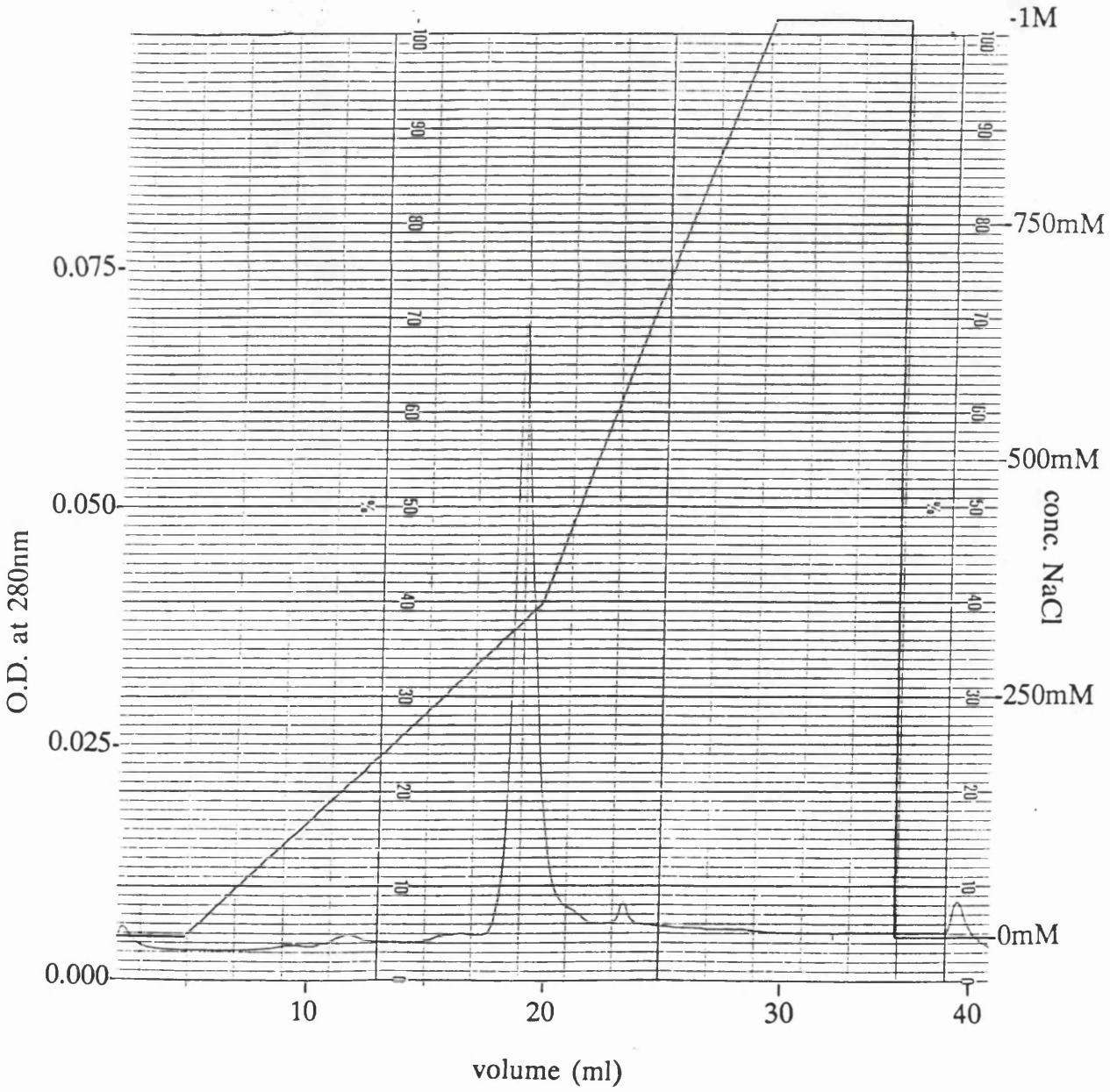

140-
94-
67-
kD
43-
30-
20.1-
1
2
3
4
5
6
7
8

FIGURE 12

SAMPLE:	*Biomphalaria glabrata* 1444 10μl in 490μl buffer A
COLUMN:	MONO Q HR5/5 anion exchange
BUFFER A:	20mM Tris pH 7.0
BUFFER B:	20mM Tris pH 7.0 + 1M NaCl
FLOW RATE:	1ml.min^{-1}
SENSITIVITY:	0.1



necessary to modify the characteristics of the eluting gradient of sodium chloride in order to attempt to resolve the polypeptides identified by SDS PAGE. Figure 14 shows the trace resulting from a shallower eluent gradient (250-350mM NaCl over 10 minutes) for *B. nasutus* 1214 cell-free haemolymph. It is now apparent that the single peak in Figure 13 has been resolved into three distinct peaks. The three peaks show that different proteins (either individual polypeptides or sets of different polypeptides) are being resolved by elution from the column at slightly different concentrations of salt, i.e.the first peak is eluting at 290mM, the second at 305mM and the third at 320mM salt. The eluent gradient was further reduced (250-350mM NaCl over 25 minutes), and the elution profile under these conditions is depicted in Figure 15. Several peaks are now evident, indicating the differential elution of proteins with increasing salt concentration. The proteins are being eluted between 250mM and 320mM salt, the same range as with the first elution gradient (Figure 13), but due to the new gradient occurring over a greater time interval (20 minutes as opposed to 2 minutes) individual proteins can be specifically eluted without the interference of other proteins that will elute soon after. Under the first elution gradient conditions, proteins were eluted concurrently, as there was insufficient time to allow the minor variations in charge to facilitate elution into separate fractions.

Using the MONO Q column alone, it was not possible to isolate the agglutinating molecules in a single peak. The agglutinating fractions were passed onto a Superose 12 gel filtration column, but even at the highest sensitivity, only very small peaks were obtained, none of which had any detectable agglutinating activity. The dilution occurring during this two column procedure was too great for the small volumes of haemolymph available for application to the columns (i.e. 10μl samples were used), and thus alternative purification procedures were sought.

FIGURE 13

SAMPLE:	*B. nasutus* 1214 10μl in 490μl buffer A
COLUMN:	MONO Q HR5/5 anion exchange
BUFFER A:	20mM Tris pH 7.0
BUFFER B:	20mM Tris pH 7.0 + 1M NaCl
FLOW RATE:	1ml.min^{-1}
SENSITIVITY:	0.1

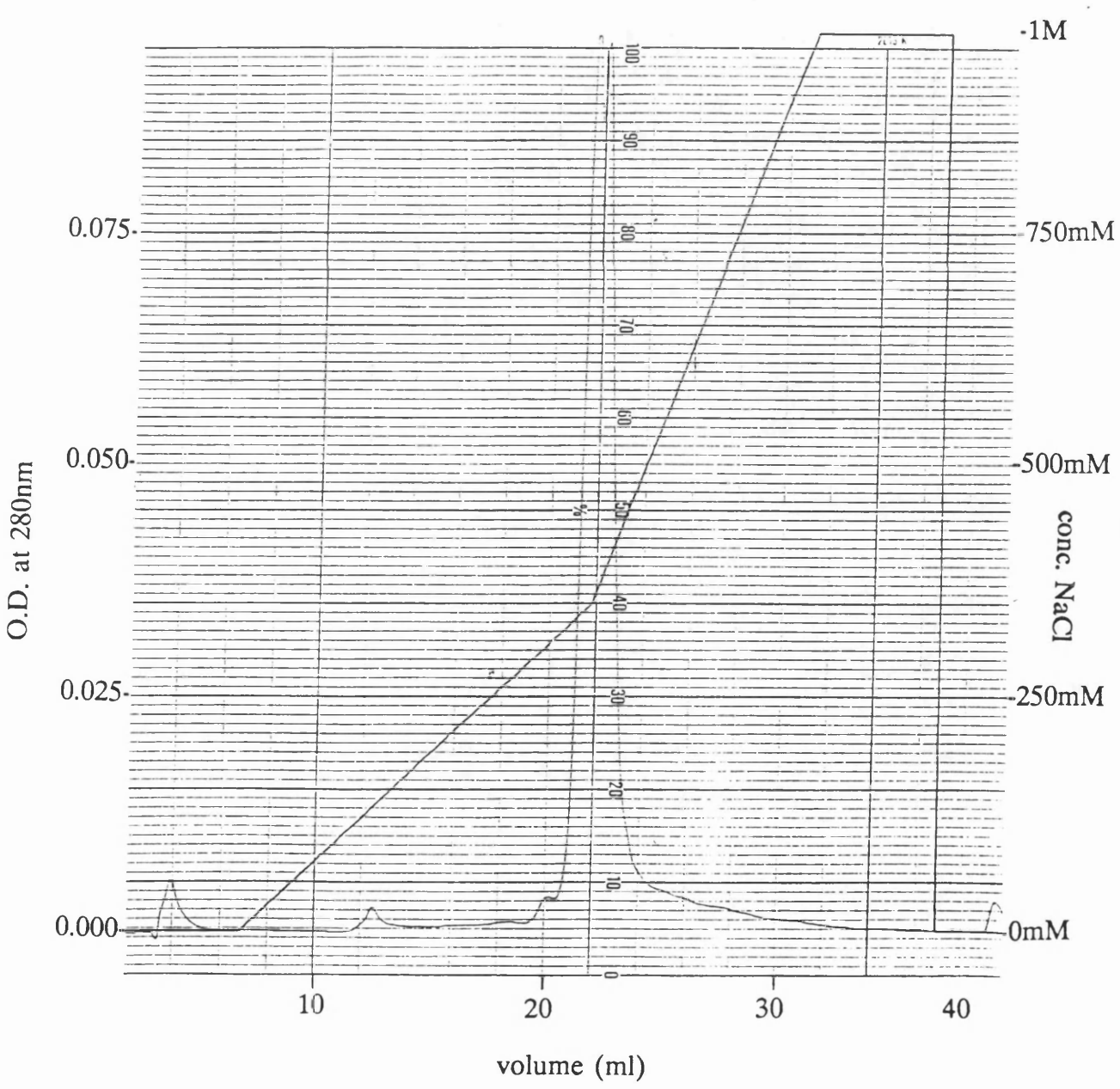
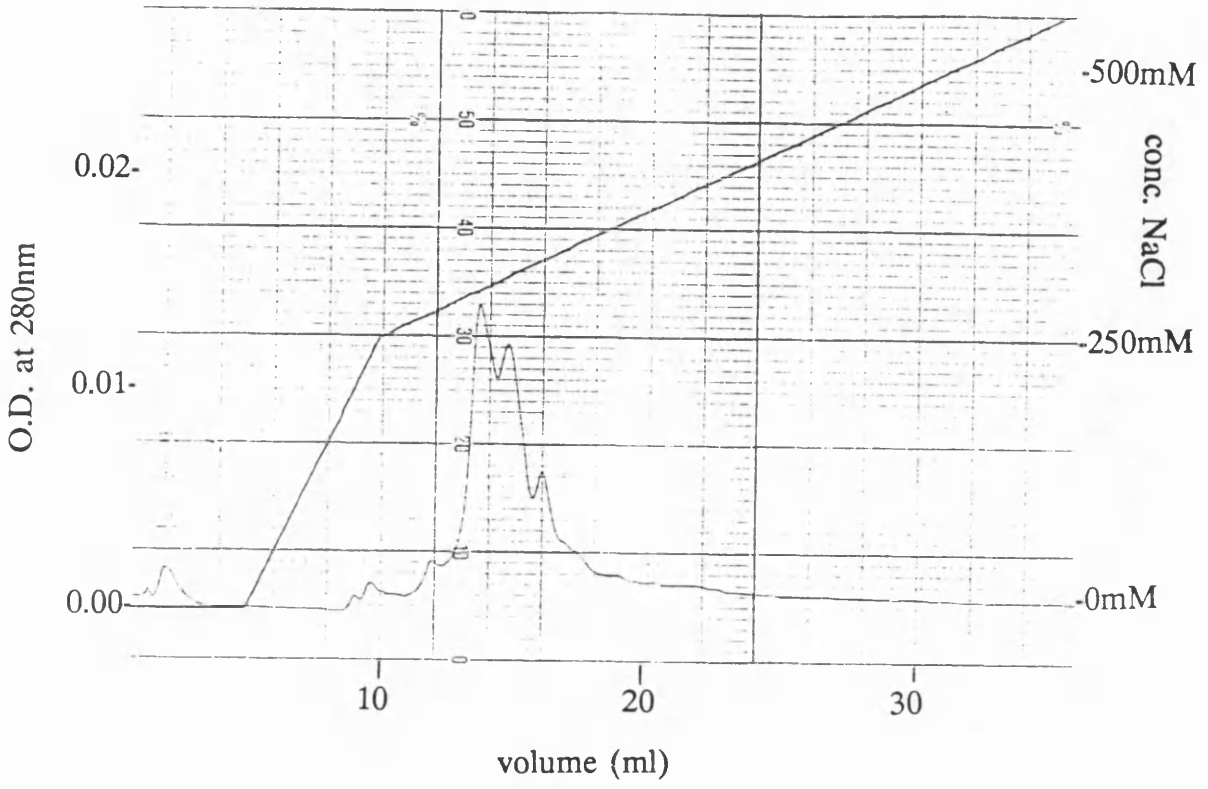


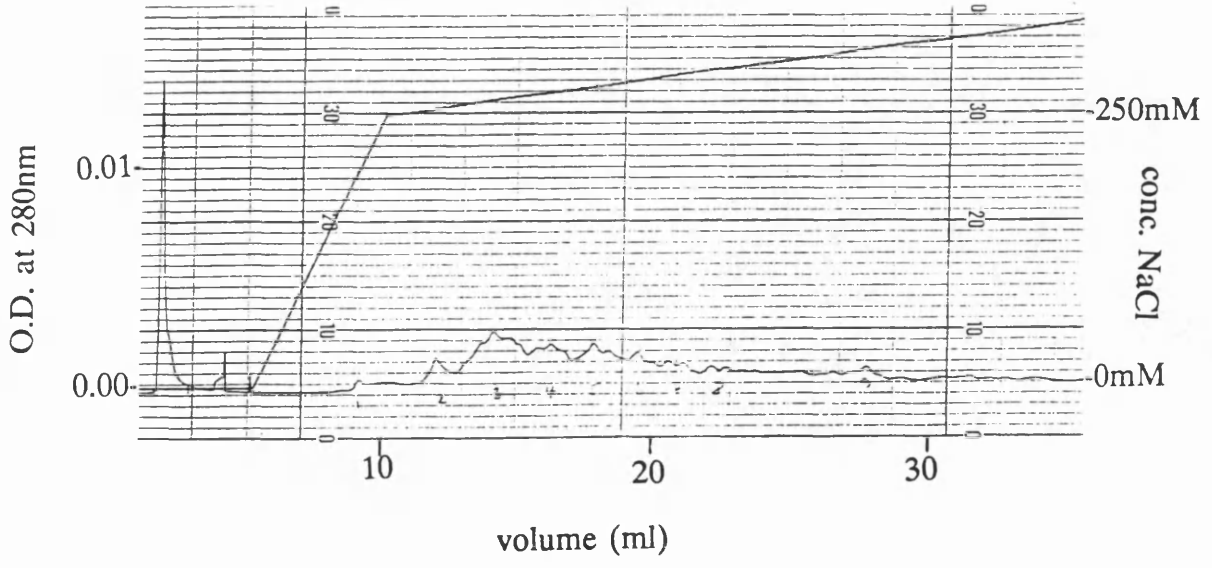
FIGURE 14

SAMPLE:	*B. nasutus* 1214 10μl in 490μl buffer A
COLUMN:	MONO Q HR5/5 anion exchange
BUFFER A:	20mM Tris pH 7.0
BUFFER B:	20mM Tris pH 7.0 + 1M NaCl
FLOW RATE:	$1ml.min^{-1}$
SENSITIVITY:	0.2

FIGURE 15

SAMPLE:	*B. nasutus* 1214 5μl in 20μl buffer A
COLUMN:	MONO Q HR5/5 anion exchange
BUFFER A:	20mM Tris pH 7.0
BUFFER B:	20mM Tris pH 7.0 + 1M NaCl
FLOW RATE:	$1ml.min^{-1}$
SENSITIVITY:	0.5


-500mM
conc. NaCl
0.02-
0.01-
0.00-
-250mM
-0mM
O.D. at 280nm
10
20
30
volume (ml)


-250mM
conc. NaCl
0.01-
0.00-
-0mM
O.D. at 280nm
10
20
30
volume (ml)

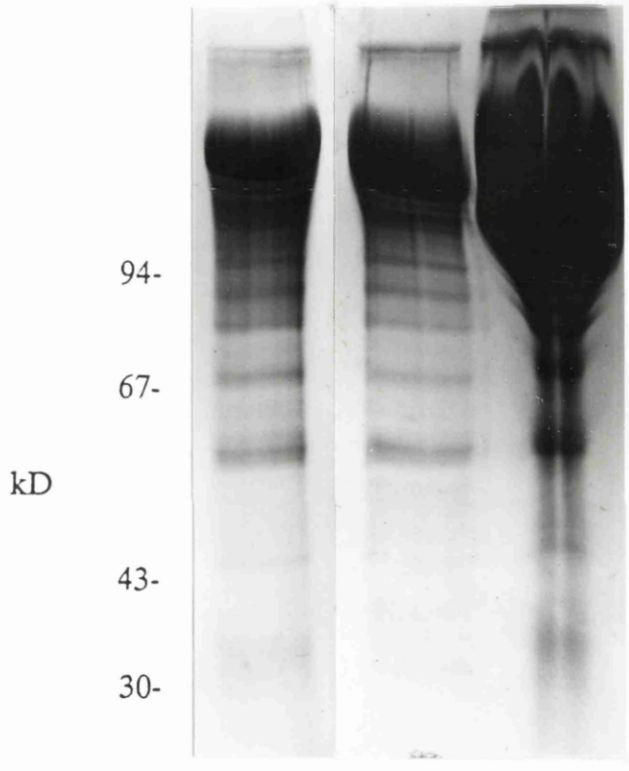
ULTRACENTRIFUGATION SEDIMENTS HAEMOGLOBINS.

SDS PAGE shows that the polypeptide composition of haemolymph is dominated by haemoglobins. These smear the gel and distort the resolution of other lower M_r bands. The abundance and physical properties of snail haemoglobins have been a stumbling block so far in all previous attempts to analyse the soluble proteins of haemolymph. The range of plasma proteins and their respective roles in the haemolymph of red-blooded snails is therefore still undetermined. Ultracentrifugation was employed in an attempt to remove the high molecular mass proteins, the majority of which are haemoglobin molecules, thus facilitating resolution of all other plasma components. Haemoglobins were indeed present in the resulting pellet fraction following this procedure as this was clearly red coloured, and the supernatant appeared colourless. Mammalian haemoglobin is not pelleted under identical conditions.

The pellet and supernatant fractions of cell-free haemolymph of *Biomphalaria glabrata* 1144 resulting from centrifugation at 178000 x g for 30 minutes were analysed by SDS PAGE (Figure 16). It is clear that there are a very high concentration of proteins in the pellet sample, individual protein bands not being clearly visible. The proteins in the supernatant fraction also appear to have been concentrated as compared with diluted whole haemolymph (lanes 1 and 2) as shown by the increased intensity in staining. However, the volumes of the respective pellet and supernatant samples has been reduced compared to that of the whole haemolymph sample, and thus one would expect there to be a greater intensity of staining of the former samples due to this concentration effect. The apparent smearing of both the pellet and supernatant lanes on the gel also reflects the degradation of certain proteins. This is further emphasised by the occurrence of

FIGURE 16. SDS PAGE 7.5% Separation of plasma components by ultracentrifugation.

Cell-free haemolymph of *Biomphalaria glabrata* 1144 (lane 1) was ultracentrifuged at 178 000 xg for 30 minutes. Samples of the resultant supernatant (lane 2) and pellet (lane 3) fractions were loaded at identical dilution to the control. Gel stained with Coomassie Brilliant Blue.


94-
67-
kD
43-
30-
1
2
3

many more lower M_r bands in the pellet and supernatant fractions as compared to the whole haemolymph sample.

As each sample analysed by SDS PAGE looked essentially similar, the effect of ultracentrifugation has not been clarified. Therefore, the pellet and supernatant fractions were analysed by FPLC gel filtration on Superose 12 which fractionates polypeptides according to their M_r between $1x10^3$ and $3x10^5$k. Whole haemolymph was also applied to the column by way of comparison. Figure 17a shows the trace for whole cell-free haemolymph of *B. natalensis* 272. The major peak is the void peak (i.e. M_r greater than $2x10^5$k), but a number of lower molecular mass components were also present, as represented by the numerous smaller peaks. Figures 17b and 17c show the traces of the pellet and supernatant fractions following ultracentrifugation of the initial sample. The majority of the proteins appear in the void peak of the pellet fraction. With the supernatant fraction, there is a small void peak, and all the lower molecular mass peaks are evident. Superimposition of the pellet and supernatant traces on the trace for whole haemolymph shows a perfect agreement of the separation of high and low molecular mass components into the pellet and supernatant fractions respectively.

Cell-free haemolymph from *B. globosus* 1381 was used in identical experiments, and the traces for whole haemolymph, pellet and supernatant fractions following gel filtration are shown in Figures 18a, 18b and 18c respectively. For the whole haemolymph sample, the majority of the protein is again registered in the void peak, with numerous lower molecular mass peaks (with higher protein concentrations than their *B. natalensis* 272 counterparts) in evidence. For the pellet fraction, the void peak is very large, but unlike with the *B. natalensis* 272 pellet fraction, lower molecular mass peaks are evident with the *B. globosus* 1381 pellet fraction. These

FIGURE 17a

SAMPLE:	*B. natalensis* 272 200µl 1/64 diluted haemolymph
COLUMN:	SUPEROSE 12 HR10/10 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE:	0.5ml.min^{-1}
SENSITIVITY:	0.05

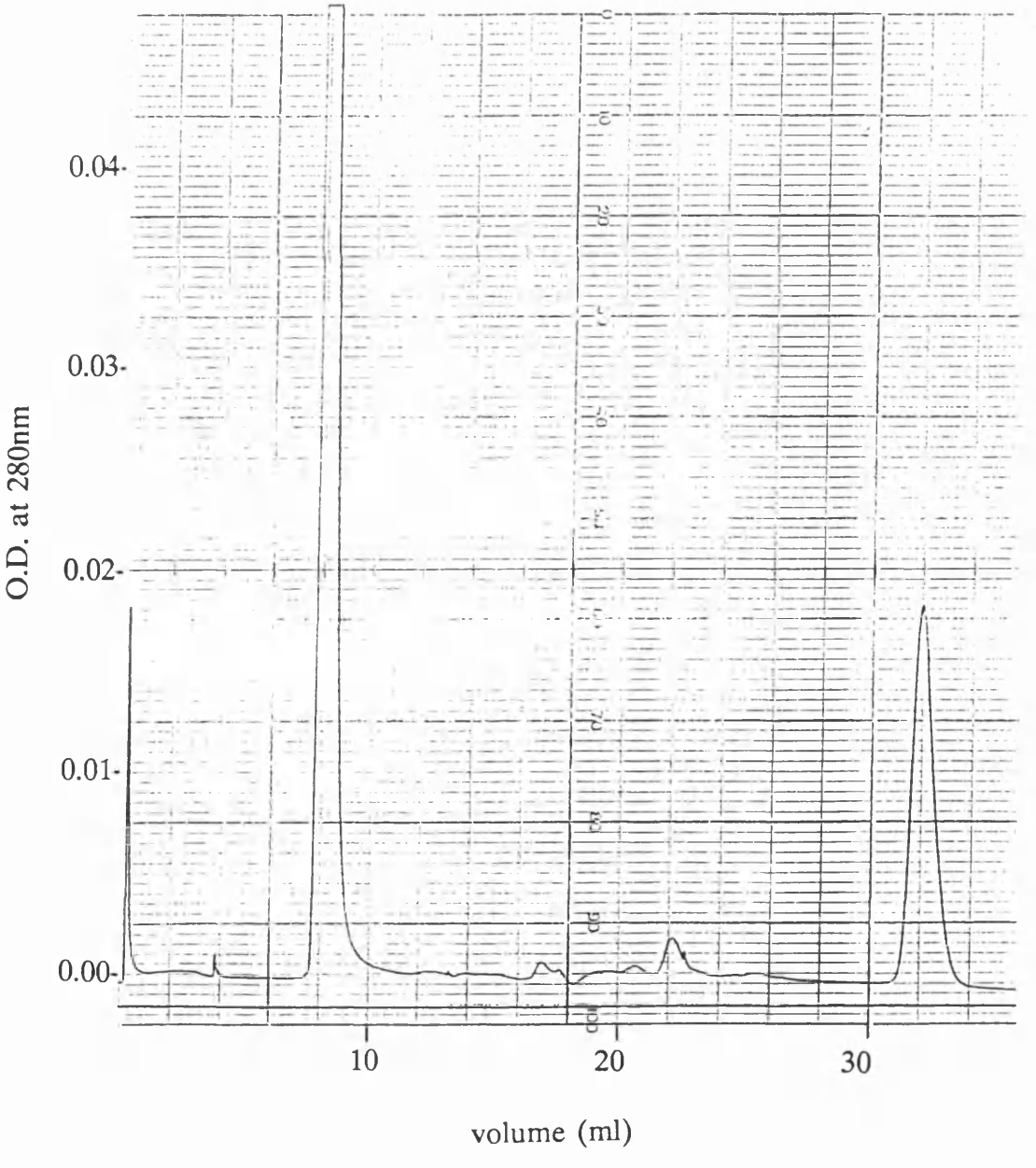
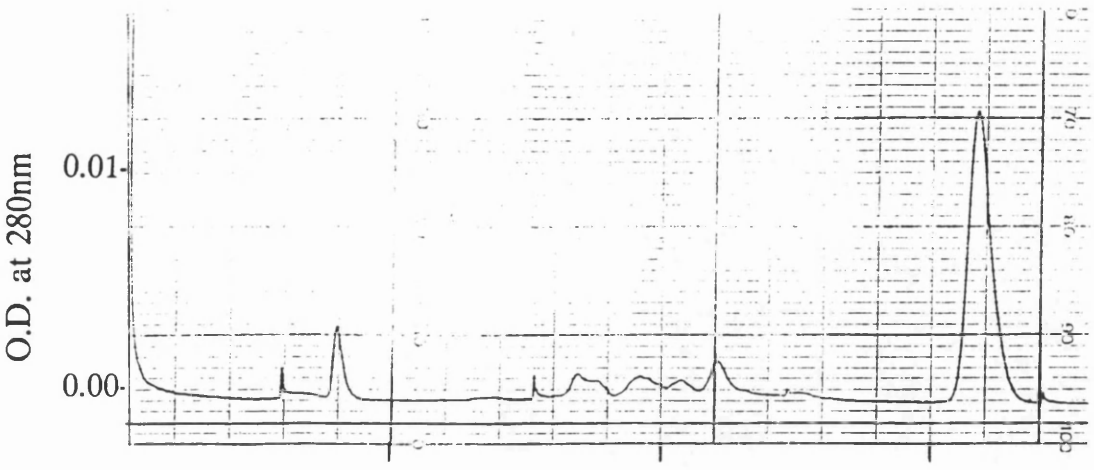


FIGURE 17b

SAMPLE:	*B. natalensis* 272 200μl 1/64 airfuge supernatant
COLUMN:	SUPEROSE 12 HR10/30 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE:	$0.5ml.min^{-1}$
SENSITIVITY:	0.05

FIGURE 17c

SAMPLE:	*B. natalensis* 272 100μl airfuge pellet
COLUMN:	SUPEROSE 12 HR10/30 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE:	$0.5ml.min^{-1}$
SENSITIVITY:	0.02


O.D. at 280nm
0.01
0.00

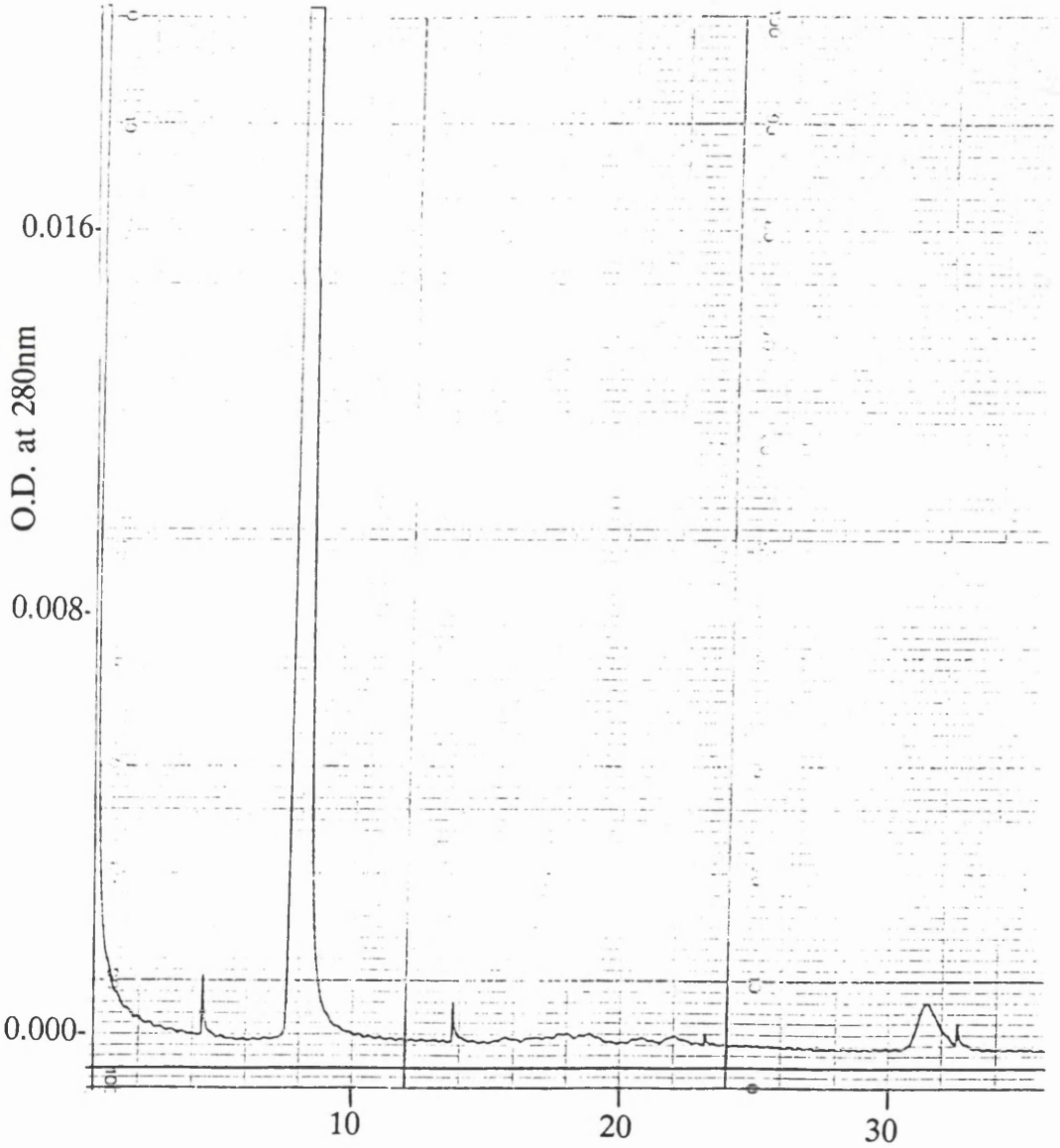

O.D. at 280nm
0.016
0.008
0.000
10
20
30
volume (ml)

FIGURE 18a

SAMPLE:	*B. globosus* 1381 100μl 1/64 diluted haemolymph
COLUMN:	SUPEROSE 12 HR10/10 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150 mM NaCl
FLOW RATE:	0.5ml.min^{-1}
SENSITIVITY:	0.05

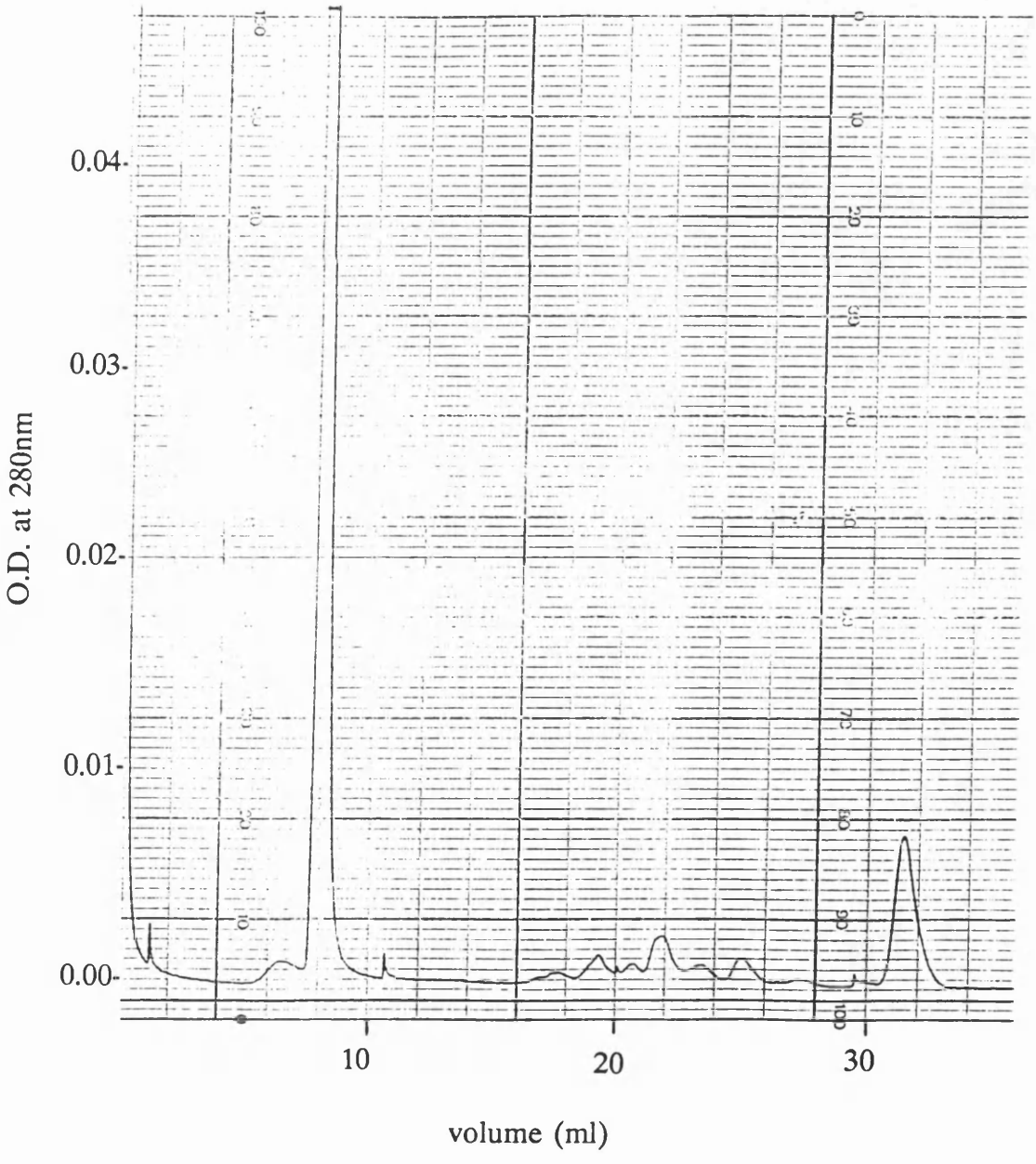
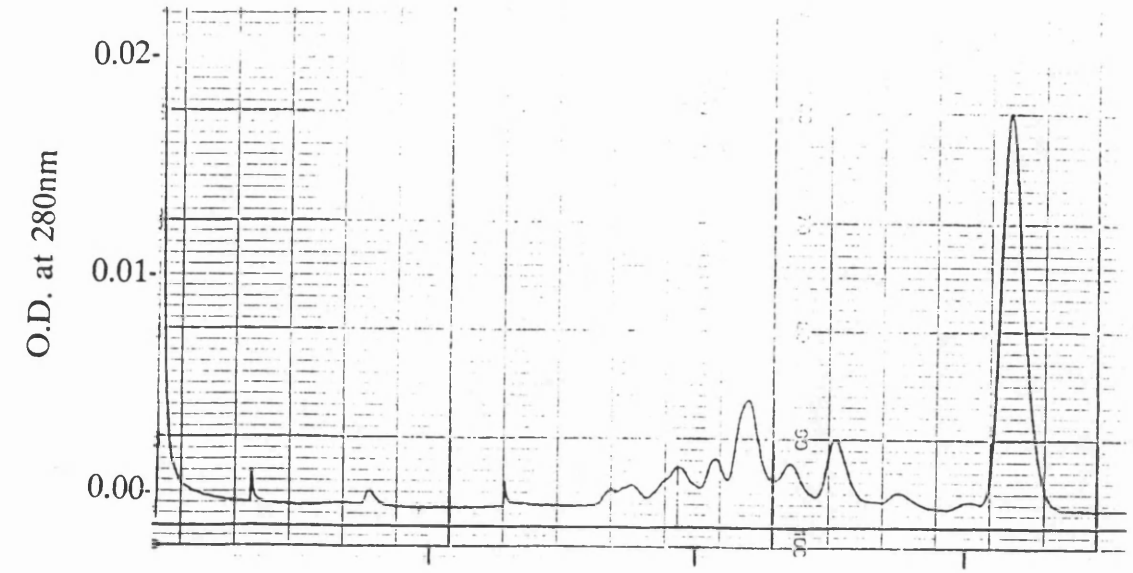


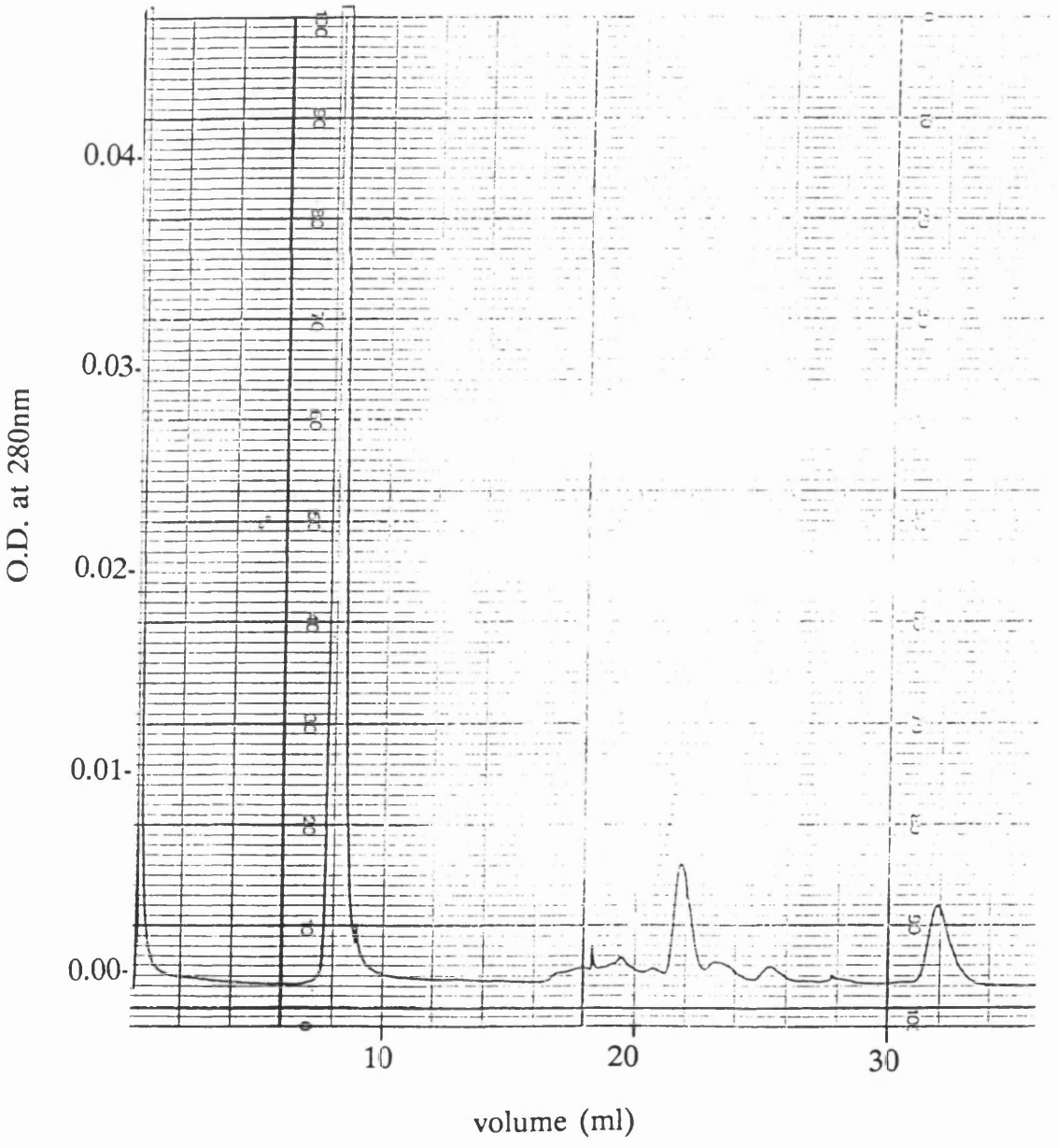
FIGURE 18b

SAMPLE:	*B. globosus* 1381 200μl 1/50 diluted airfuge supernatant
COLUMN:	SUPEROSE 12 HR10/30 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE:	0.5ml.min.$^{-1}$
SENSITIVITY:	0.05

FIGURE 18c

SAMPLE:	*B. globosus* 1381 100μl 1/50 diluted airfuge pellet
COLUMN:	SUPEROSE 12 HR10/30 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE:	0.5ml.min^{-1}
SENSITIVITY:	0.05


O.D. at 280nm
0.02
0.01
0.00


O.D. at 280nm
0.04
0.03
0.02
0.01
0.00
10
20
30
volume (ml)

lower molecular mass peaks are even more apparent in the *B. globosus* 1381 supernatant fraction, in which a void peak is hardly present.

The elution profiles for the pellet and supernatant fractions of *B. nasutus* 1214 cell-free haemolymph subjected to size chromatography give a similar picture (Figures 19a, 19b and 19c). The void peak in the pellet fraction shows the major concentration of protein. The relatively sizeable lower molecular mass peaks in the supernatant fraction may reflect degradation products, as this haemolymph sample was a stored (frozen) sample, while all the previous samples had been collected fresh for the experiments. When the eluted fractions for both *B. nasutus* 1214 pellet and supernatant fractions were assayed for agglutinating activity, activity was recorded in the void peaks of both, none being recorded in any of the resolved lower molecular mass peaks.

The use of ultracentrifugation was intended to help resolve plasma components, and in particular to separate the dominating haemoglobins from the agglutinin(s). Ultracentrifugation did not achieve the required level of resolution. Haemoglobin was confined to the pellet following this procedure, thus allowing resolution of components in the supernatant free from haemoglobin contamination. However, as agglutinating activity was detected in the pellet fraction for *B. nasutus* 1214 haemolymph, it appears that the agglutinin(s) are not being separated from the haemoglobin molecules by this treatment. The presence of agglutinating activity in the pellet fraction could be explained in several ways (i) the agglutinin(s) are of sufficiently high molecular mass to cause their own sedimentation; (ii) during the sedimentation of haemoglobin molecules, the agglutinin(s) are forcibly sedimented due to the abundance of the former type; or (iii) there are molecular interactions

FIGURE 19a

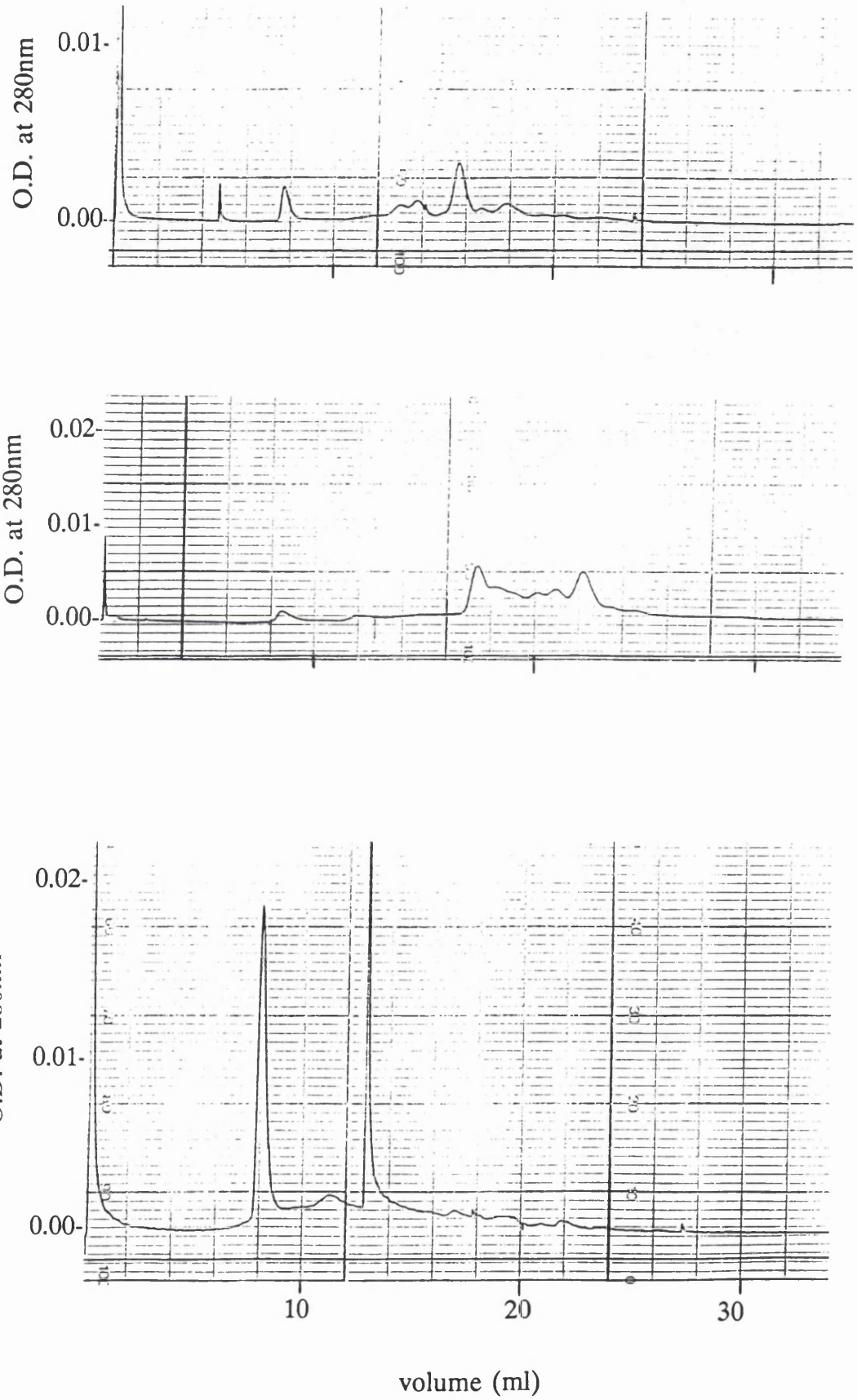
SAMPLE: *B. nasutus* 1214 100μl 1/64 diluted
COLUMN: SUPEROSE 12 HR10/30 gel filtration
BUFFER: 50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE: 0.5ml.min^{-1}
SENSITIVITY: 0.05

FIGURE 19b

SAMPLE: *B. nasutus* 1214 150μl in 350μl buffer 1/64 diluted airfuge supernatant
COLUMN: SUPEROSE 12 HR10/30 gel filtration
BUFFER: 50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE: 0.5ml.min^{-1}
SENSITIVITY: 0.1

FIGURE 19c

SAMPLE: *B. nasutus* 1214 20μl in 100μl buffer 1/64 diluted airfuge pellet
COLUMN: SUPEROSE 12 HR10/30 gel filtration
BUFFER: 50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE: 0.5ml.min^{-1}
SENSITIVITY: 0.05


O.D. at 280nm
0.01-
0.00-
O.D. at 280nm
0.02-
0.01-
0.00-
O.D. at 280nm
0.02-
0.01-
0.00-
10
20
30
volume (ml)

between the haemagglutinin and haemoglobins which are not disrupted by ultracentrifugation. Purification of the *B. nasutus* 1214 agglutinin therefore required a different approach.

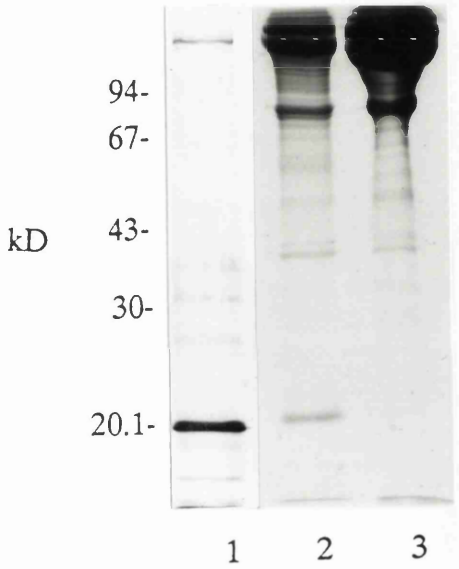
PURIFICATION OF THE *B. nasutus* 1214 AGGLUTININ.

The concanavalin A-sepharose 4B beads became coloured red following incubation for 30 minutes with *B. nasutus* 1214 haemolymph, and after two repeats of such treatments the haemolymph supernatant was colourless. Agglutinating activity was recorded in the supernatant, although the titre was reduced threefold from 1:512 to 1:64. This reduction in titre was due either to dilution of the haemolymph during the bead incubation, or due to loss of agglutinating molecules onto the beads by specific or non-specific interactions with haemoglobin molecules bound to the beads. This adsorption was inhibited by 200mM α-methyl-mannoside. The lentil lectin-sepharose 4B beads were also coloured red after incubation with *B nasutus* 1214 haemolymph, but after two such treatments the supernatant was still coloured red, indicating the presence of haemoglobin. On the other hand, unmodified sepharose 4B beads, used as a control for non-specific absorption to the bead matrix did not become coloured red following incubation with *B. nasutus* 1214 haemolymph, nor was there any loss in agglutinating titre of the supernatant. SDS PAGE analysis of these samples is shown in Figure 20.

This indicates that with the lectin-conjugated beads, specific reactions are occurring between haemoglobin molecules and the concanavalin A molecules on the surface of the beads. One presumes that these specific reactions involve the sugar residues, and in particular the mannose residues, of the haemoglobin molecules.

FIGURE 20. SDS PAGE 7.5% Adsorption of plasma components by sepharose 4B beads.

Samples of haemolymph of *B. nasutus* 1214 were incubated with concanavalin A-sepharose 4B, lentil lectin-sepharose 4B, or unconjugated sepharose 4B beads for 30 minutes. The beads were sedimented, and aliquots of the resultant supernatants applied to the gel at equivalent dilution; con A bead supernatant (lane 1), lentil lectin bead supernatant (lane 2), control (lane 3). Gel stained with Coomassie Brilliant Blue.


kD
94-
67-
43-
30-
20.1-
1
2
3

When the concanavalin A-sepharose 4B bead supernatant was applied to a Superose 12 gel filtration column (optimal separation $1x10^3$-$3x10^5$k), agglutinating activity was detected only in the fractions comprising the void volume (Figure 21). When the void peak fractions were passed over a Superose 6 gel filtration column (optimal separation range $5x10^3$-$5x10^6$), a single peak was observed at an apparent M_r of 210k (Figure 22). The eluent fractions containing this peak showed agglutinating activity (Titre 1:512). Following SDS PAGE analysis (using 7.5% resolving gels) of the concanavalin A sepharose 4B bead supernatant and FPLC gel chromatography purified fractions (Figure 23) a number of protein bands are recorded in the former sample, with a single band at an apparent M_r of 135k with the latter sample. As this FPLC purified fraction agglutinated human A2 erythrocytes, this sample is deduced to be a homogenous preparation of the *B. nasutus* 1214 agglutinin.

Using the two step purification of concanavalin-A sepharose 4B and Superose 6, the haemagglutinin present in the haemolymph of *B. nasutus* 1214 has been purified to homogeneity. The result of each of these steps is clearly visible by reference to SDS PAGE analysis (Figure 23). The cell-free haemolymph is seen to comprise of a very complex mixture of proteins, with an extensive range of M_r (lane 1). There is a great concentration of proteins especially in the high M_r range. The supernatant resulting from the treatment with Concanavalin A-sepharose 4B beads clearly shows that the majority of proteins have been adsorbed onto the beads, leaving a major band at an M_r of 135k, and a few other bands of lower M_r (lane 2). The final gel filtration by FPLC on Superose 6 shows a single band at an M_r of 135k (lane 3).

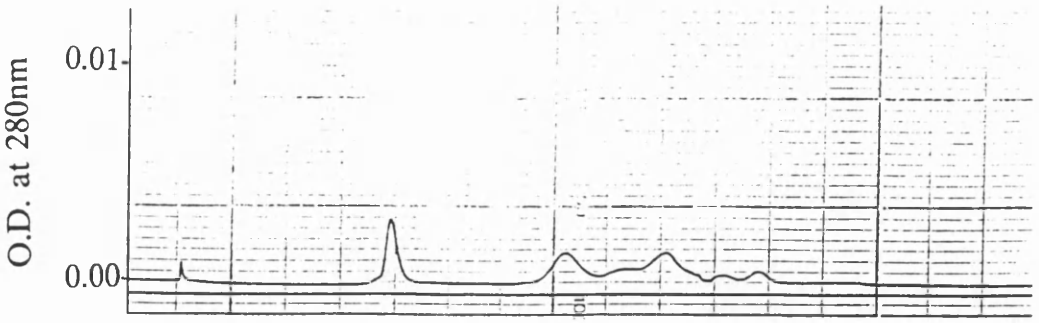
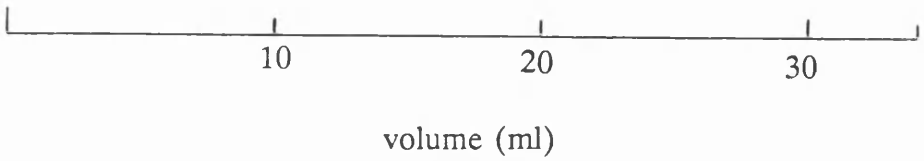


FIGURE 21

SAMPLE:	*B. nasutus* 1214 200μl Con A supernatant
COLUMN:	SUPEROSE 12 HR10/10 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150 mM NaCl
FLOW RATE:	0.5ml.min^{-1}
SENSITIVITY:	0.05



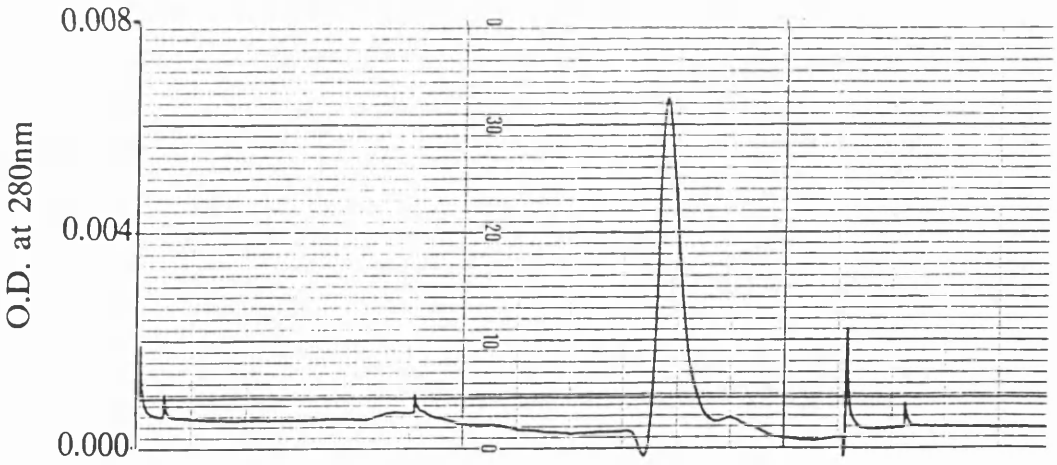
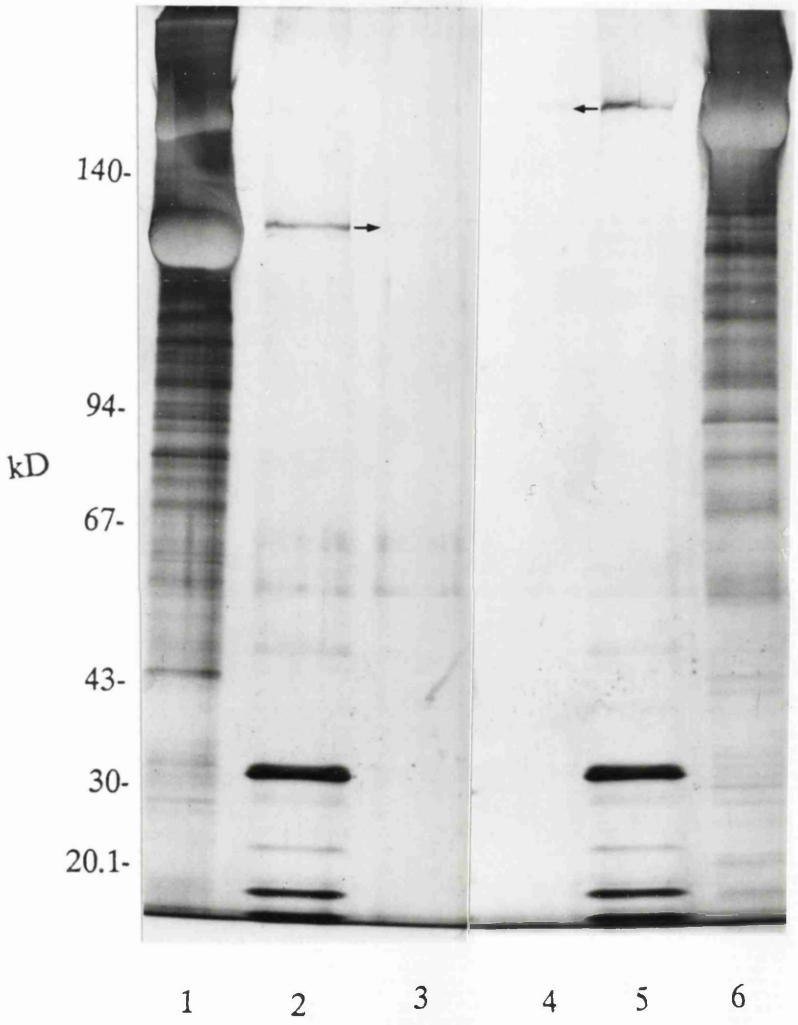


FIGURE 22

SAMPLE:	200μl void peak (above)
COLUMN:	SUPEROSE 6 HR10/10 gel filtration
BUFFER:	50mM Tris pH 7.0 + 150mM NaCl
FLOW RATE:	0.35ml.min^{-1}
SENSITIVITY:	0.02

FIGURE 23. SDS PAGE 7.5% Summary of purification of *B. nasutus* 1214 haemagglutinin.

Cell-free haemolymph (lanes 1 and 6) of *B. nasutus* 1214 was rotated with con A-sepharose 4B beads for 30 minutes. Following sedimantation of the beads, the supernatant (lanes 2 and 5) was applied to a Superose 6 gel filtration column in an FPLC (Pharmacia) system. A single peak of agglutinating activity (lanes 3 and 4) resulted. Samples were prepared in the presence (lanes 1, 2 and 3) or absence (lanes 4, 5 and 6) of the reducing agent dithioerythritol (DTE). The haemagglutinin (arrowed) is present at an M_r of 135k (reduced) or 210k (nonreduced). Gel silver stained.


140-
94-
kD
67-
43-
30-
20.1-
1
2
3
4
5
6

Under non-reducing conditions, whole cell-free haemolymph is again seen to comprise mainly of high M_r components (lane 6). Following purification with concanavalin A-sepharose 4B and gel filtration with Superose 6, the haemagglutinin is seen to be a single band present at an M_r of 210k (lane 4).

CHARACTERISTICS OF THE PURIFIED AGGLUTININ.

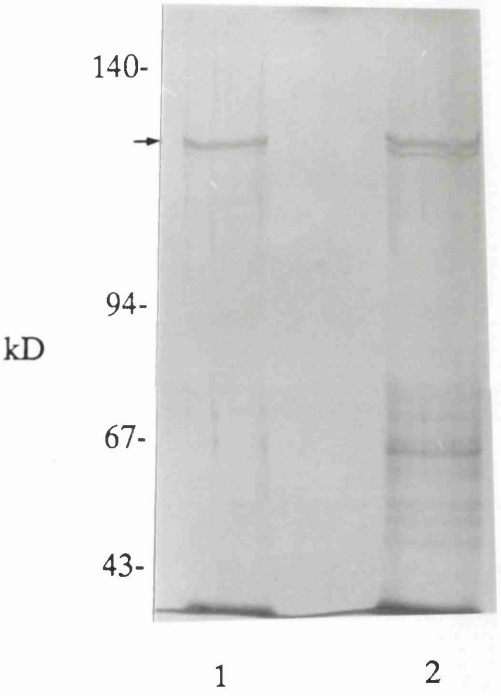
The purified agglutinin was stable at 4°C for up to 14 days. Agglutinating activity could still be detected after two rounds of freezing and thawing, although the titre was very much reduced. Consistent with the results obtained for the haemagglutination of the whole haemolymph, the purified agglutinin was sensitive to heat and mucins, and was capable of agglutination of the same range of erythrocyte types.

PURIFICATION OF PROTEINS FROM THE HAEMOLYMPH OF *Bulinus spp*.

Although human A_2 haemagglutinating activity was apparently confined to *B. nasutus* 1214, one could not discount the possibility of the presence of equivalent proteins being present in the haemolymph of other *Bulinus* species. The concanavalin A sepharose 4B bead adsorption treatment was used with cell-free haemolymph from *B. natalensis* 272. SDS PAGE analysis (5% resolving gel) of the resulting supernatant was compared with an identically prepared supernatant of *B. nasutus* 1214, and a purified *B. nasutus* 1214 agglutinin preparation. Figure 24 shows that the protein banding patterns for both the *B. nasutus* 1214 and *B. natalensis* 272 concanavalin A sepharose 4B bead supernatants are essentially similar, with a doublet of M_r 135k and 132k being the most prominent bands. The apparent "extra banding" with the *B. natalensis* 272 preparation compared to that of *B. nasutus* 1214 presumably reflects

FIGURE 24. SDS PAGE 7.5% Purification of 135k proteins.

Cell-free haemolymph of *B. nasutus* 1214 and *B. natalensis* 272 were rotated for 30 minutes with con A-sepharose 4B beads. Following centrifugation, samples of the resultant supernatants were loaded at equivalent dilution. *B. nasutus* 1214 (lane 1), *B. natalensis* 272 (lane 2). Gel silver stained.


140-
94-
kD
67-
43-
1
2

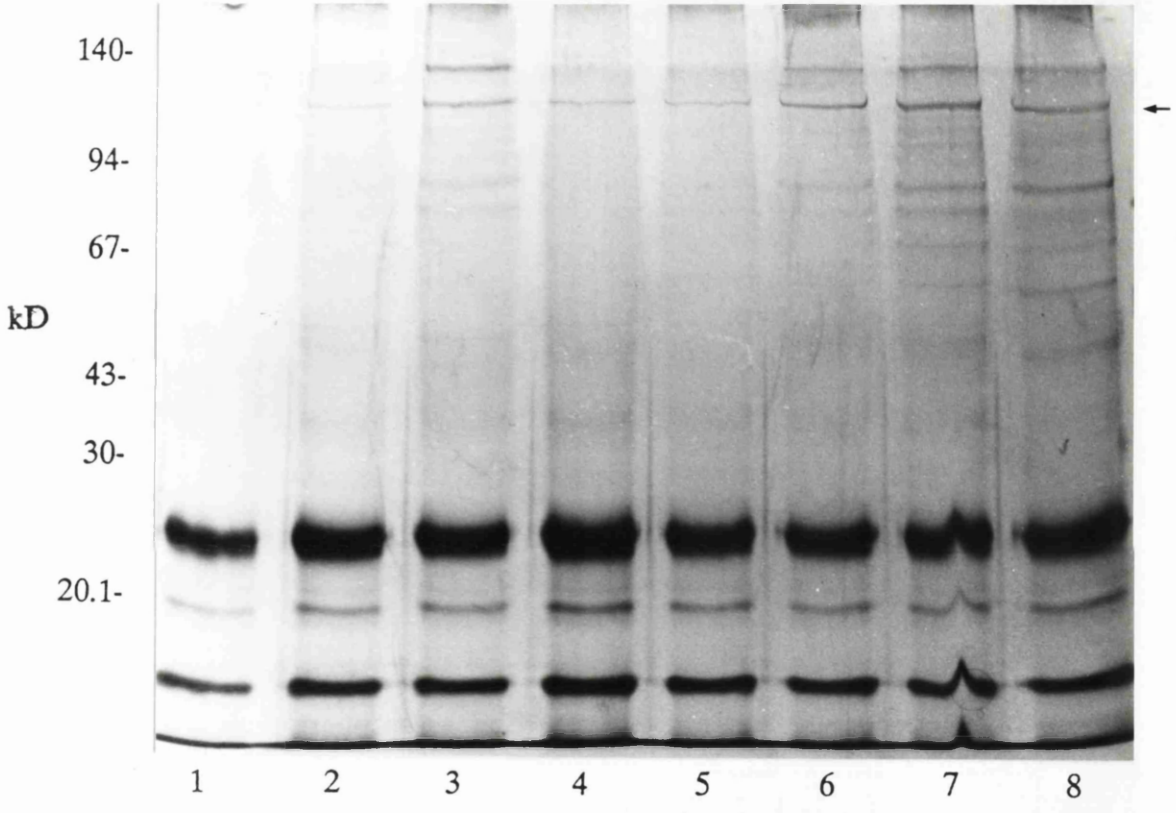
that the former haemolymph was collected fresh for the experiment, while the latter was a stored, previously collected preparation. The single 135k band with the FPLC purified agglutinin corresponds to the 135k band in the *B. natalensis* 272 preparation, which, it should be emphasised, does not agglutinate human erythrocytes.

A range of bulinid species were available for experimentation. As the two main test species, *B. nasutus* 1214 and *B. natalensis* 272 appeared to have similar protein profiles on SDS PAGE analysis, cell-free haemolymphs from other *Bulinus spp.* were used in identical procedures. Figure 11 shows the total protein profiles of 1/4 diluted haemolymph of seven bulinid snails. The protein banding for these preparations are essentially very similar. The majority of the proteins for each species are located at an M_r of 140k. These are almost certainly haemoglobins. There are then visible a set of characteristic protein bands at M_r of 118k, 115k, 100k, 94k, 87k, 74k and 60k, although there are noticeable variations in the size and intensity of the lower molecular mass bands in particular. Major differences between species are apparent in the M_r range less than 40k, where protein bands are only visible in the *B. africanus* 1227, *B. obtusispira* 1476 and *B. globosus* 1381 samples (all *africanus* group snails).

Cell-free haemolymph from each of these bulinid species was subjected to the concanavalin A sepharose 4B adsorption procedure, and the protein profiles following SDS PAGE analysis (7.5% resolving gel) of the resulting supernatants are shown in Figure 25. For the seven species used, it is clear that again the preparations are very similar. A prominent band is evident at an apparent M_r of 135k in each species, although the intensity of staining of this protein band varies between species. The strongly staining bands at M_r 33k, 25k, 22.5k and 18k are a property of the

FIGURE 25. SDS PAGE 7.5% Comparison of con A-sepharose 4B supernatants of *Bulinus spp.*

Haemolymph of bulinid species was rotated for 30 minutes with con A-sepharose 4B beads. Following sedimentation of the beads, samples of the resulting supernatants were loaded at equivalent dilution. Control con A-sepharose 4B supernatant (lane 1), *B. truncatus* 1521 (lane 2), *B. ugandae* 1250 (lane 3), *B. africanus* 1227 (lane 4), *B. tropicus* 28 (lane 5), *B. umbilicatus* 478 (lane 6), *B. obtussispira* 1476 (lane 7), *B. globosus* 1381 (lane 8). Gel silver stained.


140-
94-
67-
kD
43-
30-
20.1-
1
2
3
4
5
6
7
8

beads themselves, as the control lane indicates. Glycoprotein staining of these samples indicates that the 135k bands are glycosylated proteins (Figure 26).

THE POLYPEPTIDE PORTIONS OF ALL *Bulinus spp.* 135k PROTEINS ARE SIMILAR.

The presence of a protein band at 135k in each of the purified preparations of *Bulinus spp.* haemolymph indicated that these proteins might be similar. Cleveland mapping was conducted in order to provide polypeptide fingerprints for the 135k proteins from each of the species tested, and these are shown in Figure 27.

It is immediately apparent that the profiles for all the sixteen bulinid species represented are the same. The major polypeptide bands occur at apparent M_r of 30k and 28k. The other intensely staining polypeptide bands occur at 19k and 16k. Minor differences are evident in certain samples. For instance, an extra band is conspicuous in the *B. truncatus* 1521 profile at an apparent M_r of 15k. However, these minor differences might reflect the varied concentrations of proteins in the initial gel slices, due to the variable quantities of haemolymph from each species available to prepare the 135k bands.

The overall picture is that the protein portions of the 135k glycoproteins isolated from genetically and geographically distinct species of *Bulinus* are identical.

ANTISERA RAISED TO 135k PROTEINS ARE CROSS-REACTIVE WITHIN SPECIES COMPLEXES.

An antiserum was obtained from Balb/C mice immunized with *B. truncatus* 1521 homogenised SDS PAGE gel slice 135k protein. The preparations of *B. natalensis* 272 and *B. globosus* 1381 135k proteins were presumably not immunogenic enough

FIGURE 26. Glycoprotein staining of con A-sepharose 4B supernatants of *Bulinus spp.* haemolymph.

Samples of BSA (1mg.ml^{-1}), con A (1mg.ml^{-1}) and of *Bulinus spp.* haemolymph con A-sepharose 4B bead supernatants were stained with a glycan detection kit following transfer from 7.5% SDS PAGE gels onto nitrocellulose paper. BSA (lane 1), con A (lane 2), *B. beccarii* 1416 (lane 3), *B. ugandae* 1250 (lane 4), *B. forskalii* 1162 (lane 5), *B. umbilicatus* 1555 (lane 6), *B. guernei* 1126 (lane 7), *B. rohlfsi* 1326 (lane 8), *B. trigonis* 755 (lane 9), *B. truncatus* 1521 (lane 10), *B. obtussispira* 1476 (lane 11), *B. coulboisi* 1087 (lane 12), *B. permembranaceus* 1256 (lane 13) and *B. octoploidus* 1077 (lane 14).

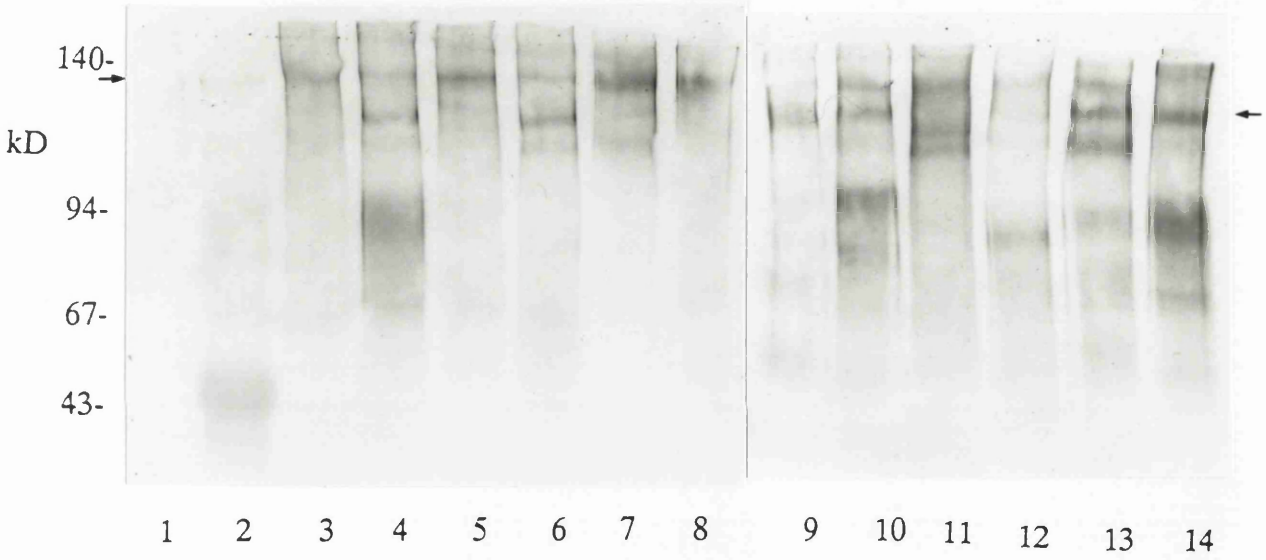
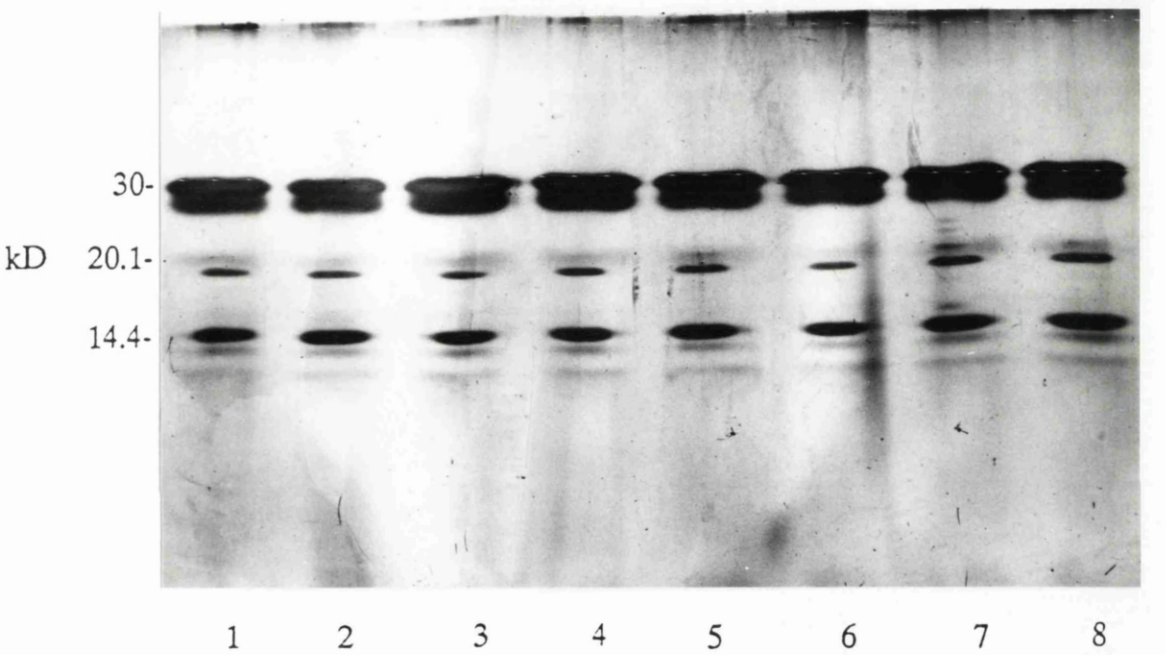

140-
kD
94-
67-
43-
1
2
3
4
5
6
7
8
9
10
11
12
13
14

FIGURE 27. SDS PAGE 15% Cleveland Mapping of *Bulinus spp.* 135k proteins. 135k gel slices were loaded into individual wells of a 15% gel, and overlain with Cleveland's buffer containing the proteolytic enzyme SV-8. The resultant polypeptide patterns are for *B. permebranaceus* 1256 (lane 1), *B. ugandae* 1250 (lane 2), *B. trigonis* 755 (lane 3), *B. umbilicatus* 1555 (lane 4), *B. rohlfsi* 1326 (lane 5), *B. guernei* 1126 (lane 6), *B. truncatus* 1521 (lane 7) and *B. forskalii* 1162 (lane 8). Gel silver stained.


kD
30-
20.1-
14.4-
1
2
3
4
5
6
7
8

to elicit a good response, even with the acrylamide to act as an adjuvant.

The antiserum to *B .truncatus* 1521 was reactive to *B. truncatus* 1521 and *B. natalensis* 272 135k proteins, but not to that of *B. globosus* 1381 (Figure 28). When the range of test species was extended, positive cross-reactions were observed with preparations of *B. guernei* 1126, *B. rohlfsi* 1326, *B. coulboisi* 1087, *B. permebranaceus* 1256, *B. octoploidus* 1077 and *B. trigonis* 755. All these positively reacting haemolymphs are from members of the **truncatus/tropicus** species complex. Preparations from *B. ugandae* 1250, *B. umbilicatus* 1555 and *B. obtusispira* 1476 (all **africanus** group snails), *B. beccarii* 1416 and *B. forskalii* 1162 (both **forskalii** group snails), and *I. exustus* 1275 did not cross-react with the same antiserum.

Thus cross-reactivity was restricted to **truncatus/tropicus** species complex members when a **truncatus/tropicus** species antiserum was applied.

ISOELECTRIC FOCUSSING.

While the SDS PAGE protein profiles of haemolymphs from *Bulinus spp.* showed very little variability, it was expected that isoelectric focussing would highlight any differences between species. The haemolymph protein profiles following isoelectric focussing are shown in Figure 29. These include three bulinid species, *B. truncatus* 1381, *B. nasutus* 1214 and *B. tropicus* 28 (all of the truncatus/tropicus complex), and two geographically distinct Planorbid species, *I. exustus* 1275 and *Biomphalaria glabrata* 1144. It is immediately apparent that the majority of the haemolymph components have pI values within the range 4-5.5. For each species, the most intensely staining regions occur at the most acidic pI values. Fundamental differences are also apparent when one compares the profiles for different species. For the three bulinid species, the pI values of proteins from *B. nasutus* 1214 are more acidic than

FIGURE 28. Western Immunoblot. *Bulinus spp.* 135k proteins recognized by an antiserum raised against *B. truncatus* 1521 135k.

Con A-sepharose 4B bead supernatants of *Bulinus spp.* haemolymph were run on a 7.5% SDS PAGE gel and blotted onto nitrocellulose paper. The blot was probed with a polyclonal antiserum raised in Balb C mice against *B. truncatus* 1521 135k protein. *B. globosus* 1381 (lane 1), *B. coulboisi* 1087 (lane 2), *B. octoploidus* 1087 (lane 3), *B. rohlfsi* 1326 (lane 4), *B. truncatus* 1521 (lane 5), *B. guernei* 1126 (lane 6), *B. natalensis* 272 (lane 7), *B. ugandae* 1250 (lane 8) and *B. forskalii* 1162 (lane 9).

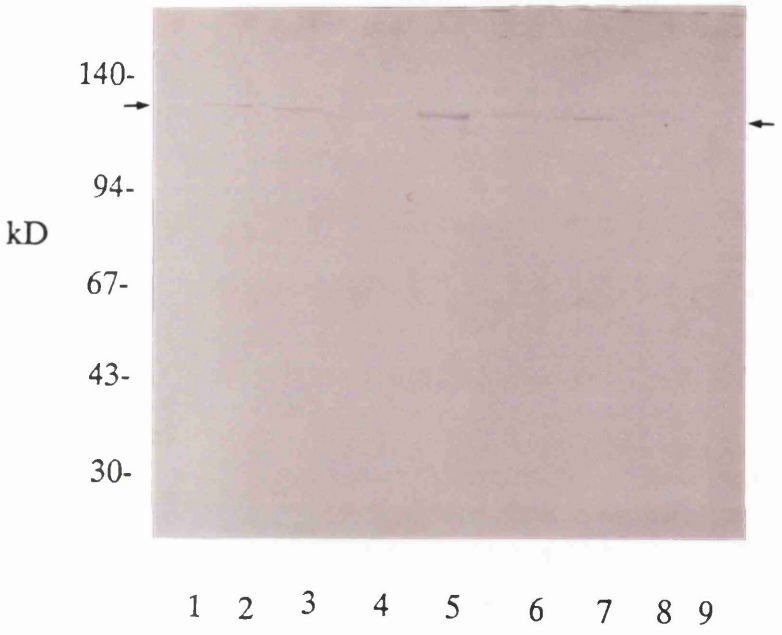
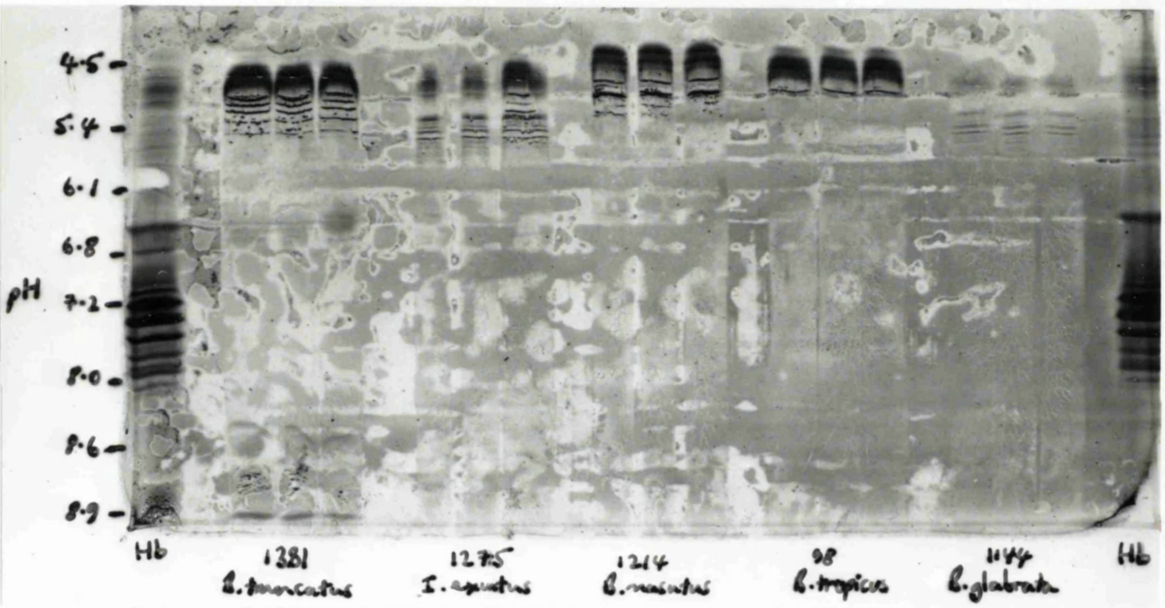

140-
94-
kD
67-
43-
30-
1 2 3 4 5 6 7 8 9

FIGURE 29. Isoelectric focussing of *Bulinus spp.* haemolymph.

Samples of cell-free haemolymph of *B. truncatus* 1521, *I. exustus* 1275, *B. nasutus* 1214, *B. tropicus* 98 and *Biomphalaria glabrata* 1144 were separated in a pH gradient of 3.5-9.5. Gel stained with Coomassie Brilliant Blue.


pH
4.5
5.4
6.1
6.8
7.2
8.0
8.6
8.9
Hb
1381
1275
1214
98
B. tropicus
1144
B. glabrata
Hb

those of *B. tropicus* 28, and those of *B. truncatus* 1381 have higher values than either of the other two species. The profile of *I. exustus* 1275 compares very well to those of *B. truncatus* 1381 and *Biomphalaria glabrata* 1144. A tighter pH interval, over the range 3-7 would serve better to resolve the finer differences between species.

ELEVATION OF PLASMA PROTEIN LEVELS ON INJECTION.

Injection of snails following anaesthesia proved a viable methodology for the introduction of foreign substances. Snails took on average 3 hours to fully recover mobility, after several changes of snail conditioned water. Some haemolymph was extruded on injection, which resulted in loss of some of the injected material.

The level of haemolymph proteins increased on injection of saline. However, due to the inevitable damage caused by the injection procedure, and the subsequent prolonged interval before haemolymph could be collected, the stimulus that provokes this increase is unclear. SDS PAGE analysis showed the elevation in plasma protein levels clearly (Figure 30), with reference to a comparison of lanes 2 and 3. It appears that the higher molecular mass components are especially marked in the samples from injected animals. Interestingly, a comparison of haemolymph from *E. togoensis* infected snails and haemolymph from unchallenged individuals show equivalent levels of protein staining. This could be due to the fact that the penetration of the echinostomes was unsuccessful.

In a separate study, specimens of *B. africanus* 1583 were wounded by piercing with a needle, in an attempt to mimic miracidial penetration. The percentage changes in plasma protein levels compared to the resting state (as measured spectroscopically) over a 96 hour period, are given in Figure 31. The percentage changes in the level of plasma haemoglobin are similarly given Figure 32. It is

FIGURE 30. SDS PAGE 10% Elevation in plasma protein levels due to injection and infection.

Specimens of *Biomphalaria glabrata* 1144 were mechanically wounded by fine-point piercing, and sampled 3 hours post-wounding; or were challenged with miracidia of *E. togoensis* and sampled 5 days post-infection. Samples of injected (lane 1), infected (lane 2) and unchallenged (lane 3) haemolymph were applied at equivalent dilution. Gel stained with Coomassie Brilliant Blue.

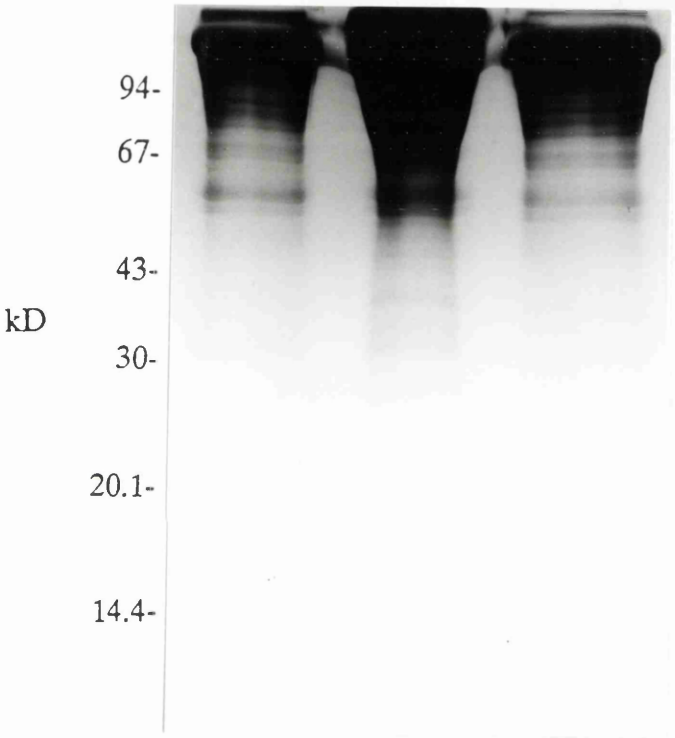

94-
67-
43-
kD
30-
20.1-
14.4-
1
2
3

FIGURE 31. VARIATION OF PLASMA PROTEIN LEVELS ON WOUNDING

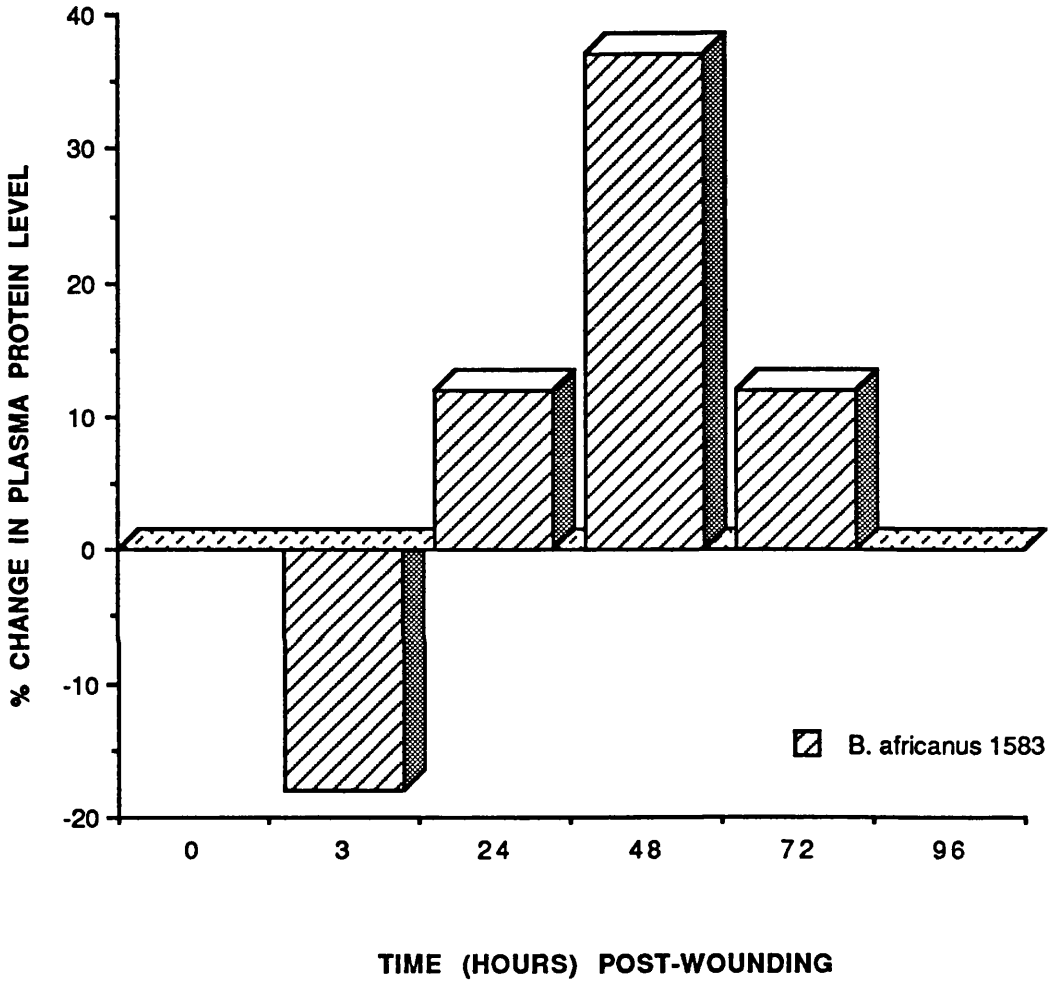
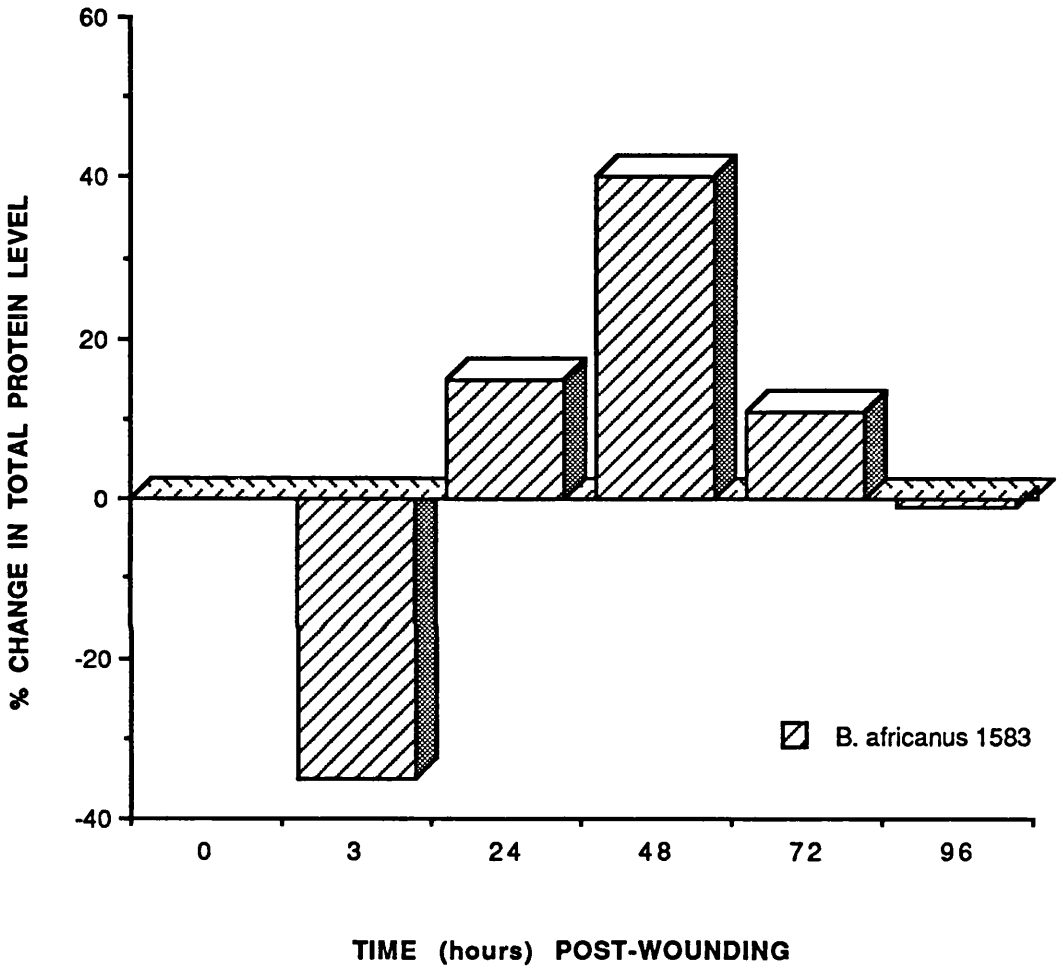


FIGURE 32. VARIATION OF PLASMA HAEMOGLOBIN LEVELS ON WOUNDING



immediately apparent that the trends of both graphs are identical, with a decrease at 3 hours post-wounding, a rise to a maximum level at 48 hours post-wounding, and a return to the resting level by 96 hours post-wounding. SDS PAGE analysis of these samples is shown in Figure 33. No obvious differences are apparent in the banding patterns of total cell-free haemolymph. With supernants of con A-sepharose 4B purified haemolymph, no differences could be discerned in samples collected at different times post-wounding, with the 135k band remaining prominent throughout.

PLASMA PROTEIN LEVELS ALTER DURING INFECTION.

Figure 30 shows a comparison of haemolymphs from snails infected with *S. margrebowiei* miracidia and from uninfected *B. natalensis* 272, recovered 5 days post-infection. It is immediately apparent that the level of plasma proteins is higher in haemolymph from the infected snails. It was due to these high concentrations of the higher molecular mass components that the purification protocol designed for the isolation of the *B. nasutus* 1214 involved unchallenged snail specimens.

In a separate infection experiment the levels of plasma proteins were investigated during the course of an infection. By the end of the incubation period, no free-swimming miracidia could be seen in the crystallizing dishes, indicating that all the miracidia had either attached to, or had penetrated the snails. None of the challenged *B. umbilicatus* 478 snails showed evidence of cercarial shedding by day 30 post-infection. There was no shedding during the subsequent 14 days in these snails either. Not all of the challenged *B. natalensis* 272 snails were shedding cercariae by day 30 post-infection. The "shedders" were separated from the "non-shedders" when the haemolymphs were pooled. The unchallenged "resting" total haemolymph protein concentration is consistent for three bulinid species at approximately 1.81mg.ml^{-1}.

FIGURE 33. SDS PAGE 7.5% Effect of wounding on *B. africanus* 1583 plasma protein levels.

Specimens of *B. africanus* 1583 were pierced with a needle and haemolymph was sampled at 3, 24, 48, 72 and 96 hours post-wounding. Samples of the pool of 5 specimens were loaded at equivalent dilution. Unchallenged (lane 1), 3 hours (lane 2), 24 hours (lane 3), 48 hours (lane 4), 72 hours (lane 5), 96 hours (lane 7). Gel stained with Coomassie Brilliant Blue.

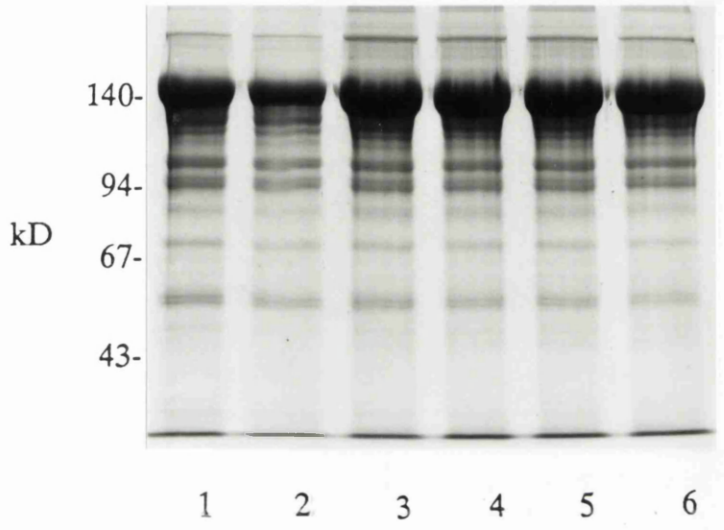
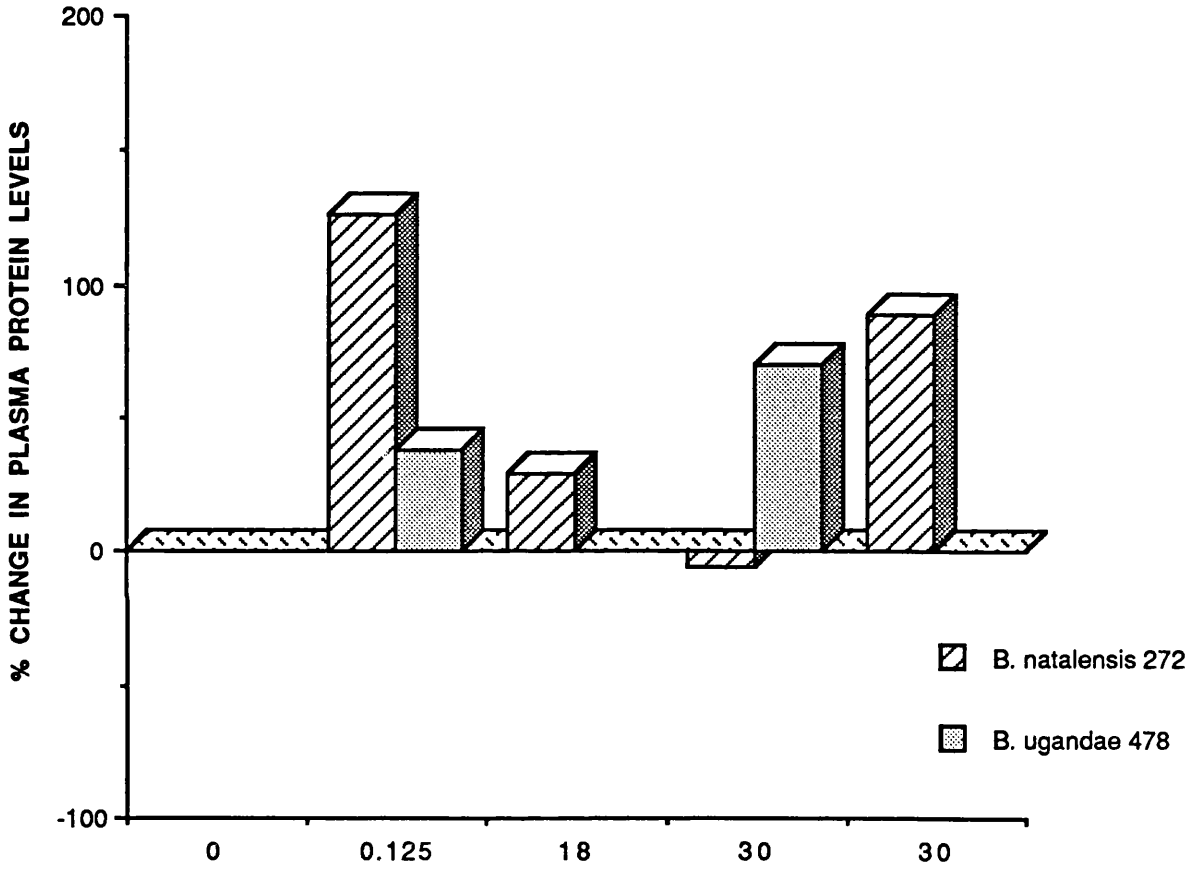

140-
94-
kD
67-
43-
1
2
3
4
5
6

FIGURE 34. VARIATION OF PLASMA PROTEIN LEVELS DURING THE COURSE OF INFECTION



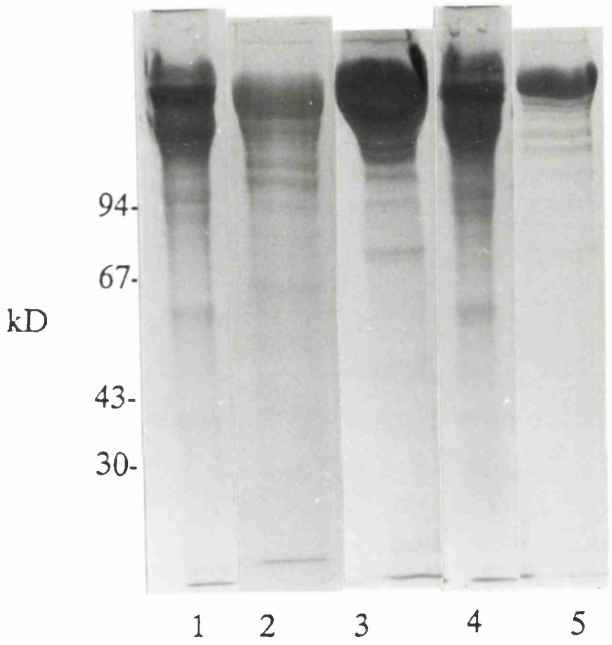
With both *B. umbilicatus* 478 and *B. natalensis* 272 there was a significant increase in protein concentration at 3 hours post-infection (Figure 34). This increase is more marked with *B. natalensis* 272 (127%) than with *B. umbilicatus* 478 (38%). At 21 days post-infection the haemolymph protein level in *B. natalensis* 272 had fallen to 2.35mg.ml^{-1}, which is still 30% higher than the resting level. At the time of cercarial shedding, the protein levels of *B. natalensis* 272 "nonshedders" (1.72mg.ml^{-1}) was just below the resting level, while that of the "shedders" was 90% higher than the resting level. For *B. umbilicatus* 478, the protein level at this sample time is below the resting level (but this may reflect the fact that these were the same specimens that had been bled previously, and hence their haemolymph may have not had sufficient time to return to normal composition).

The general trends with the susceptible *B. natalensis* 272 therefore are for (1) an initial great elevation in haemolymph protein concentration during miracidial penetration and transition to sporocysts, (2) a gradual return towards the resting level during sporocyst maturation and division, and (3) a final level equivalent to the resting level during cercarial shedding. For the resistant *B. umbilicatus* 478, the same initial elevation is observed, but this is not as accentuated as with the susceptible bulinid species.

SDS PAGE analysis of haemolymphs collected from challenged snails are shown in Figure 35. After three hours post-infection, there are no obvious differences between the protein banding profiles of *B. umbilicatus* 478 (resistant) and *B. natalensis* 272 (susceptible). Compared to the lane of haemolymph from unchallenged *B. natalensis* 272, the haemolymphs from both challenged snails appear to have elevated protein concentrations. After 21 days post-infection, the difference in protein concentration of haemolymphs from infected and unchallenged *B. natalensis*

FIGURE 35. SDS PAGE 7.5% Variation in level of plasma proteins during the course of infection.

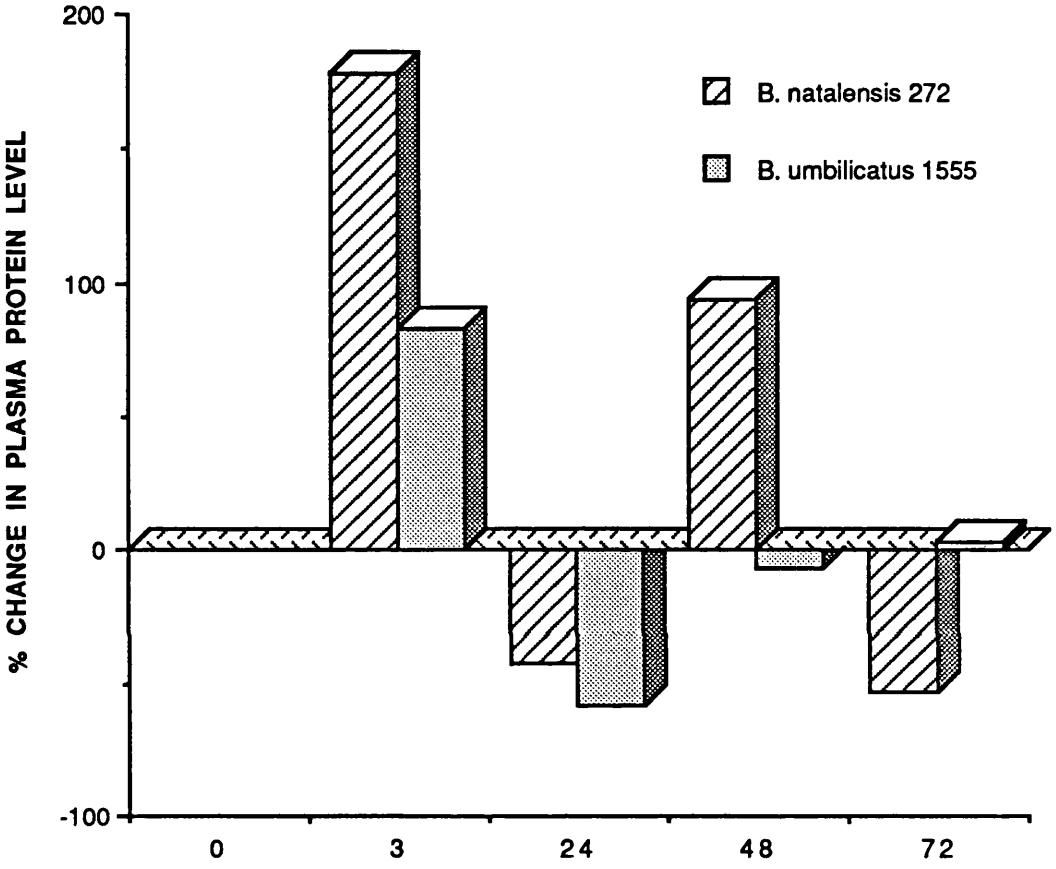
Haemolymph was sampled from susceptible *B. natalensis* 272 (lanes 1, 2 and 3) and resistant *B. ugandae* 478 (lanes 4 and 5) at 3 hours (lanes 1 and 4), 21 days (lane 2) and 30 days (lanes 3 and 5) post-infection. Gel stained with Coomassie Brilliant Blue.


kD
94-
67-
43-
30-
1
2
3
4
5
BN272
BU478

272 is less accentuated. At 30 days post-infection, no difference in intensity of protein banding was observed for haemolymphs from *B. umbilicatus* 478 and *B. natalensis* 272, or between cercarial shedding and non-shedding *B. natalensis* 272.

In order to investigate the initial elevation in plasma protein levels observed in this study, another experiment was conducted, wherein haemolymph was sampled at 3, 24, 48 and 72 hours post-challenge. These time points were selected in order to encompass the serial events of miracidial penetration, transformation to sporocyst and sporocyst destruction in resistant hosts. The % changes in haemolymph protein levels are given in Figure 36 for both *S. margrebowiei*- susceptible *B. natalensis* 272 and *S. margrebowiei*- resistant *B. umbilicatus* 1555. There was an initial increase in protein levels of hamolymph of both species, the increase in the susceptible *B. natalensis* 272 being greater (178%) than that of the resistant *B. umbilicatus* 1555 (82%). At 24 hours post-challenge there was a decrease in plasma protein levels, with a higher decrease in resistant *B umbilicatus* 1555 (59%) than in susceptible *B. natalensis* 272 (43%). At 48 hours post-infection the level of haemolymph proteins in the resistant species had returned to the resting (unchallenged) level, where it remained at 96 hours post-challenge. With the susceptible *B. natalensis* 272, at 48 hours post-infection there was a second elevation in plasma protein levels (93%) and then a second decrease at 72 hours post-challenge (54%). SDS PAGE analysis of samples following adsorption with con A-sepharose 4B beads are shown in Figure 37. With the *B. natalensis* 272 samples, the 135k band is absent at 3 and 24 hours post-infection, but reappears at 48 hours and is also present at 72 hours post-infection. At the two first time points (3 and 24 hours) there are a variety of proteins banding. With similarly produced samples of *B. umbilicatus* 1555, the 135k band is present throughout, except at 48 hours post-infection when a variety of protein bands

FIGURE 36. VARIATION OF PLASMA PROTEIN LEVELS DURING INITIAL STAGES OF INFECTION



are evident.

It is clear that there are differences in the haemolymph protein levels in susceptible and resistant snails given the same stimulus (schistosome infection).

THE *B. natalensis* 272 135k PROTEIN RECOGNISES INVADING PARASITES.

Specimens of *B. natalensis* 272 infected with *S. margrebowiei* miracidia were prepared for cryostat sectioning 3 hours post-infection. 20μm sections were probed with the *B. truncatus* 1521 135k antiserum. Compared to control sections of unchallenged snails (Plate A), there appears to be a localisation of the antibody around an object which might be a transforming miracidium (Plate B).

CELLULAR COMPONENTS OF THE HAEMOLYMPH.

CELL COUNTS.

The number of haemocytes.ml^{-1} collected from resting specimens showed considerable variation among the snail species tested (Table 14).

The cell counts for the aquatic species varied to a similar degree as those for the terrestrial snails. The cell counts did not correlate directly with the sizes of the specimens. For instance, while *L. stagnalis* is the largest aquatic snail sampled and had the highest cell count, *A. achatina* was the largest specimen by far, yet the cell count is still lower than that for *L. stagnalis*. This might possibly reflect the methodology of haemolymph collection, the shell puncture method with the terrestrial snails not yielding haemolymph as easily as foot retraction with the aquatic snails.

FIGURE 37. SDS PAGE 7.5% Effect of infection and wounding on plasma protein levels.

Specimens of susceptible *B. natalensis* 272 (A) and *B. umbilicatus* 1555 (B) were challenged with 20 *S. margrebowiei* miracidia. Haemolymph of 10 specimens was pooled at 3 (lanes 1), 24 (lanes 2), 48 (lanes 3) and 72 (lanes 4) hours post-infection. Pooled haemolymph of 5 specimens of *B. africanus* 1583 (C) wounded by piercing was collected at the same time intervals. Samples of each pool were incubated for 30 minutes with con A-sepharose 4B beads. The beads were sedimented, and samples of the resulting supernatants were loaded at equivalent dilutions. Thus variations in levels of proteins, and in particular of 135k proteins can be compared in susceptible (A) and resistant (B) hosts at different times during the early stages of infection (1-4). The differences in stimulation by injection (C) as opposed to infection (A and B) can also be seen over the same time period. Gel stained with Coomassie Brilliant Blue.

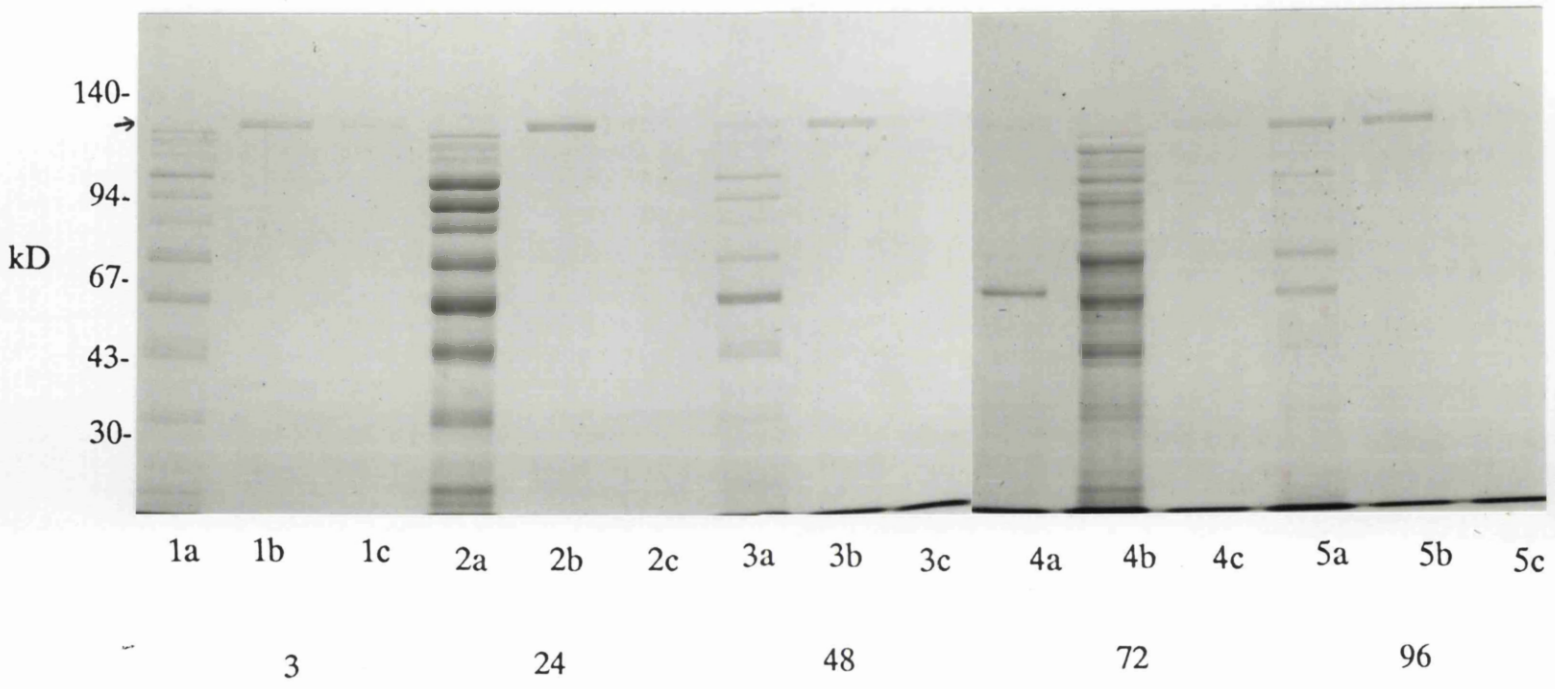

140-
94-
kD
67-
43-
30-
1a
1b
1c
2a
2b
2c
3a
3b
3c
4a
4b
4c
5a
5b
5c
3
24
48
72
96

PLATE A. Cryostat sections of resting *B. natalensis* 272 probed with an antiserum raised against *B. truncatus* 1521 135k protein.

20μm sections were cut from the head-foot region of specimens of *B. natalensis* 272 (scale bar 40μm). The phase contrast image (upper) shows a regular organisation of cells, with an ordered body wall (w). The fluorescence image (lower) shows a natural fluorescence of mucus at the surface of the foot (arrowed).

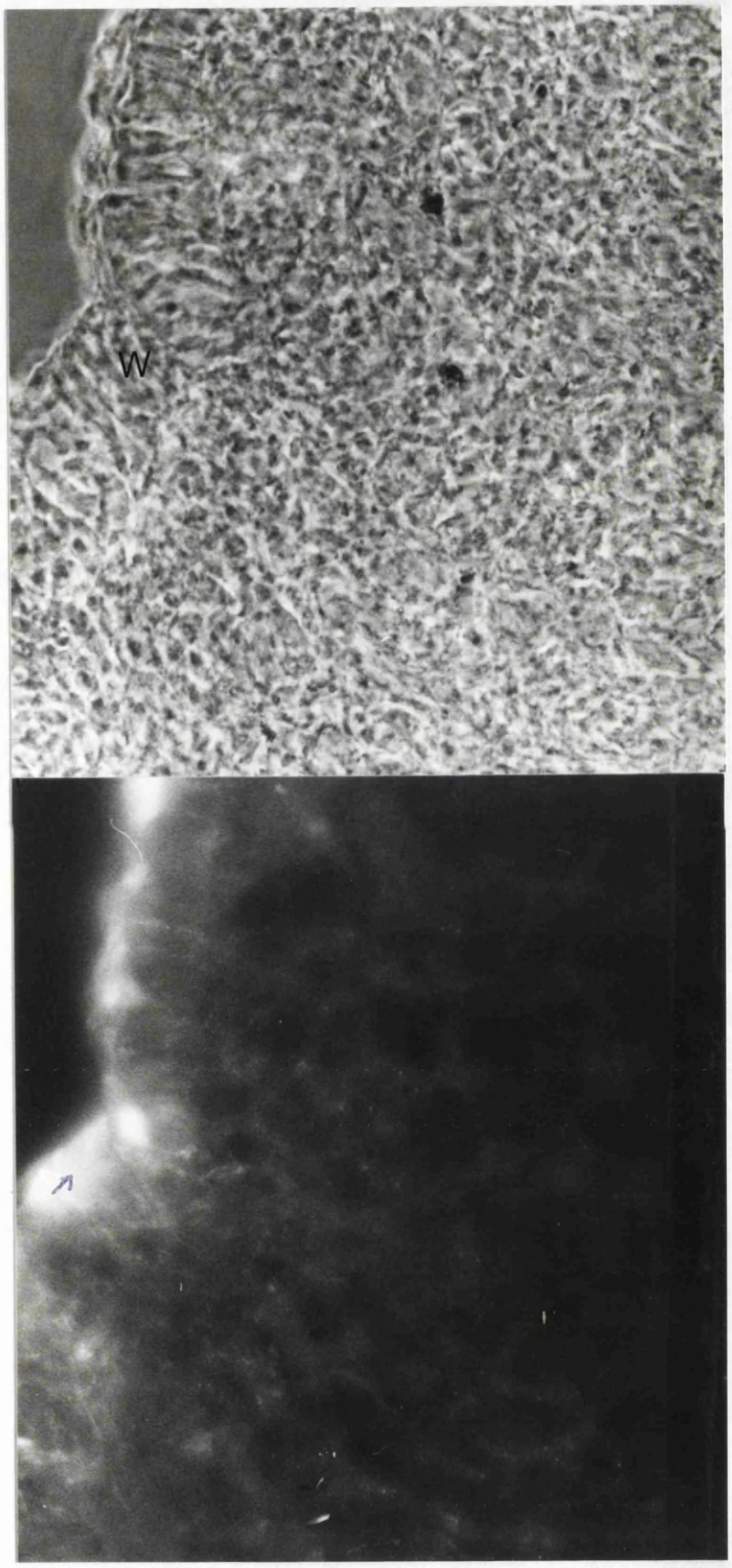

W

PLATE B. Cryostat sections of *B. natalensis* 272 3 hours post-infection with *S. margrebowiei* miracidia.

20μm sections were cut from the head-foot region of specimens of *B. natalensis* 272 3 hours post-infection with 20 *S. margrebowiei* miracidia (scale bar 40μm). The phase-contrast image (upper) shows the presence of an oval-shaped structure measuring 15μm in length, which is distinst from the surrounding snail tissue (arrowed). The fluorescence image (lower) shows a localisation of the antibody at the surface of what is probably a transforming miracidium.

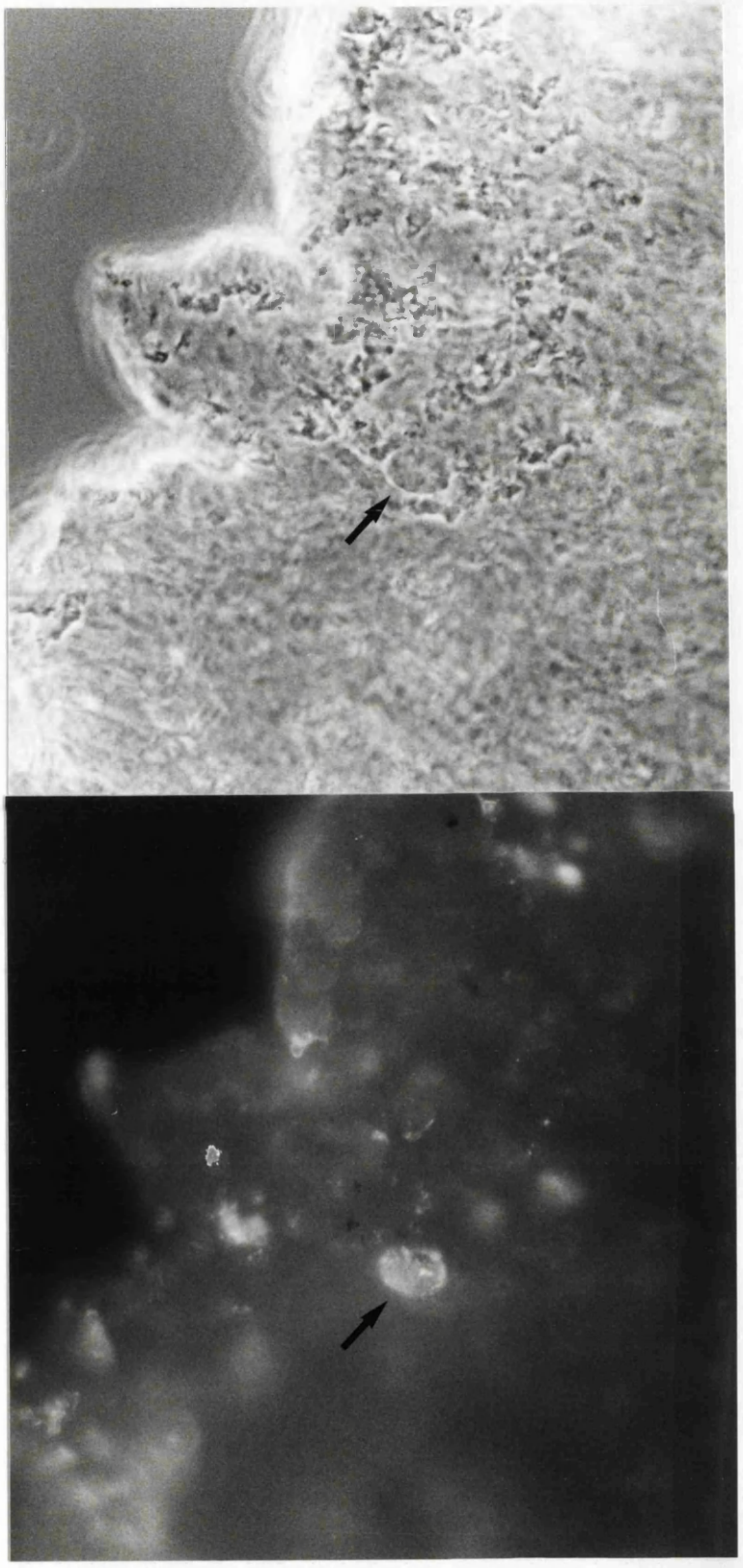


TABLE 14 COMPARISON OF HAEMOCYTE COUNTS FOR SIX SNAIL SPECIES

SNAIL SPECIES	No. HAEMOCYTES. ML^{-1}
Aquatic	
Biomphalaria glabrata	$2.5 \pm 0.7 \times 10^4$
Bulinus natalensis 272	$5.7 \pm 0.1 \times 10^5$
Lymnaea stagnalis	$1.5 \pm 0.1 \times 10^6$
Terrestrial	
Achatina achatina	$4.6 \pm 0.2 \times 10^5$
Achatina fulica	$1.9 \pm 0.1 \times 10^5$
Helix aspersa	$5.0 \pm 0.1 \times 10^6$

Number of haemocytes.ml^{-1} of haemolymph for each snail species was determined with a Burker-Turk counting chamber. Values are the mean +/- standard deviation (n=4).

HAEMOCYTE MORPHOLOGY.

Haemocyte populations of all snail species were seen to adhere to glass and to spread in the SSS buffer. No significant differences were evident when comparing live cells with fixed cells. The majority of cells in each species were large, spreading cells with a high cytoplasm-to-nucleus ratio. Very few round, small cells with a high cytoplasm-to-nucleus ratio were observed. The terrestrial snail haemocytes were generally larger than their aquatic species counterparts (Plate 1).

Haemocytes of *A. achatina* were very large cells, often showing great longitudinal extension while spreading. Haemocytes of *A. fulica* were smaller and became less elongated, with broader pseudopods. The glass adherent haemocytes of *Biomphalaria glabrata* had jagged, spiky extensions. Those of *B. natalensis* 272 were very small and showed broad pseudopodia. *H. aspersa* spreading haemocytes were quite large, exhibited broad extensions and had characteristic oval-shaped nuclei. In *L. stagnalis* haemocyte morphology ranged from small round cells, through intermediate shaped to large spreading cells.

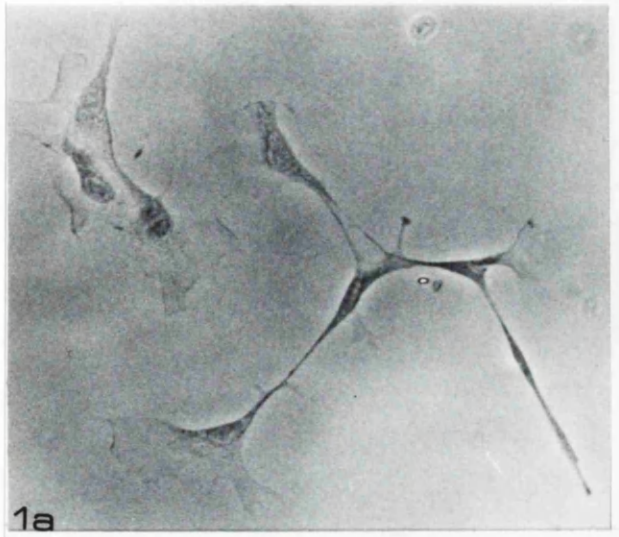
These qualitative differences in haemocyte morphologies may reflect the reactions of the cells to the SSS buffer. This buffer has been specifically developed for haemocytes of *L. stagnalis*, and while there was no destructive effect of this buffer on the haemocytes of all the other snail species, their shapes may have reflected for compensations in osmotic differences between their intracellular environments and that of the SSS buffer.

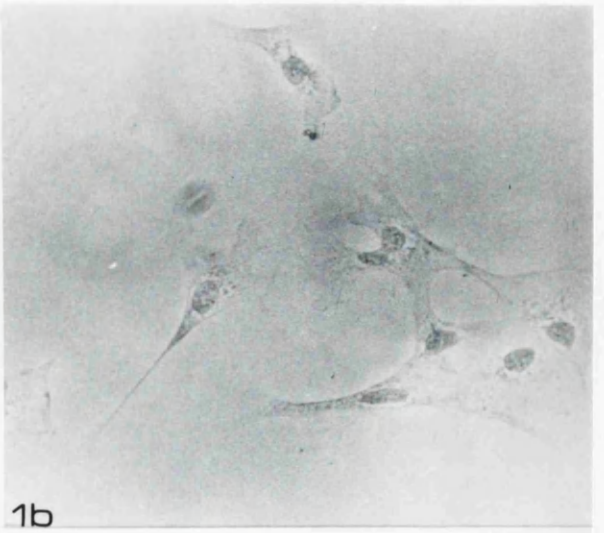
LECTIN STAINING OF HAEMOCYTES.

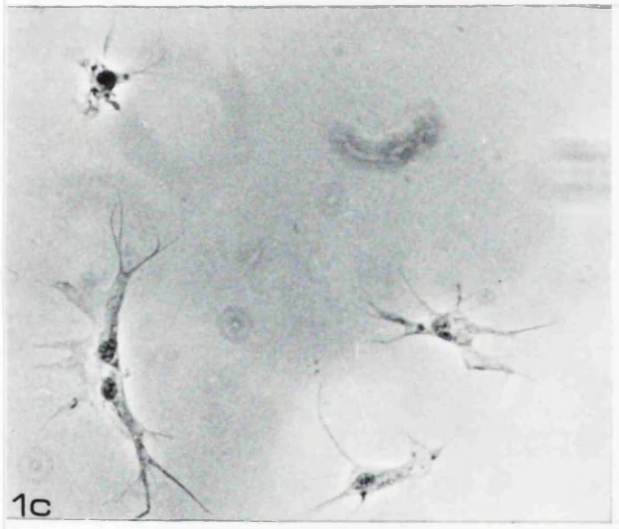
Of the five lectins used in haemocyte staining experiments, only Concanavalin A and Wheat germ agglutinin gave positive reactions, and to varying degrees for

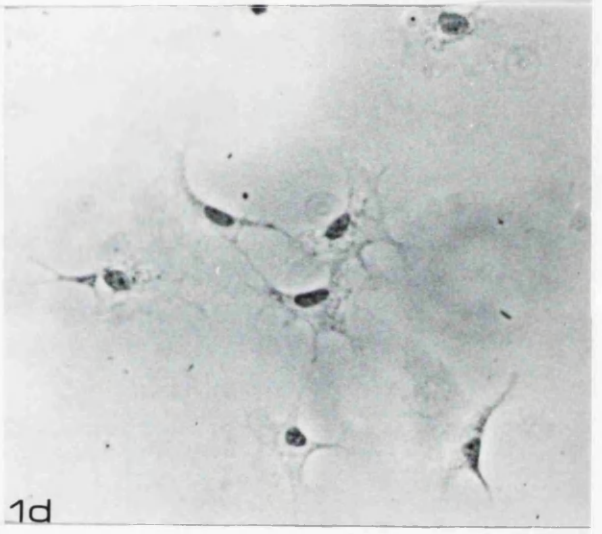
PLATE 1. Morphology of haemocytes.

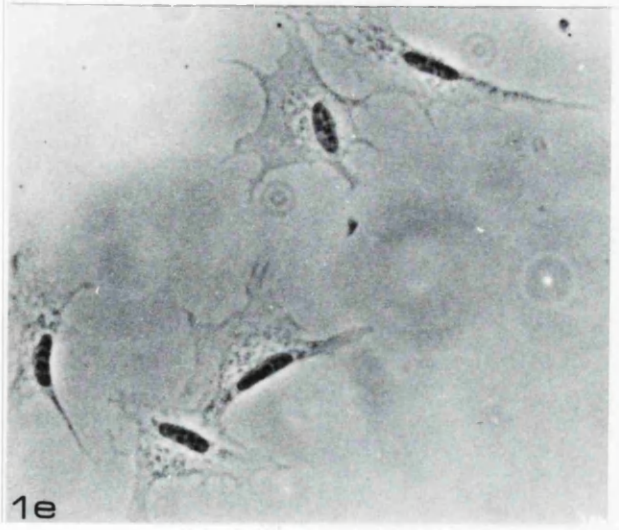
Morphology of glass adhering haemocytes in SSS derived from six snail species is shown. Depending on the species, the haemocytes displayed different characteristics of morphology. Haemocytes of land snails are larger than those of aquatic species. Magnification 40x. 1a: *Achatina achatina*; 1b: *A. fulica*; 1c: *Biomphalaria glabrata*; 1d: *Bulinus natalensis* 272; 1e: *Helix aspersa*; 1e: *Lymnaea stagnalis*.


1a


1b


1c


1d


1e

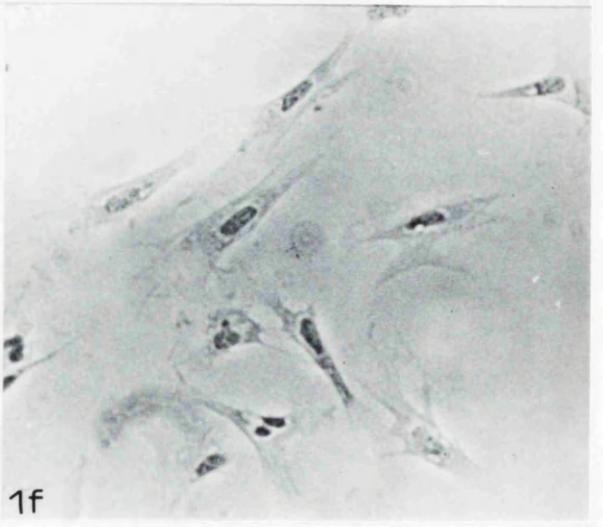

1f

TABLE 15 LECTIN STAINING OF SNAIL HAEMOCYTES

SNAIL SPECIES	LECTIN	
	Concanavalin A	Wheat germ
Aquatic		
Bulinus natalensis	+	-
Lymnaea stagnalis	-	++
Terrestrial		
Achatina achatina	+/-	+/-
Achatina fulica	+	+
Helix aspersa	+	-

Lectins used at a concentration of 1mg.ml^{-1}. Haemocyte monolayers were constructed using pooled haemolymph from two specimens.

Reaction intensity: NO STAIN - ; WEAK STAIN +/- ;STRONG STAIN ++

species (Table 15).

All but *L. stagnalis* stained with concanavalin A, indicating a common specificity of surface sugar determinants on haemocyte surfaces (α-D-mannose, α-D-glucose). *L. stagnalis* haemocytes were strongly stained with Wheat germ agglutinin however, indicating a preponderance of N-acetyl glucosamine and neuraminic acid residues. Haemocytes of *A. achatina* did not give as strong staining as those of *A. fulica*, yet both stained with both lectins, implying similarities in surface components.

MONOCLONAL ANTIBODY STAINING OF HAEMOCYTES.

All of the monoclonals utilised in this study (LS1, LS3, LS8, YII and YIII) have been characterised as being differential-staining of haemocyte subpopulations of the snail species which they were raised against. Thus while LS1, LS3 (for *L. stagnalis*), YII and YIII (for *Biomphalaria glabrata*) are all described as surface markers, LS8 is a granule marker of *L. stagnalis* haemocytes. Tables 16 and 17 gives a descriptive account of the results of the study, and plates C, D, E, F, G and H illustrate the results of staining with LS8. It is apparent that monoclonal antibodies raised to haemocyte epitopes of one species of snail were capable of recognising determinants on haemocytes of other snail species. The degree, location and subpopulation specificity of staining differed for the monoclonals employed. Thus LS1 stained haemocytes of all six species, but only differentiated subpopulations of *L. stagnalis* and *A. fulica*; LS3 only differentiated haemocytes of *L. stagnalis* and stained all haemocytes of all other snail species; with LS8 a range of staining was observed, with granules being recognised in haemocytes of *L. stagnalis*, surfaces of haemocytes of *Biomphalaria glabrata*, *B. natalensis* 272, *A. achatina* and *H. aspersa*, but haemocytes of *A. fulica* did not stain; with YII, haemocyte surface staining of

TABLE 16 MACROSCOPIC SCORING OF STAINING OF HAEMOCYTES WITH IMMUNOREAGENTS

	IMMUNOREAGENT						
	LS1	LS3	LS8	YII	YIII	B1	B2
SPECIES							
Aquatic							
Biomphalaria glabrata	-	-	-	++	+	-	+++
Bulinus natalensis 272	-	+	-	+	+	-	++
Lymnaea stagnalis	+	+++	-	+++	+	-	+++
Terrestrial							
Achatina achatina	++	-	-	+	-	-	++
Achatina fulica	++	-	-	+	-	-	+++
Helix aspersa	++	-	-	-	-	-	+++

KEY : NO STAINING - ; WEAK STAINING + ; STRONG STAINING +++

TABLE 17 MICROSCOPIC OBSERVATIONS OF HAEMOCYTE STAINING BY IMMUNOREAGENTS

Monoclonal LS1(surface)	
Biomphalaria glabrata	weak staining of all haemocytes
Bulinus natalensis 272	faint staining of all haemocytes
Lymnaea stagnalis	small rounded haemocytes only stained
Achatina achatina	all haemocytes stained
Achatina fulica	strong staining of rounded cells
Helix aspersa	all haemocytes stained
Monoclonal LS3 (surface)	
Biomphalaria glabrata	intermediate spreading cells only stained
Bulinus natalensis 272	all haemocytes stained
Lymnaea stagnalis	small rounded haemocytes only stained
Achatina achatina	all haemocytes stained
Achatina fulica	all haemocytes faintly stained
Helix aspersa	all haemocytes faintly stained
Monoclonal LS8 (granule)	
Biomphalaria glabrata	rounded haemocytes only stained
Bulinus natalensis 272	all haemocytes faintly stained
Lymnaea stagnalis	granules stained
Achatina achatina	all haemocytes stained
Achatina fulica	no staining
Helix aspersa	all haemocytes faintly stained
Monoclonal YII (surface)	
Biomphalaria glabrata	large haemocytes surface stained, granules in small
Bulinus natalensis 272	small haemocytes surface stained, granules in large
Lymnaea stagnalis	granules stained
Achatina achatina	all haemocytes stained
Achatina fulica	granules stained
Helix aspersa	all haemocytes stained
Monoclonal YIII (granule)	
Biomphalaria glabrata	granules in small haemocytes strongly stained
Bulinus natalensis 272	rounded haemocytes only stained
Lymnaea stagnalis	no staining
Achatina achatina	all haemocytes stained
Achatina fulica	all haemocytes stained
Helix aspersa	no staining

Antiserum B1 (135k protein)

Biomphalaria glabrata	all haemocytes faintly stained
Bulinus natalensis 272	all haemocytes faintly stained
Lymnaea stagnalis	small haemocytes faintly stained
Achatina achatina	all haemocytes strongly stained
Achatina fulica	no staining
Helix aspersa	no staining

Antiserum B2 (surface)

Biomphalaria glabrata	all haemocytes weakly stained
Bulinus natalensis 272	all haemocytes stained
Lymnaea stagnalis	strong staining of granules
Achatina achatina	all haemocytes strongly stained
Achatina fulica	partial staining of haemocyte surfaces
Helix aspersa	no staining

LEGEND FOR PLATES C-H

Haemocyte monolayers were prepared for each snail species. The results of staining with the monoclonal LS8 are shown in the plates. For each species, the first two images, numbered X_1 are controls, and the two images on the facing pages, numbered X_2 are the stained cells. The upper image in each case, labelled A is a phase contrast image, and the lower is the bright field image.

PLATE C *Biomphalaria glabrata* Scale bar 10μm

PLATE D *Bulinus natalensis* 272 Scale bar 10μm

PLATE E *Lymnaea stagnalis* Scale bar 10μm

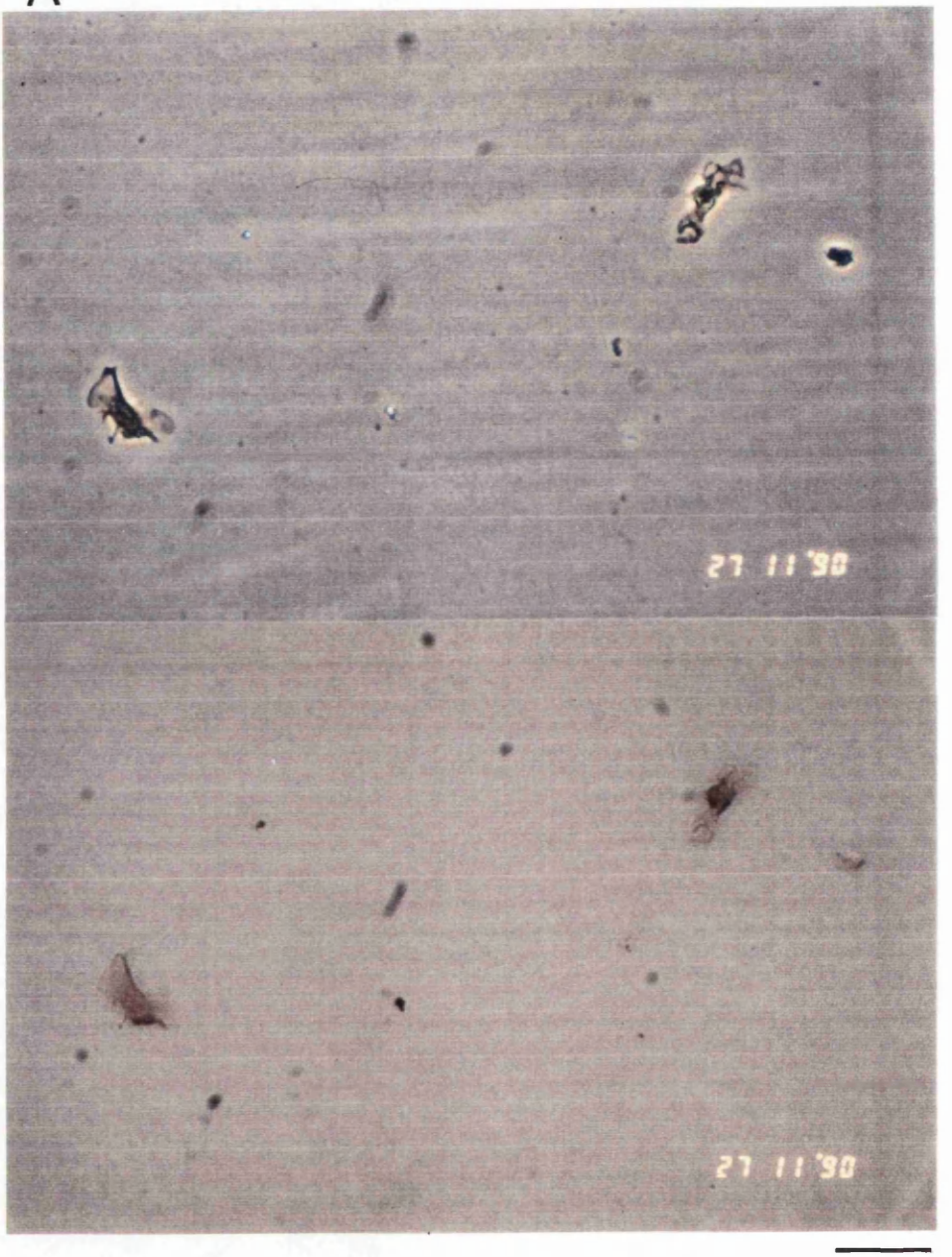
PLATE F *Achatina achatina* Scale bar 15μm

PLATE H *Achatina fulica* Scale bar 15μm

PLATE I *Helix aspersa* Scale bar 15μm

C_1

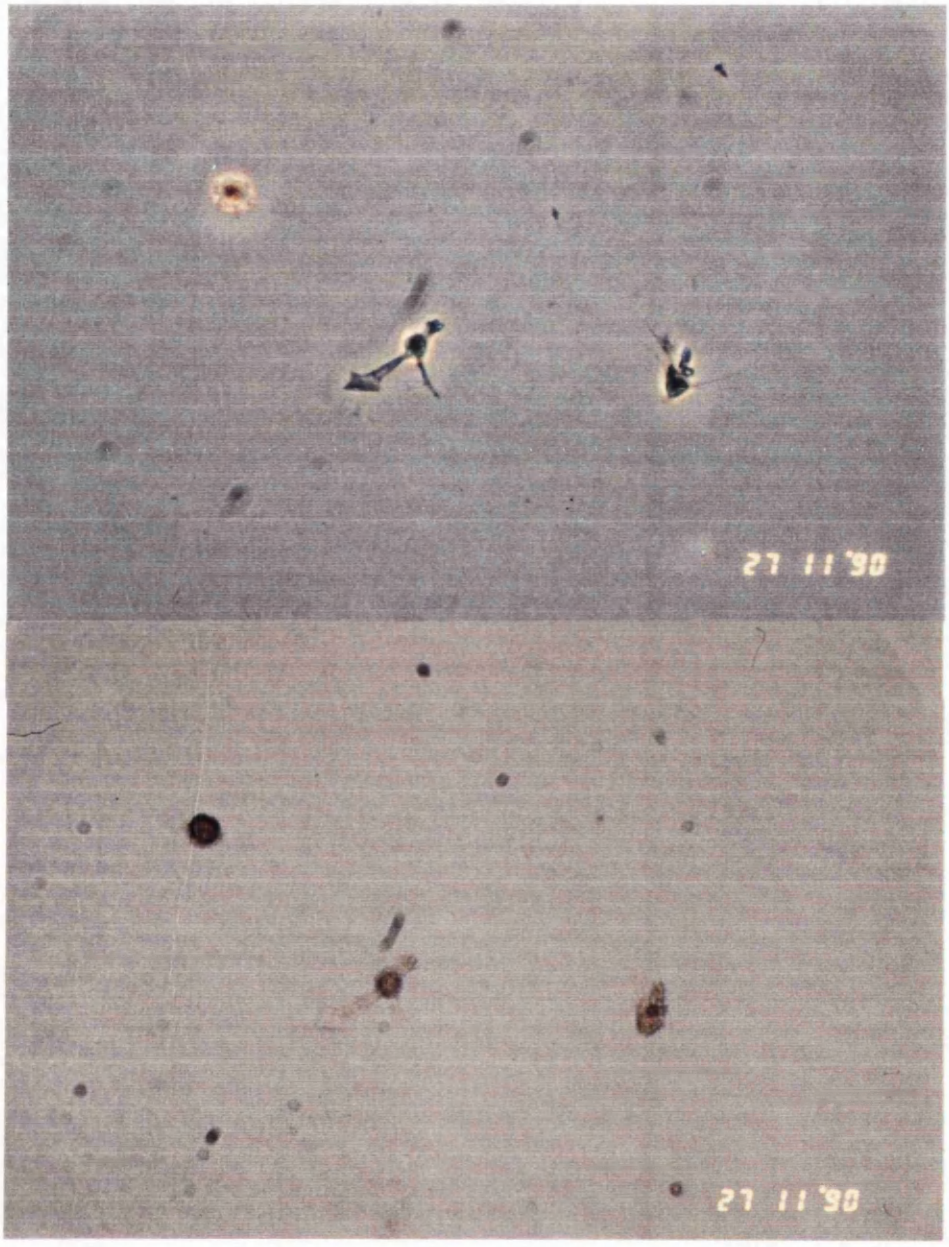
A



B

C_2

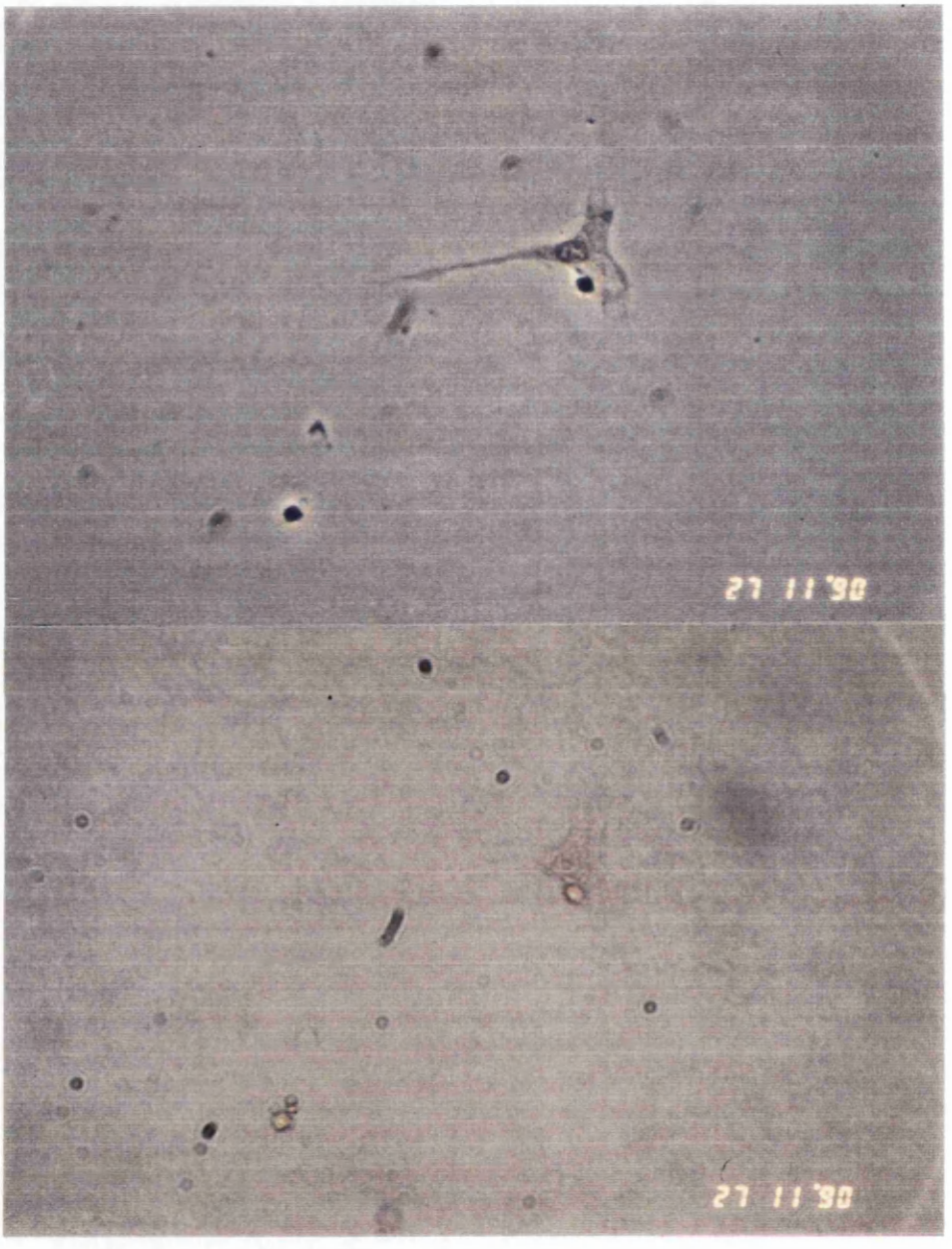
A



B

D_1

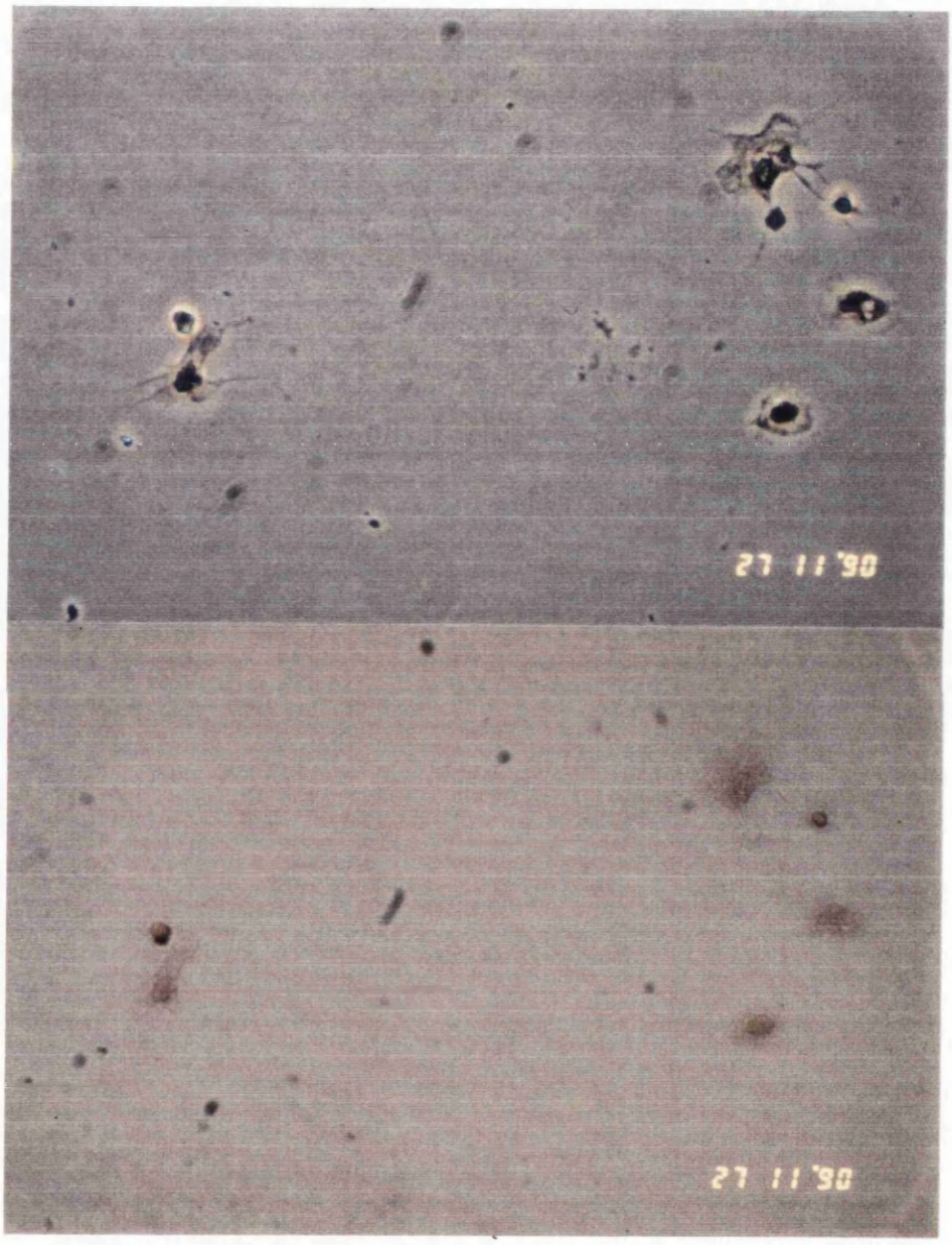
A



B

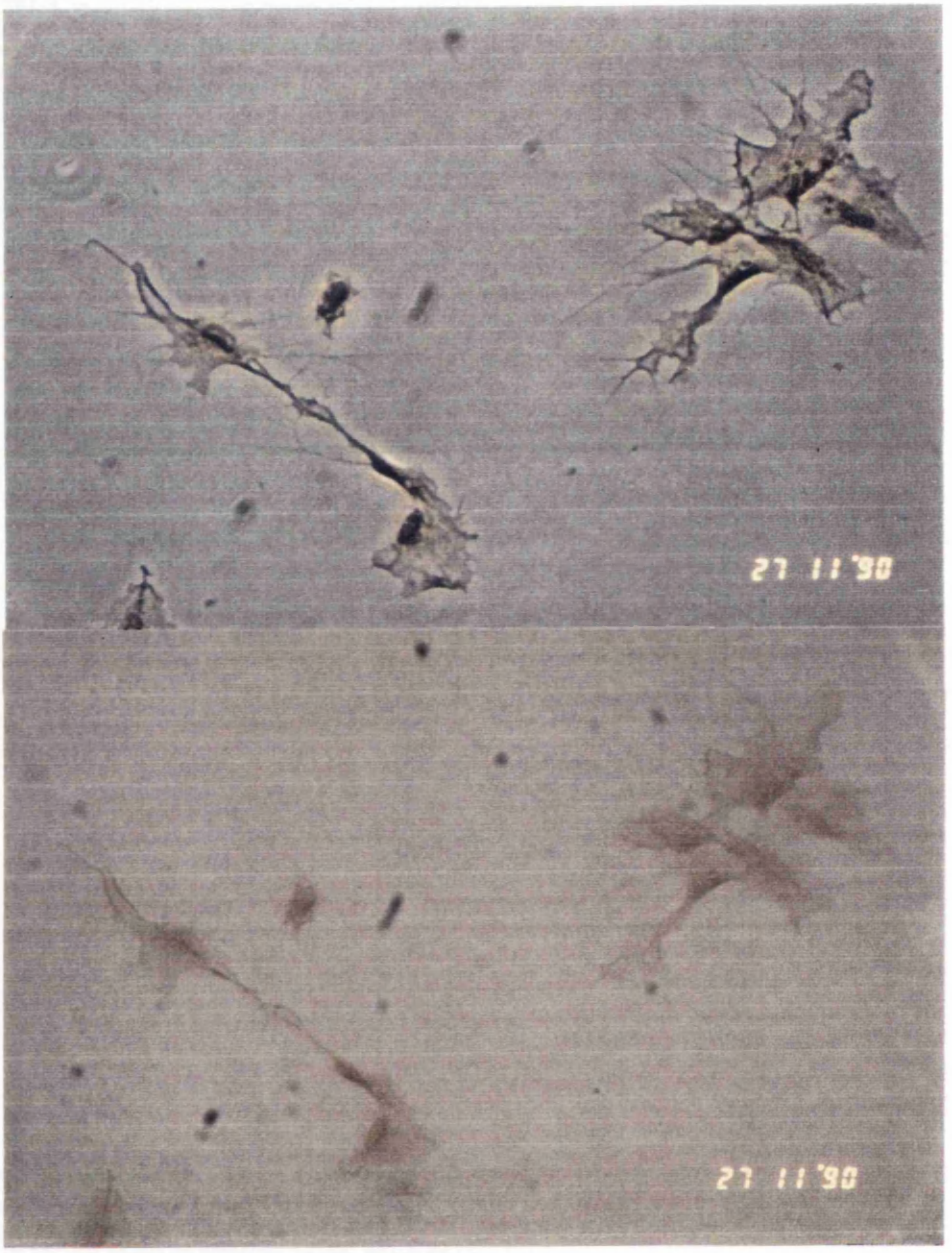
D_2

A



B

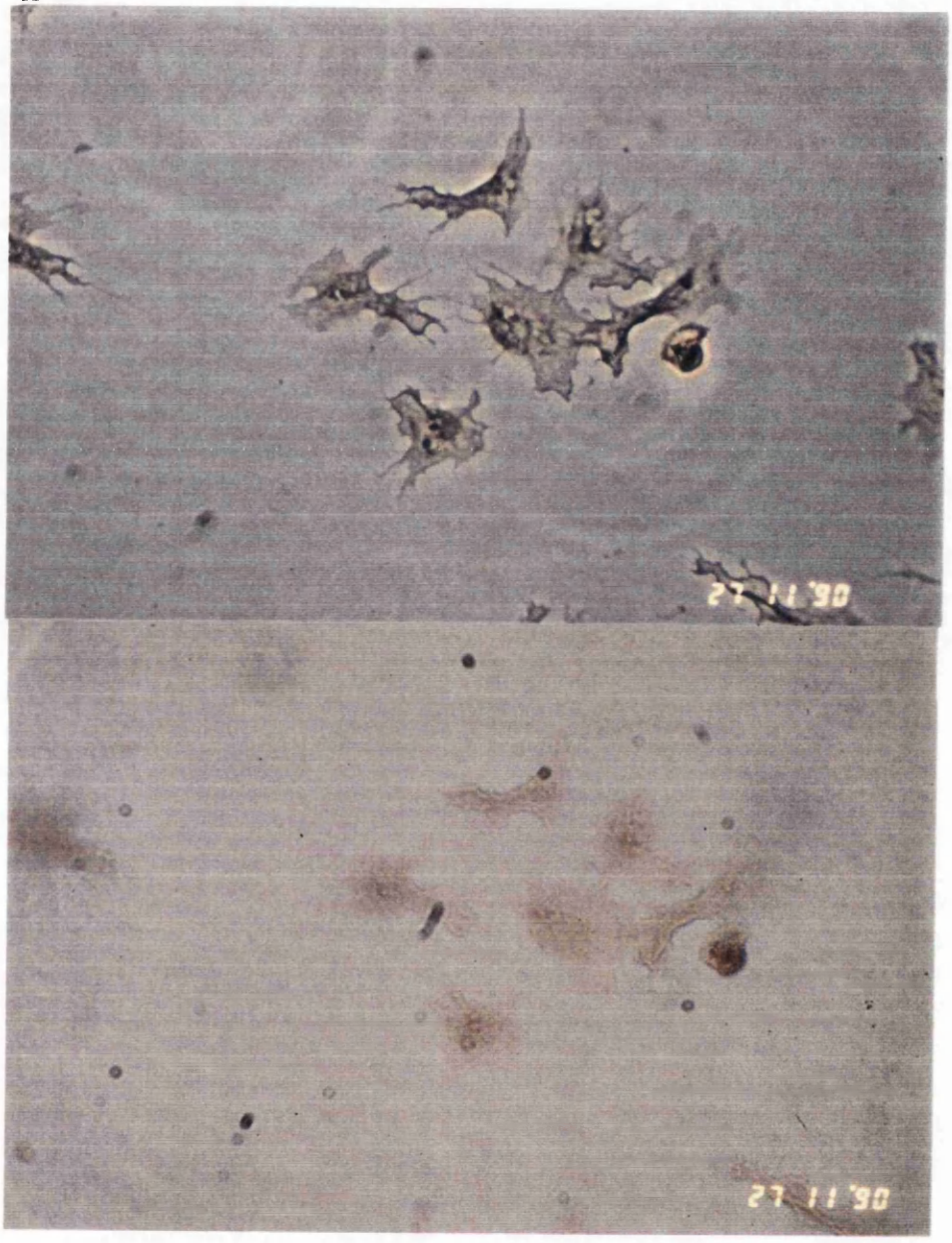
A



B

E_2

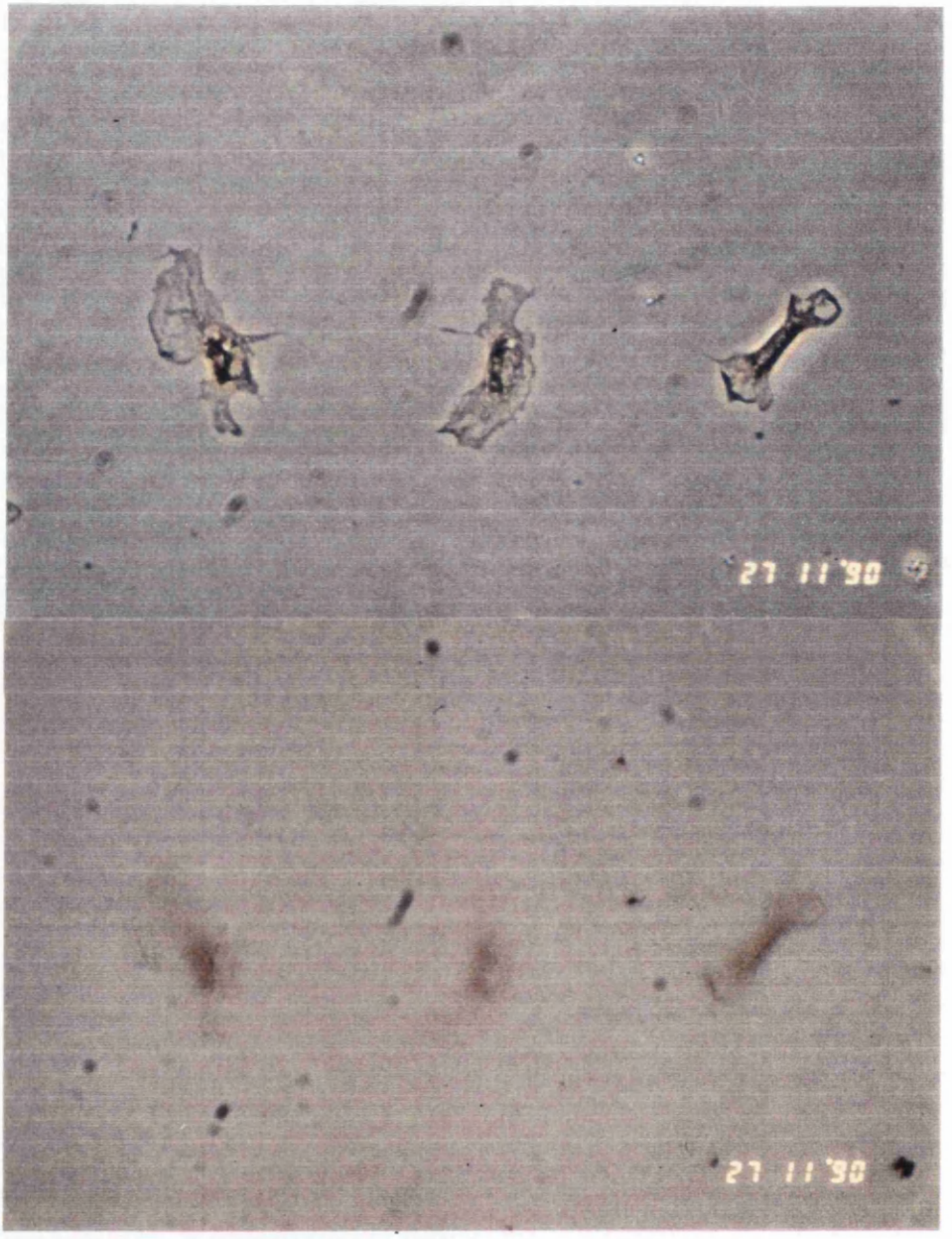
A



B

F_1

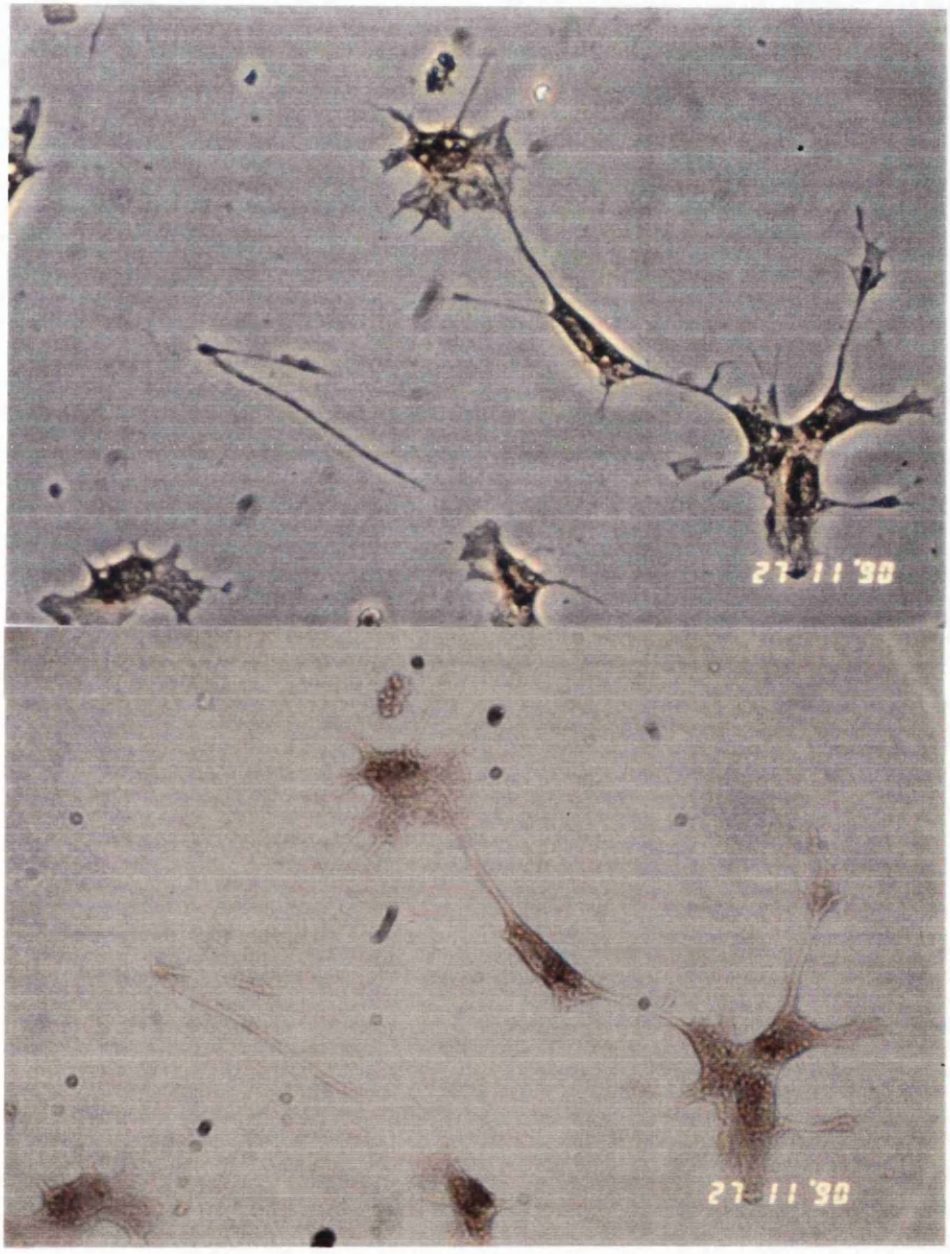
A



B

F_2

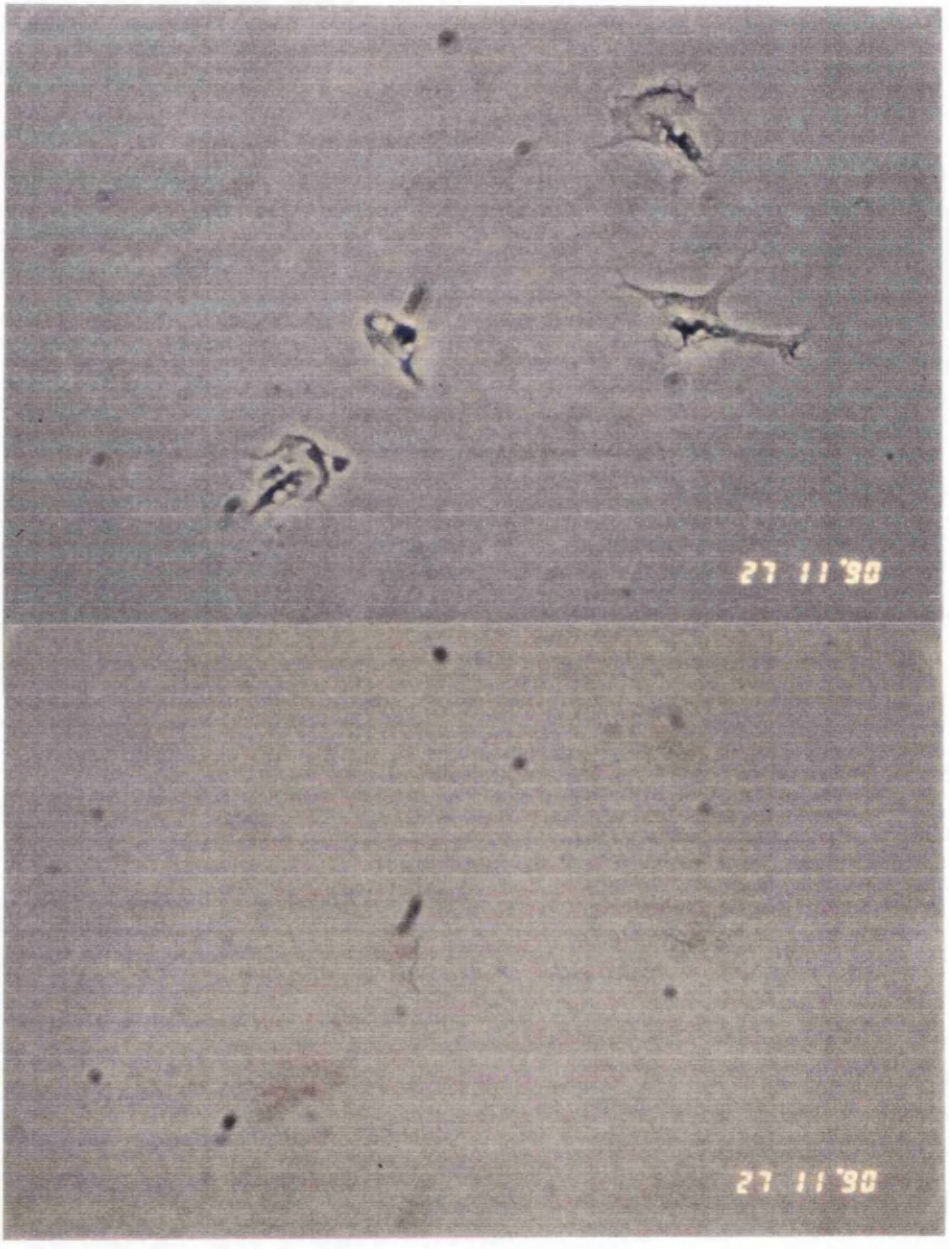
A



B

H_1

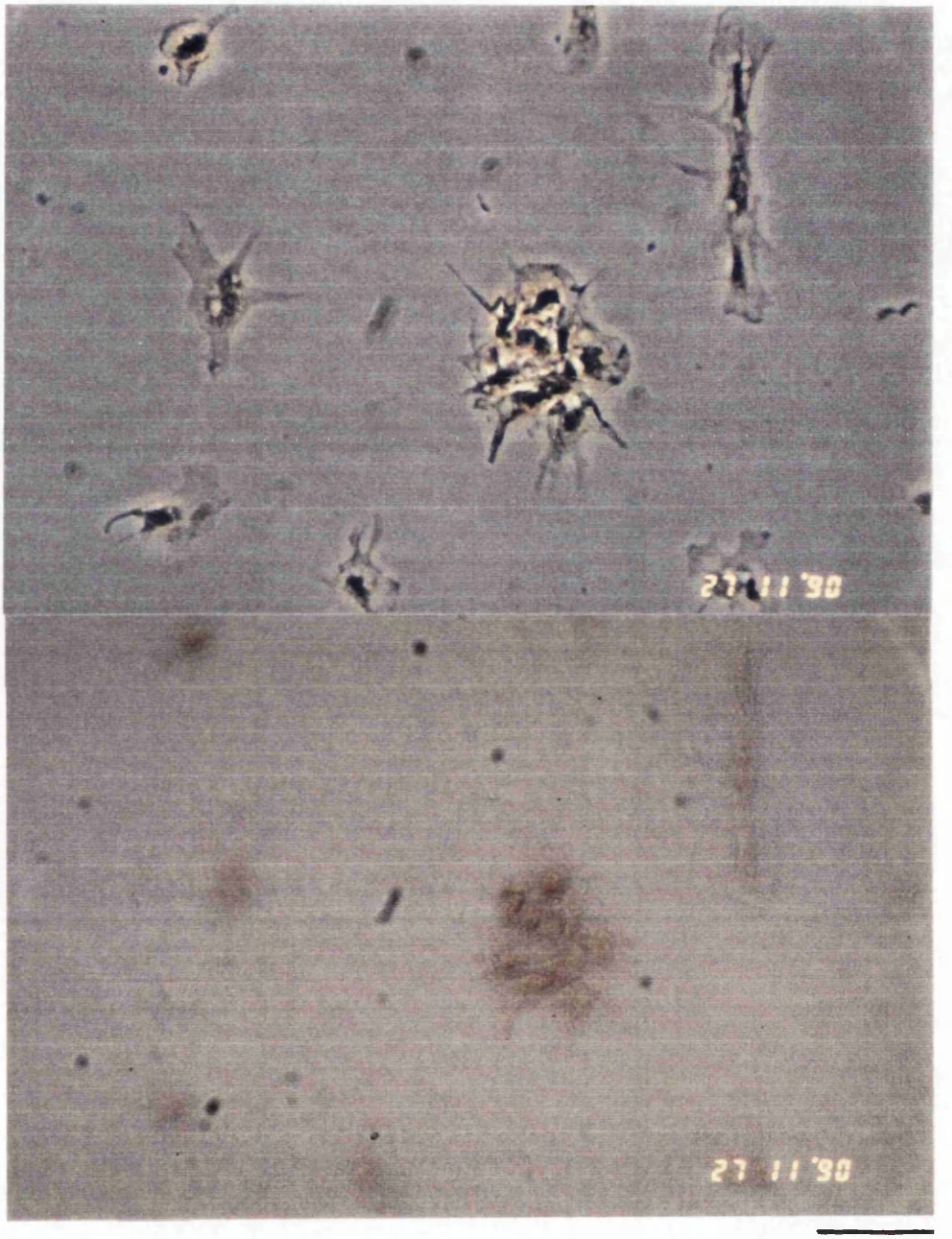
A



B

H_2

A



B

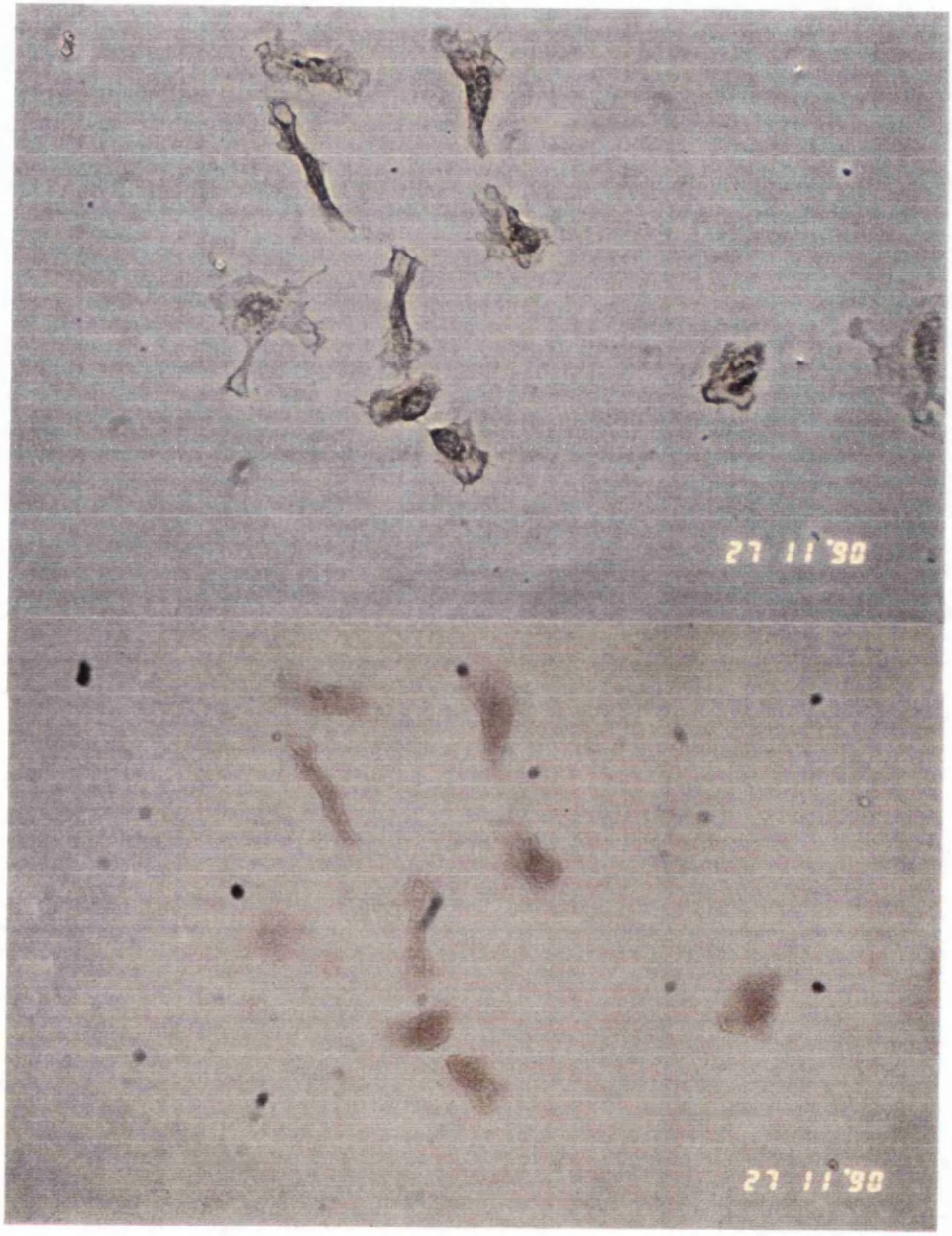
I_1

A

B

I_2

A



B

Biomphalaria glabrata and *B. natalensis* 272 was similar, and granule staining of *A. achatina* and *H. aspersa* was observed; with YIII, *L. stagnalis* and *H. aspersa* haemocytes were again not recognized, while differentiation of subpopulations was observed with *Biomphalaria glabrata* and *B. natalensis* 272. Two antisera were also used in staining experiments, B1 which had been raised against *B. truncatus* 1521 135k protein, and B2 which had been raised against *A. achatina* haemocytes. Again, the results of staining of haemocytes of the six snail species showed the same diversity as was obtained with the monoclonal antibodies.

ENDOGENOUS PEROXIDASE ACTIVITY.

Endogenous peroxidase activity was observed in haemocyte preparations of *B. natalensis* 272, *L. stagnalis*, *H. aspersa* and *A. achatina*, but not in those of *A. fulica* and *Biomphalaria glabrata* (Table 18, Plate 2). The fine morphology of the cells was lost when staining was performed on live cells, as the haemocytes tended to round up. However, peroxidase staining of fixed haemocytes gave weaker intensity reactions, although the morphology was unaltered, allowing localization of the activity within the cells. No endogenous peroxidase activity was detected in the control preparations for each species.

HAEMOCYTES PHAGOCYTOSE ZYMOSAN.

Haemocytes of all snail species were observed to phagocytose zymosan in SSS buffer. Ingestion of the particles rather than adherance to the surface was substantiated by focussing through the plane of the cells (Plate 3). The number of particles phagocytosed by particular haemocytes varied from one to five (with *L. stagnalis*), and the percentage of phagocytosing cells is given in Table 19.

TABLE 18 ENDOGENOUS PEROXIDASE ACTIVITY IN HAEMOCYTES OF SIX SNAIL SPECIES

SPECIES	NO.SNAILS	FIXED CELLS		LIVE CELLS	
		% POSITIVE	INTENSITY	% POSITIVE	INTENSITY
Aquatic					
Biomphalaria glabrata	5	0	-	0	-
Bulinus natalensis 272	15	7 +/- 1	+	10 +/- 1	
Lymnaea stagnalis	5	89 +/- 1	+	96 +/- 2	
Terrestrial					
Achatina achatina	2	75+/- 11	+	96 +/- 4	
Achatina fulica	3	0	-	0	
Helix aspersa	5	6 +/- 3	+/-	17 +/- 1	

Monolayers were made using pooled haemolymph. Endogenous peroxidase activity was assayed by DAB reaction in both live and fixed cells. The percentage of positive cells is given as a mean +/- standard deviation, n=3.

Scoring of reaction intensity: NO ACTIVITY -
WEAK ACTIVITY +/-
CLEAR ACTIVITY +
STRONG ACTIVITY ++
VERY STRONG ACTIVITY +++

PLATE 2. Endogenous peroxidase activity.

Fixed snail haemocytes were incubated with diaminobenzidine (DAB) and hydrogen peroxide. The presence of endogenous peroxidase in the haemocytes was demonstrated by dark blue deposits (arrows). Magnification 100x. 2a: *Achatina achatina*; 2b: *Bulinus natalensis* 272; 2c: *Helix aspersa*; 2d: *Lymnaea stagnalis*.

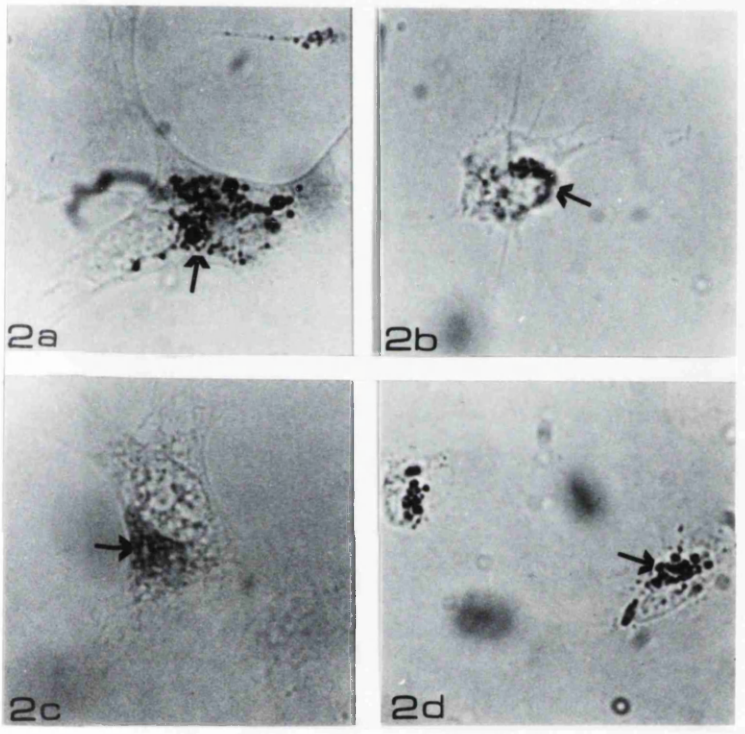

2a
2b
2c
2d

PLATE 3. Phagocytosis of zymosan particles.

Glass adherent haemocytes were incubated with zymosan particles in SSS. After one hour the haemocytes were washed to remove non-haemocyte-associated particles and fixed. A number of haemocytes of all snail species had ingested zymosan particles (z). Magnification 40x. 3a: *Achatina achatina*; 3b: *A. fulica*; 3c: *Biomphalaria glabrata*; 3d: *Bulinus natalensis* 272; 3e: *Helix aspersa*; 3f: *Lymnaea stagnalis*.

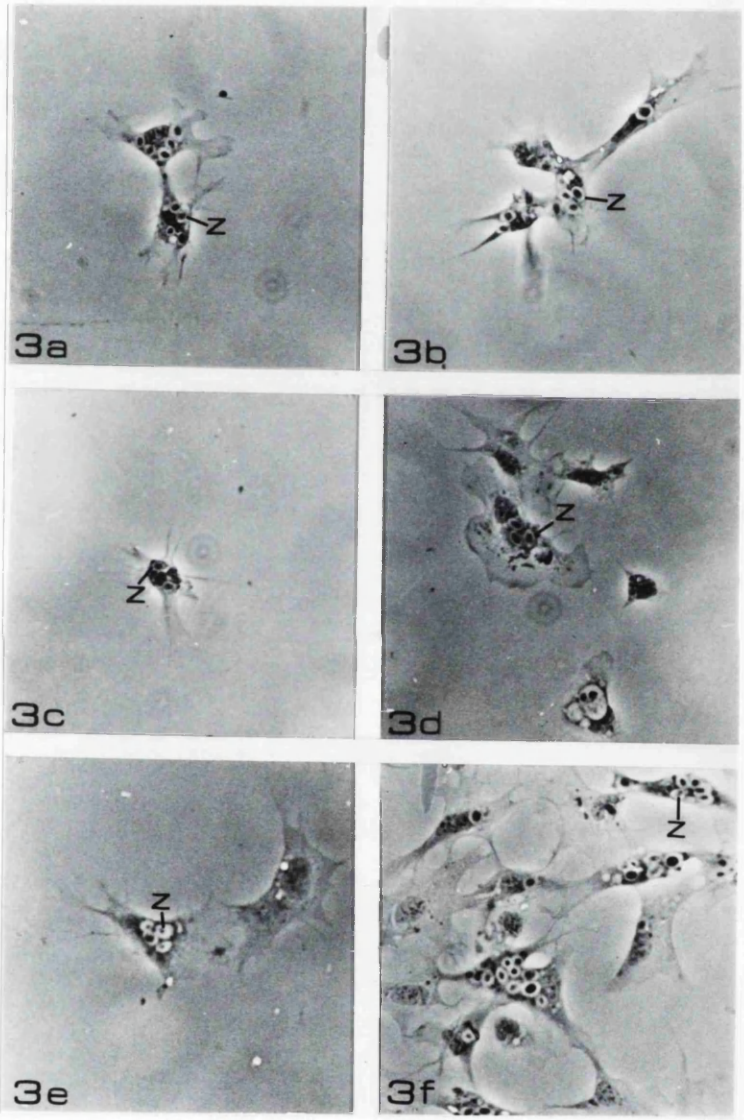

Z
3a
Z
3b
Z
3c
Z
3d
Z
3e
Z
3f

TABLE 19 PHAGOCYTOSIS OF ZYMOSAN PARTICLES BY HAEMOCYTES

SPECIES	NO.SNAILS	PERCENTAGE PHAGOCYTOSING CELLS
Aquatic		
Biomphalaria glabrata	5	32 +/- 9
Bulinus natalensis 272	15	8 +/- 2
Lymnaea stagnalis	5	66 +/- 22
Terrestrial		
Achatina achatina	2	79 +/- 22
Achatina fulica	3	75 +/- 13
Helix aspersa	5	20 +/- 12

Monolayers were made using pooled haemolymph. Following a 1 hour incubation with zymosan particles at room temperature, the percentage of phagocytosing haemocytes was determined. The results of four separate experiments are given as a mean +/- standard deviation.

HAEMOCYTES PRODUCE SUPEROXIDES.

Haemocytes of all snail species were observed to reduce nitrobluetetrazolium to insoluble formazan upon phagocytic stimulation, indicating the production of superoxide molecules (Table 20, Plate 4). This was clearly indicated by the blue deposits present around ingested zymosan particles. The reaction product was not observed in the control incubations, in which haemocytes were not stimulated or in which superoxidedismutase (SOD) had been added to scavenge for superoxide.

LUMINOL DEPENDENT CHEMILUMINESCENCE.

Luminol dependent chemiluminescence (LDCL) activity was demonstrated with zymosan stimulated haemocytes of *A. achatina* and *A. fulica*. Resting haemocytes displayed a very low activity whereas SOD inhibited the chemiluminescence of activated haemocytes strongly, the activity being marginally higher than the resting level. The observed activity was higher for *A. achatina* (Figure 38) than for *A. fulica* (Figure 39), although both these activities were significantly smaller than that of *L. stagnalis* (Figure 40) haemocyte monolayers. The differences in activities may again reflect the fact that the incubation buffer was specifically designed for *L. stagnalis* haemocytes. Haemocytes of both *Achatina* species might be capable of equivalent levels of LDCL given a buffer which is osmotically balanced with their intracellular environments.

TABLE 20 SUPEROXIDE PRODUCTION ASSAY

SPECIES	NO.SNAILS	PERCENTAGE NBT REDUCTION POSITIVE HAEMOCYTES	RELATIVE ACTIVITY
Aquatic			
Biomphalaria glabrata	5	30 +/- 14	+
Bulinus natalensis 272	15	4 +/- 1	+
Lymnaea stagnalis	5	8 +/- 3	+
Terrestrial			
Achatina achatina	2	97 +/- 1	+++
Achatina fulica	3	39 +/- 2	++
Helix aspersa	5	69 +/- 30	+

PLATE 4. Superoxide production by haemocytes.

Phagocytosing haemocytes were incubated with nitrobluetetrazolium (NBT). In haemocytes of all snail species tested superoxide was generated as indicated by the dark formazan deposits (arrows) surrounding phagocytosed zymosan particles (z) in haemocytes. Magnification 100x. 4a: *Achatina achatina*; 4b: *A. fulica*; 4c: *Biomphalaria glabrata*; 4d: *Bulinus natalensis* 272; 4e: *Helix aspersa*; 4f: *Lymnaea stagnalis*.

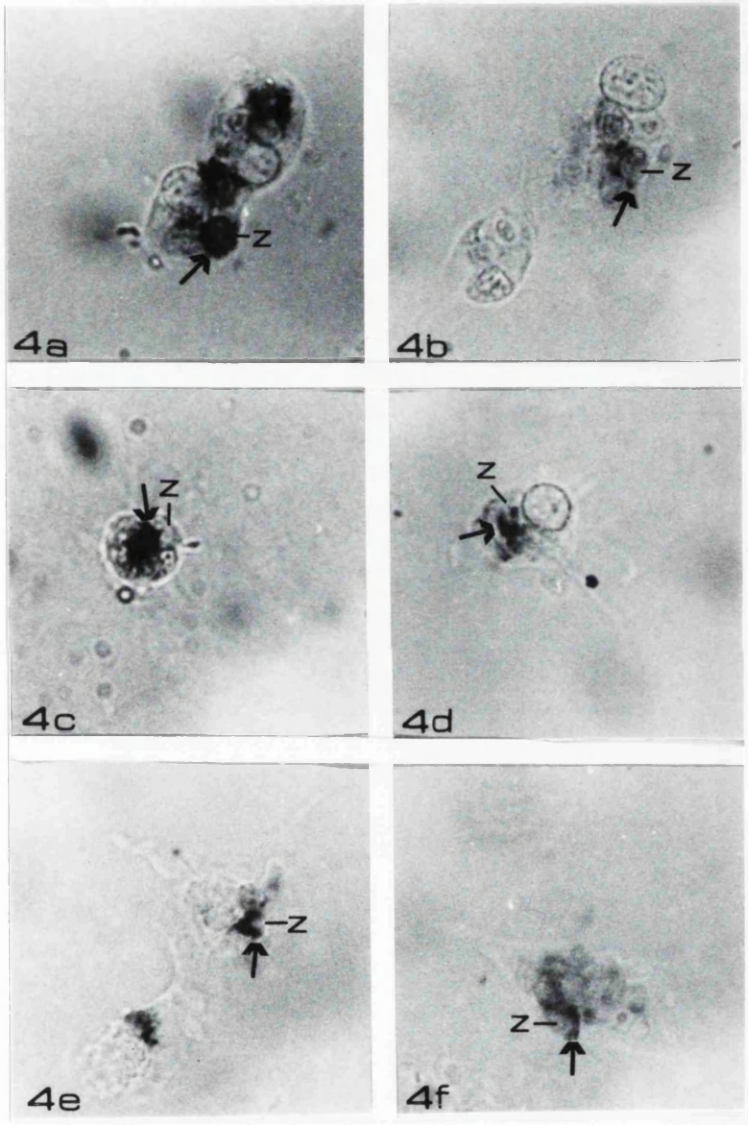

z
4a
z
4b
z
4c
z
4d
z
4e
z
4f

FIGURE 38. Luminol-dependent chemiluminescence (LDCL) activity of *Achatina achatina* haemocytes upon phagocytic stimulation by zymosan particles.
LDCL activities of stimulated haemocytes (Aa1) and resting haemocytes (Aa3) are shown. The LDCL response of stimulated haemocytes in the presence of superoxide dismutase (SOD), a specific scavenger for superoxide, is also shown (Aa2). The data given are for one experiment.

FIGURE 38.
LDCL ACTIVITY OF Achatina achatina HAEMOCYTES

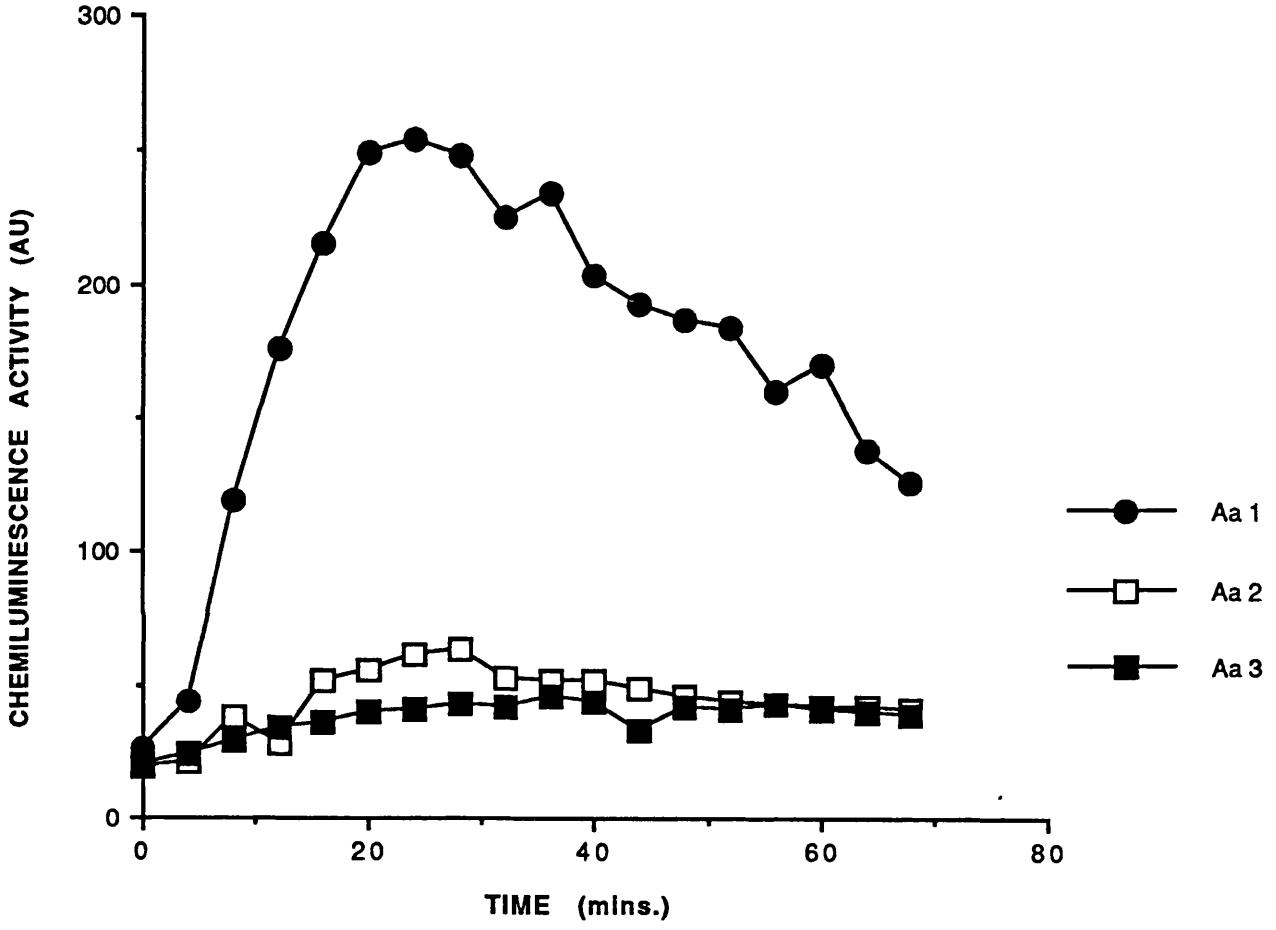


FIGURE 39. Luminol-dependent chemiluminescence (LDCL) activity of *Achatina fulica* haemocytes upon phagocytic stimulation by zymosan particles.
LDCL activities of stimulated haemocytes (Af1) and resting haemocytes (Af3) are shown. The LDCL response of stimulated haemocytes in the presence of superoxide dismutase (SOD), a specific scavenger for superoxide, is also shown (Af2). The data given are for one experiment.

FIGURE 39:
LDCL ACTIVITY OF Achatina fulica HAEMOCYTES

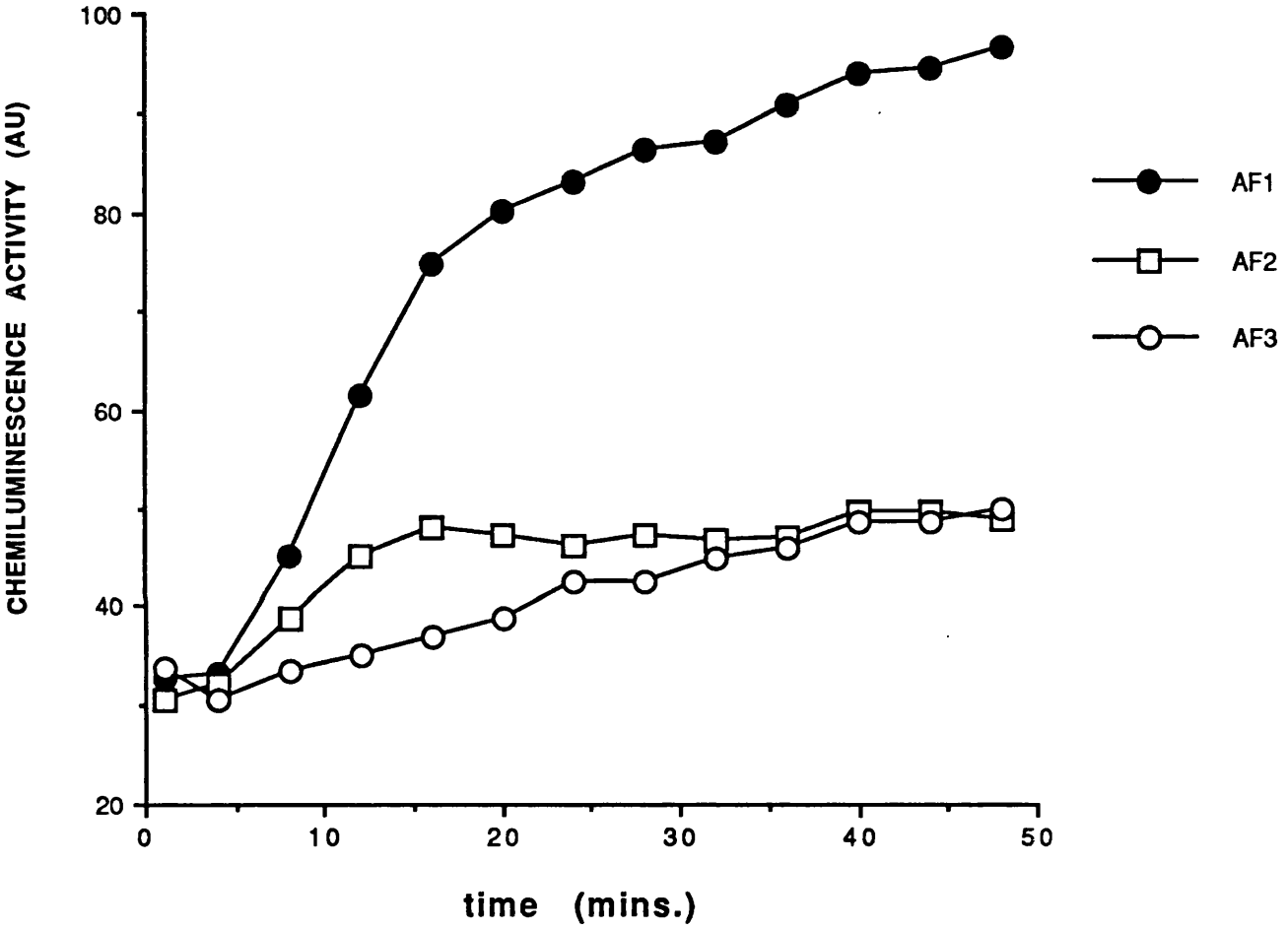
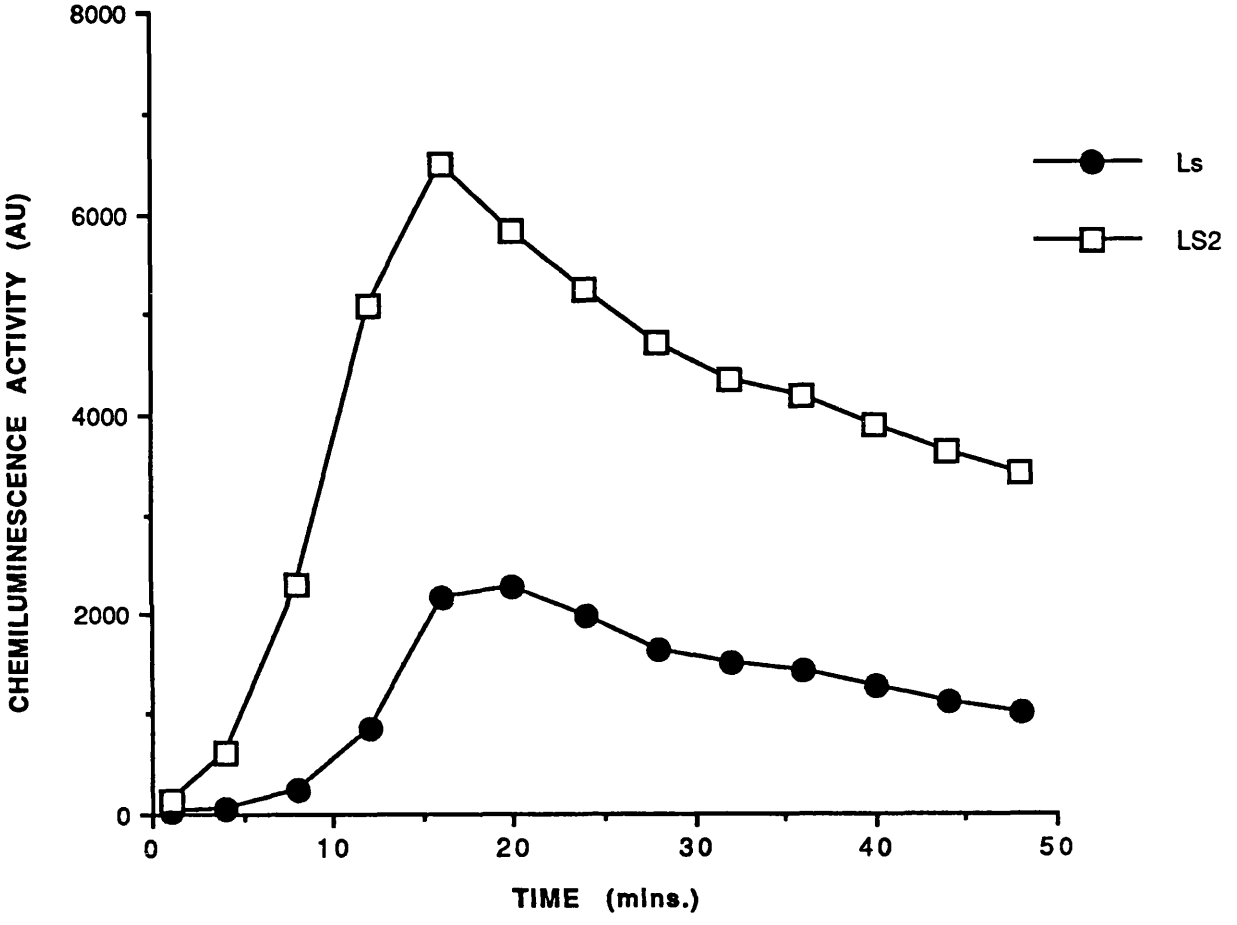


FIGURE 40. Luminol-dependent chemiluminescence (LDCL) activity of *Lymnaea stagnalis* haemocytes upon phagocytic stimulation by zymosan particles.

LDCL activities of stimulated haemocytes (Ls1) and (Ls2) in the two separate experiments are given. Haemocytes of this species were used a control for the investigation of *Achatina spp.* haemocyte LDCL, but due to their much higher values, they are shown separately. Variation in the experimental results given the same haemocytes and the same conditions is also demonstrated.

FIGURE 40.
LDCL ACTIVITY OF Lymnaea stagnalis HAEMOCYTES



DISCUSSION.

The defence reactions elicited by molluscs in response to physical injury or infection by unicellular or metazoan parasites involve combinations of cellular and plasma effector components. Successful defence is only achieved when there is an effective integration of both cellular and humoral factors, and these interactions may require a high degree of specificity. Penetration of freshwater snails by larval schistosomes, their subsequent development and migration inflicts a considerable stress on the intended host. Successful destruction of these invading parasites requires not only an effective recognition by the snail of nonself and subsequent mobilisation of haemocytes, but also an ablilty to circumvent any constraints the parasites may impose on the normal functioning of these processes. In the natural host, either effective recognition does not occur, or the parasite is successful in subverting the host defence responses. Recently, experimental studies of the interactions between larval schistosomes and snails have been confined to the *Biomphalaria glabrata-S. mansoni* and *Lymnaea stagnalis-Tricobilharzia ocellata* associations. The functional specificities and diversities of haemocytic populations and the precise roles of humoral factors such as haemagglutinins in such interactions are still undetermined.

The present study has confirmed the presence of haemagglutinating activity in the haemolymph of the African freshwater snail *Bulinus nasutus* 1214. Agglutinating activity is not detectable in the egg masses of this, or any other bulinid species tested, but has been shown for other snail species (*Helix pomatia* and *Helix aspersa* Hammarstrom & Kabat, 1969; *Arianta arbustorum* Renwrantz and Berliner, 1978; and

Biomphalaria glabrata Stein and Basch, 1979) (see Table 5) . With *B. nasutus* 1214 plasma, a variety of vertebrate erythrocytes were agglutinated, and maximum titres were higher than in most other invertebrate species which have been studied. The highest reactivity was recorded for human A_2 type red blood cells. The terminal sugar residues present on the surface of mammalian erythrocytes are well characterised (Figure 41), and the variation in titres observed with different human cell types reflects the saccharide specificity of the agglutinin binding site (i.e. human A_1 cells have α-Gal-NAc but B_1 cells have α-Gal). A preference for a given cell type therefore implicates the carbohydrate specificity of an agglutinating molecule. However, the inability of any single simple sugar to inhibit agglutination suggests that agglutination involves more than a single carbohydrate specificity. The efficiency of inhibition of agglutination by mucins, which are glycoproteins with multiple carbohydrate specifities, lends support to this hypothesis. The inability of combinations of simple sugars to inhibit agglutinating activity suggests that not only are multiple specificities involved in binding, but that the steric arrangement of these saccharides is also an important factor. Inactivation of porcine mucin inhibition by heat further illustrated this point. The majority of haemagglutinins which have been recorded from invertebrate groups are inhibited by galactose or galactose-derivatives. This is particularly true of the Insecta, of which an extensive range of species have been studied (Table 4). The inhibition of agglutinating activity of *B. nasutus* 1214 haemolymph with mucins is in agreement with the study of Renwrantz and Stahmer, (1983) with *H. pomatia,* in which no single carbohydrate gave inhibition, but mucins gave complete inhibition. The involvement of the constituent galactose moieties of mucins would make an interesting study. Determination of agglutinin sugar specificity is important for two reasons. First, knowledge of a single specificity should allow a

FIGURE 41.
CARBOHYDRATE CHAINS ON THE SURFACE OF MAMMALIAN ERYTHROCYTES.

Human type A -

α-GalNAc(1-3)-ß-Gal(1-3, or 1-4)-GluNAc.....
α-1,2-fucose

Human type B -

α-Gal(1-3)-ß-Gal(1-3, or 1-4)-GluNAc.....
α-1,2-fucose

Human type O -

ß-Gal(1-3, or 1-4)-GluNAc.....
α-1,2-fucose

Sheep -

α-GalNAc(1-3)-GalNAc(ß-1-3)-Gal(α-1-4)-Gal(ß-1-4)-CERAMID

Rabbit -

α-Gal(1-3)-Gal(1-3)-αGalNAc(1-3)-Gal(1-4)-CERAMID

single-step purification of the molecule. Second, the carbohydrate composition of the surface of many pathogenic microorganisms are known, thus the interactions of such organisms with agglutinins in both *in vitro* and *in vivo* studies can be predicted.

B. nasutus 1214 agglutinating activity was sensitive to temperature and proteolytic digestion, and was independent of the presence of divalent cations. Most invertebrate agglutinins have been shown to be thermally unstable, with a loss of activity generally occurring at temperatures in excess of 50°C, indicating their proteinaceus nature. However, lipoidal lectins, which are not inactivated by heating have been shown to be present in some invertebrates (e.g. *Styela plicata* Fuke and Sugai, 1972). Dependence on the presence of divalent cations varies among agglutinins of invertebrate phyla. They have been shown to be necessary for reactivities of haemagglutinins of some Crustacea and Insecta, but among the Mollusca the requirement is particularly variable; among the Helicidae, for example, there has been no report of a divalent cation dependence for agglutinating activity (Renwrantz & Berliner, 1978). Divalent cations are postulated as being required for maintenance of the structural stablilty of the haemagglutinating molecules, there being an increased tolerance to heat inactivation for instance, in the presence of cations for dependent species. Maintenance of the structural stablity of agglutinins independent of the presence of divalent cations must be accomplished (if at all) by some other means.

Preadsorption experiments were conducted in order to investigate the possibility that agglutinating activity was being achieved by heteroagglutinins This resulted in a complete loss of agglutinating activity when the second erythrocyte type was employed (with any combination of the mammalian red cell types), indicating that a single agglutinating type is present within the haemolymph of *B. nasutus* 1214. This appears to be a common phenomenon among gastropod molluscs, but among other

invertebrate groups, the tunicates in particular, the presence of multiple agglutinins within the body fluids is the rule. The existence of multiple agglutinins within the body fluids of certain phyla and not others is intriguing. With multiple agglutinins, a range of binding specificities would be available for recognition purposes, possibly encompassing the great range of epitopes presented by a diverse group of invading pathogens. However, within the Mollusca the carbohydrate specificities of their haemolymph agglutinins has indicated multiple binding specificities on a single molecule (e.g. mucins are the only abrogators of agglutinating activities in some animals, such as *B. nasutus* 1214). It is a matter of debate whether a single lectin with multiple specificities can be as effective as a complement of lectins each with a single, different specificity in the recognition of nonself. There may also be evolutionary parallels of possession of several lectins with designated functional specificity, and the infinite variety of antibody specificity in vertebrates. Conversely the possession of fewer molecules with multiple functions might be metabolically more conservative, and could be construed as being of superior technical and evolutionary design. However, a complement of lectins would not necessarily require the existence of a complement of structurally dissimilar molecules. A variety of binding specificities could be achieved on a single backbone structure, identical to each lectin. Thus as with vertebrate antibodies, variable (Fab) and constant (Fc) portions of a molecule could enable functional diversity.

The agglutinating titre of *B. nasutus* 1214 plasma was not elevated either on wounding or on invasion by trematode parasites, indicating that the agglutinating agent in *B. nasutus* 1214 is normally present in the haemolymph, and its level is not induced on challenge. Levels of lectins may be increased in some invertebrate species, given the appropriate stimulus, as has been reported in the Insecta (e.g.

Sarcophaga peregrina Komano *et al*, 1980), but within the Mollusca this does not appear to be a typical phenomenon. However, this apparent lack of inducibility of the *B. nasutus* 1214 agglutinin might be explained by experimental criteria such as the nature of the applied stimulus and the extent of the time interval thereafter before body fluids are collected and analysed. With *B. nasutus* 1214, there was no change in agglutinating titres 3 hours post-wounding, or 3, 6 and 24 hours post-challenge with *S. margrebowiei* miracidia.

Agglutinating activity has been recorded in a number of snail species. Table 5 shows a summary of the characteristics of the purified agglutinins. A total of 28 populations of *Biomphalaria glabrata* have exibited agglutinating activity, but only 15 of these were shown to have agglutinating activity in the haemolymph. The agglutinating activity in extracts of egg masses and albumin glands is of unknown significance; however, it has been suggested that agglutinins might play a protective role. The albumin gland produces a secretion which coats eggs as they are laid. The presence of agglutinins might account for the lack of invasion of egg masses by microorganisms. Although this remains as yet hypothetical, such a postulated role has gained currency such that the term "protectins" has been coined for agglutinins found in this association (Prokop, Uhlenbruck & Kohler, 1968). The similarity of albumin gland/egg mass and haemolymph agglutinins is still unclear. In *A. fulica* for example, different agglutinins have been isolated and characterised from the albumin gland and haemolymph (Sarkar, Bachhawat & Mandal, 1984; Mitra & Sarkar, 1988), but in *Biomphalaria glabrata* a single agglutinating type is reported from all extracts, and is assumed to be a single protein (Jeong, Sussman, Rosen, Lie & Heyneman, 1981) (see Table 5). Only one strain of *Biomphalaria glabrata* (10-R2) was reported as

having no agglutinating activity against a test panel of erythrocytes (Boswell & Bayne, 1984). Of the conducted studies with *Biomphalaria glabrata*, certain characteristics of the agglutinating plasmas vary between strains, such as erythrocyte specificity and dependence on the presence of divalent cations. The carbohydrate specificity of the agglutinating glycoprotein has been determined in one study as N-acetyl-galactosamine (Boswell & Bayne, 1984), but there was no information of the range of inhibitory substances utilised (Boswell and Bayne, 1984). In another, N-acetyl-galactosamine was reported as definitely being non-inhibitory for agglutinating plasmas of different strains of *Biomphalaria glabrata* (Michelson and Dubois, 1977). All studies are in agreement with the thermal instability of the agglutinating agents.

These similarities and differences in the biological characteristics of haemagglutinins in different strains of *Biomphalaria glabrata* indicate that a heterogenous population of humoral lectins exist within this single species. Humoral haemolymph agglutinins have only been recorded in four other gastropods, *Otala lactea* (Boyd and Brown, 1965), *Arianta arbostorum*, (Renwrantz & Berliner, 1978), *Lymnaea stagnalis* (van der Knaap, Doderer, Boerrigter-Barendsen and Sminia, 1982), and *Achatina fulica* (Mitra and Sarkar, 1988), and in *L. stagnalis* the presence of heteroagglutinins was reported. The erythrocyte specificities of these haemagglutinins encompass human and rabbit types, and the carbohydrate specificities involve galactose derivatives. Each of these studies has been concerned with a single species, or in the case of *Biomphalaria glabrata* a single strain. Other members of the genera *Lymnaea* and *Biomphalaria* have not been investigated. A comparative study of the recognition factors present within these genera would increase the significance of the characterisations of the agglutinins present in the haemolymphs of the investigated species.

This study reports the presence of agglutinating activity in haemolymph of *Bulinus spp.*. The confinement of this potent activity to a single species, and a single isolate of that species (i.e. haemolymph of *B. nasutus* isolates 476 and 1583 did not agglutinate the same panel of erythrocytes) is puzzling. This observation may reflect the sensitivity of the agglutination assay. There are a considerable number of factors which could contribute to variations in titres expressed in agglutination assays - (i) the age of the test cells; (ii) the saline in which the suspensions were made; (iii) the temperature at which assays were performed; and (iv) quantitative errors in constructing the dilution series. In attempting to compare the agglutinating activities recorded in separate studies, the sources of the test cells and the differences therein are of paramount concern due to the nature of the available carbohydrates and their organisations. The range of test cells is usually restricted to those readily available, and have included alligator and human umbilical cord types, but more usually compose only of human, rabbit and sheep types. One cannot preclude the possibility that "non-agglutinating" haemolymphs from certain species would be capable of agglutination of other, untried erythrocyte specificities, thereby indicating the presence of equivalent molecules within the haemolymph of these species. Indeed, enzyme treatment and extension of erythrocyte types proved necessary for the demonstration of agglutinating activity in the ascidian *Boltemia ovifera*, which had previously been reported as being non-agglutinating (Vasta and Marchalonis, 1984). Thus in this dissertation, the lack of agglutinating activity in all but one species of *Bulinus* is not to be taken as implying an **absence** of lectins within the haemolymph of these other snails, but rather a possible presence of agglutinins with a lack of specificity for the normal panel of erythrocyte types employed.

It is apparent that despite the reports of agglutinating activities in body fluids of a large and wide range of invertebrate species, complete characterisation of the nature of these molecules is restricted to a few isolated studies (Table 5). Very few haemagglutinins have been purified to homogeneity, and this reflects the difficulties encountered in separation of plasma factors from the respiratory pigments haemoglobin and haemocyanin which are the major constituents of invertebrate haemolymphs. Without a carbohydrate specificity, or with a low avidity carbohydrate specificity, resolution of lectins in snails has been severely restricted. Consequently the conclusions to be drawn from many studies directed at investigating specific host defence mechanisms against parasite invaders (e.g. *in vitro* killing of sporocysts) using whole plasma or whole haemolymph are speculative to say the least. Purification of possible humoral effectors is essential if hypotheses as to their precise roles are to be devised (see Points of Perspective).

In an attempt to pellet haemoglobin due to its reported high M_r, *B. nasutus* 1214 haemolymph was ultracentrifuged at 178,000 xg. Earlier studies with haemolymph of *Biomphalaria glabrata* were not in agreement as to the effectiveness of this process (Yoshino & Bayne, 1983; Granath, Spray & Judd, 1987). In the former study, proteins were subsequently isolated from the supernatant fraction following ultracentrifugation at 50000 xg for five hours, although SDS PAGE profiles were not included in this report, thus the number of proteins remaining after ultracentrifugation is unclear. In the latter study, it was reported that for all intents and purposes every haemolymph protein was pelleted following identical centrifugation procedures. In the present study, the overall effect of ultracentrifugation at 178000 xg was intermediate to these two earlier studies. Haemoglobin was indeed sedimented after 30 minutes, but never entirely. SDS

PAGE analysis (under reducing conditions) of the resulting supernatant revealed the presence of high M_r bands (>120k) and spectrophotometric recording at 410nm indicated the presence of haemoglobin. Agglutinating activity was recorded both in the pellet and supernatant fractions, but a higher activity was recorded in the pellet fraction. This could be explained in a number of ways - (i) the agglutinin is a protein of high M_r, and is sedimented by ultracentrifugation at 178,000 xg; (ii) the agglutinin is a protein of low enough M_r not to be sedimented, but is forcibly sedimented into the pellet fraction by the abundance of haemoglobin (of high M_r), a residual level of agglutinating activity in the supernatant being accounted for by agglutinin molecules that "escaped" such a fate; (iii) the agglutinin binds specifically to haemoglobin, possibly through glycosylated sites on the haemoglobin, and is sedimented due to the haemoglobin being sedimented, the binding of molecules being unaffected by the process; or (iv) the agglutinating activity is achieved by haemoglobin molecules themselves, the residual agglutinating activity in the supernatant being explained by the presence of haemoglobin molecules that it has been shown were not sedimented.

Under identical experimental conditions, the haemoglobin of vertebrates did not sediment. Vertebrate haemoglobin has been one of the most extensively studied protein molecules. In all vertebrate haemoglobins there is a considerable uniformity of both structure and function. They present a relative molecular mass of 68k, are composed of two types of peptide chain with one heme group per molecule, and have an isoelectric point between pH 6.45-7.45. Haemoglobins which exist free in the circulating fluid of some gastropods, annelids and arthropods have a molecular mass of about 1.6 or 3.2×10^6, an estimated content of 96 or 192 heme groups per molecule and an isoelectric point between pH 4.5-6.0 (Figuereido, Gomez, Heneine,

Santos and Hargreaves, 1973). The haemoglobin of *Biomphalaria glabrata* (a strain native to Belo Horizonte, Brazil) has been characterised as constituting 97% of the total haemolymph protein (Figuereido *et al*, 1973), having a pI of 4.6, a molecular mass of $1.75\pm0.06\times10^6$ (Almeida and Neves, 1974); furthermore it is a glycoprotein which contains 3% sugars and two peptide chains (Afonso *et al*, 1976). Some comparisons in amino acid content were established between this haemoglobin and human haemoglobin A (Almeida and Neves, 1974). Not all snails possess haemoglobin as a respiratory pigment. Some have haemocyanin, and the M_r of this protein in *H. pomatia* has been calculated as 6×10^6 (Svedberg, 1933). It is clearly apparent that these proteins dominate invertebrate plasmas. Their glycosylation may facilitate additional functions to oxygen carriage, and undoubtedly account for the great polymerisation which distinguishes them from their vertebrate counterparts. Some vertebrate haemoglobins (about 20% of the total in normal individuals) may be nonenzymatically glycosylated, glucose, mannose and galactose being the usual identified monosaccharides (Krishnamoorthy, Cahour, Elion, Hartmann and Labie, 1983). The glycosylation of snail haemoglobins may also account for their behaviour on ultracentrifugation. Being "sticky" molecules, under such forces they have little alternative to amalgamation and sediment. The possibility of association with other plasma components during this process, be it specific or nonspecific, is highly probable. Haemoglobin and haemocyanin are regarded by many workers as "undesirable" in studies such as this one, but I do not hold with this criticism, and believe them worthy of study. The sheer abundance of these "respiratory" proteins within the haemolymph of invertebrate animals gives much opportunity for functions which their vertebrate counterparts could never achieve within the physical constraints of an erythrocyte. As haemoglobins and other plasma factors become

purified from haemolymphs, the interactions of these proteins may prove to be of some importance.

It was the fact of glycosylation of invertebrate haemoglobin which prompted the use of lectin-conjugated beads reported here. The aim was to attempt to remove haemoglobin from haemolymph, thereby facilitating separation of the remaining components by standard chromatographic techniques. The assumption was made that the haemoglobin in haemolymph of *B. nasutus* 1214 would be structurally similar to that of *Biomphalaria glabrata* in which the presence of mannose, galactose and fucose had been reported (Afonso *et al*, 1976). A bead matrix was required which would not non-specifically adsorb proteins, and which was available both with and without conjugated lectin. As large as possible a bead size was required, to allow for maximum surface area over which adsorption could occur. Thus sepharose 4B beads were utilised. Only three lectin-conjugated beads were available which satisfied these requirements, lentil lectin (*Lens culinaris*), concanavalin A (*Canavalia ensiformis*) and wheat germ agglutinin (*Triticum vulgaris*), and these have different carbohydrate specificities. Lentil lectin and concanavalin A-conjugated beads were used in adsorption experiments with *B. nasutus* 1214 haemolymph. While with lentil lectin only some haemoglobin molecules were removed from the haemolymph, with concanavalin A the resultant supernatant following sedimentation of the beads appeared colourless, indicating that all haemoglobin had adsorbed onto the beads. With non-conjugated beads as a control, no haemoglobin adsorbed, indicating that adsorption with concanavalin A was specific, and was mediated via mannose residues on the haemoglobin. The inhibition of this adsorption in the presence of α-methyl-mannoside confirmed this hypothesis. Gel filtration of the supernatant on Superose

6 utilising an FPLC system yielded a pure preparation of the *B. nasutus* 1214 agglutinin. The agglutinin has an M_r (210k) similar to that of the humoral agglutinin reported from *A. fulica* (Mitra and Sarkar, 1988), however, the agglutinin in *B. nasutus* 1214 does not appear to be composed of subunits unlike that of *A. fulica.*

The use of Fast Protein Liquid Chromatography (FPLC) has been invaluable to this study. Due to the large volumes of running buffers and sizes of standard chromatographic columns, attempts at purifying molecules from very small sample sizes usually results in excessive dilution of the eluant proteins, and using an assay such as the agglutination assay, the maximum dilution titre is surpassed. Production of a linear, reproducible eluent salt gradient in ion exchange methodologies is also problematical using standard equipment. High Performance Liquid Chromatography (HPLC) was designed for small scale preparative work, but due to the nature of the buffers normally utilised (e.g. acetonitrile) the biological activities of certain proteins may be lost. Indeed, when *B. nasutus* 1214 plasma was applied to a reverse-phase HPLC column, no agglutinating activity could be detected in any of the eluant fractions. An FPLC system facilitates use of buffers which are not likely to abrogate the biological activity of proteins, and while the resolution of FPLC is not as great as that of HPLC, if operating conditions are optimised for FPLC then resolution can approach that attainable with HPLC. FPLC is also suited to small sample sizes, and eluent salt gradients, being computer controlled, are extremely precise and reproducible.

This study is the first to report the lectin-conjugated bead and subsequent gel filtration purification scheme. It has been designed on the assumption of the structure of bulinid haemoglobin, following the elegant biochemical studies conducted with *Biomphalaria glabrata* haemoglobin. The small volume of *B. nasutus*

1214 haemolymph available has been offset by the high agglutination titre of the agglutinin, and FPLC has ensured that over-dilution did not occur.

Analysis of native haemolymph from a range of bulinid species by SDS PAGE showed that the major patterns of protein banding were similar, if not identical in each species. This study has also shown that the haemolymphs from the genus *Bulinus* are very similar in composition to those of the genus *Biomphalaria*. Some differences in banding patterns were observed however, particularly with respect to the lower molecular mass bands. Interestingly these differences were consistent within members of specific species groups. For instance, there are identical distinguishing bands in samples of *B. africanus* 1227, *B. globosus* 1381 and *B. obtusispira* 1476, and each of these species is a member of the **africanus** species group. When identical purification procedures were conducted with haemolymph from a range of *Bulinus spp.*, a protein band at the same relative molecular mass (135k) (after SDS PAGE under reducing conditions) as the *B. nasutus* 1214 agglutinin was observed in each of the species tested (*B. natalensis* 272, *B. truncatus* 1521, *B. guernei* 1126, *B. rohlfsi* 1326, *B. coulboisi* 1087, *B. permembranaceus* 1256, *B. octoploidus* 1077, *B. trigonis* 755, *B. globosus* 1381, *B. ugandae* 1250, *B. umbilicatus* 1555, *B. obtusispira* 1476, *B. beccarii* 1416 and *B. forskalii* 1162). The presence of a band at a particular M_r on an SDS PAGE gel from different samples does not necessarily denote the presence of the same protein(s) in each sample. It only indicates that proteins with a similar migratory behaviour due to their respective relative molecular masses are present in each sample. With a range of test erythrocytes, haemagglutinating activity has only been identified in haemolymph from *B. nasutus* 1214. Thus the comparable electrophoretic mobilities of *Bulinus spp* 135k

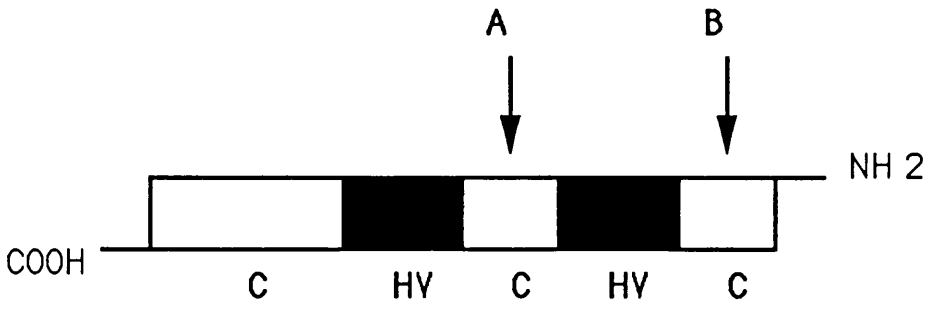
bands do not correlate with a comparable functional behaviour with regard to agglutination of these particular vertebrate erythrocytes. This does not preclude the possibility, however, that *in vivo* the biological functions of these proteins are identical. To further investigate the similarities in the structures of the 135k proteins, proteolytic digestion using SV-8 protease was conducted (Cleveland mapping) yielding polypeptide profiles for each separate 135k bulinid protein. The experiment demonstrated identical polypeptide profiles for each species tested, indicating that the polypeptide portions of all bulinid 135k glycoproteins are identical. Thus despite there only being a haemagglutinating glycoprotein present in the haemolymph of *B. nasutus* 1214, similar glycoproteins have been identified in all other *Bulinus spp.* investigated.

Homology of the 135k proteins was further tested by use of an antiserum raised against *B. truncatus* 1521 135k protein. When western blots of a range of *Bulinus spp.* 135k proteins were probed with this antiserum, a fascinating result was obtained. The antiserum would only recognise 135k proteins in species of *Bulinus* which belonged to the **tropicus/truncatus** complex species group. The 135k proteins of members of the **africanus** and **forskalii** species groups were not recognised. Given that classification of the species groups is primarily based on morphology, which is not necessarily solely genetically determined and may be influenced by environmental factors, and that the four species groups are widely distributed with overlapping ranges, then this observation is intriguing. It suggests that while the molecular mass and polypeptide backbones of all *Bulinus* 135k glycoproteins are very similar, if not identical, some antigenic determinants on these molecules differ. It is not known whether this variation in antigenic determinants is associated with the haemagglutinating properties of the respective molecules.

The polypeptide backbones cannot be assumed to be identical, as Cleveland mapping only shows that each of the bulinid 135k proteins possesses identical sites for proteolysis by SV-8 protease. One would require the use of alternative proteolytic agents to produce additional polypeptide patterns to determine investigate further the homology of the molecules. Digests were performed with papain, but the resulting peptides were so small as not resolve inefficiently on SDS PAGE gels. Thus given the maps produced by SV-8, one can postulate the basis for functional diversity in these 135k proteins. The possibility exists that within the 135k molecule there are constant regions, involved in regulation of function of the molecule, which are common to all bulinid 135k proteins, and variable regions which differ among species (Figure 42). If the hapten binding sites include the variable portions of the molecule, then this could explain the functional diversity of bulinid 135k proteins, given the same apparent molecular size. If the SV-8 digestion sites lie at "A" and "B", then identical polypeptide maps will result for every bulinid 135k protein, as the variable regions are not targets for the enzyme. Cross-reactivity of the *B. truncatus* 1521 antiserum to other **truncatus/tropicus** group members might be explained if a particular portion of the variable region, common to all **truncatus/tropicus** group members bur different to **africanus**, **forskalii** and **reticulatus** group members is an antigenic determinant that is being recognized. Alternatively, being glycoproteins, it may be postulated that the carbohydrate compositions, or rather the **organisation** of carbohydrate complements may differ on identical polypeptide backbones in different species. The antiserum raised to *B. truncatus* 1521 135k protein may therefore be recognising particular carbohydrate epitopes, or spatial arrangements of carbohydrate epitopes. The arrangement of these epitopes may be similar on the polypeptide backbones of glycoproteins of **truncatus/tropicus** species group members, but

FIGURE 42

HYPOTHETICAL MODEL OF THE Mr 135k AGGLUTININ



KEY

C = constant region

HV = hypervariable region

A & B = SV-8 protease digestion sites

different from those of other species group members. If this is the case, then it should be possible to produce specific antisera to 135k proteins of each of the four species groups, and these should only recognise members of their own species group. If these proteins are indeed recognition factors involved in the discrimination of invading schistosome larvae, then species-specificity might be partly explained by such subtle differences in carbohydrate structures on these molecules and the differences in surface carbohydrate receptors of different schistosome species. Other workers have alternatively postulated that it is a presence/absence effect of such factors which distinguishes different species. Other studies have been restricted, however, in both the range of the employed experimental approaches, and the range of test organisms utilised (i.e. most *Biomphalaria glabrata* studies have utilised the same inbred strains 13-16-1 and 10-R2). Determination of alternative assay systems has not been achieved, and thus the "absence" of certain humoral factors must be questioned.

There are many facets regarding the interaction of schistosome larvae and freshwater snails which warrant consideration. The existence of the compatibility/incompatibility concept can be attributed to a combination of ecological, physical, physiological and biochemical factors prior to participation of snail defence effectors.

Consider firstly the distributions of parasites and intermediate hosts. This discussion will pertain primarily to *Schistosoma spp.* and *Bulinus spp.* within Africa, but the concepts will be applicable to schistosome-snail combinations globally. Within the four bulinid species groups particular species are distributed over vast, overlapping ranges (see Figures 1, 2 and 3), in which permanent and seasonal waterbodies abound. The range of a particular species may be so extensive that

different populations are greatly separated from one another (Figure 4). The migration of snail species, be it active or passive, has led to the current geographical distribution of bulinid species within Africa, and undoubtedly redistribution is constantly occurring, although an active migration will tend to be over a much smaller distance than a passive one. The distribution of particular schistosome species varies and different populations of the same species may be isolated from one another. However, migration of Man and animals may extend the range of a population of schistosome, and is likely to be a much more common occurrence than intermediate host redistribution. Nevertheless, in any given area, or any given waterbody, there will exist a restricted number of snail and schistosome species that can possibly interact. Even within such a restricted locality there is still an element of chance that schistosomes and snails will be given the opportunity to interact, and this relates to the behaviour of the respective definitive and intermediate hosts. Given that within a waterbody snails and newly-hatched miracidia will eventually be in close proximity, then, and only then, do the identities of the animals become important.

There is evidence to show that miracidia exhibit taxis in host location, involving photactic, geotactic and chemotactic stimuli (see Smyth and Halton, 1983). The possibility also exists that a miracidium may encounter a snail by "accident", facilitating interaction of the two animals. Research is being conducted in order to elucidate the specificity of these miracidial tactic responses, and there appears to be variation among schistosome species. For example, miracidia of *S. mansoni* will attempt to penetrate every snail species supplied, while *S. japonicum* miracidia will only attempt penetration of *Oncomelania spp*. This implies that some species of parasite are capable of distinction of host types, and the differential ability of

schistosome species to perceive such chemical stimuli may possibly relate to differential evolution of geographically isolated species.

Many of the miracidia encountering a given snail will be unsuccessful in their attempts to penetrate that snail. This will relate not only to the parasites complement of secreted hydrolytic enzymes (this requirement will vary for different snail species) but also to the age of the miracidium. With such a short lifespan, it is imperative for miracidia to encounter a suitable intermediate host while in possession of the energy resources to enact penetration. Thus it is apparent that prior to successful penetration of a snail, all these conditions must be met in order that an interaction can take place. The chances of this happening for any individual is slight, but when one considers the vast numbers of miracidia introduced into a waterbody at a given time, and the extensive multiplication of larval schistosomes within a compatible snail host, then perpetuation of the parasite life cycle is adequately ensured.

Once miracidial invasion has occurred, the species identities of the snail and parasite become apparent. As penetration results in destruction of snail tissues, then the snail will become "defence activated" nonspecifically as to all wounding stimuli. The presence of the miracidium and subsequent shedding of ciliated plates in the process of forming the mother sporocyst will present a variety of chemical stimuli for defence activities, but whether there are specific or non-specific responses to these stimuli is questionable. If the snail species is susceptible to infection by the schistosome species, then the mother sporocyst will mature and produce daughter sporocysts and cercariae over a 4-7 week period, with a great increase in parasite numbers residing within snail tissues during this time. Cercariae will exit the snail when mature, causing physical damage to snail tissues during this process. If the snail species is resistant to infection by the schistosome species, then the mother sporocyst

is encapsulated by several layers of haemocytes and is cytolytically destroyed. The necessity for the presence of humoral factors in a successful encapsulation response of sporocysts has been shown *in vitro* by Bayne *et al* (1980b) . The presence of agglutinins in the haemolymph of many snails makes them prime candidates for recognition roles in this process. However, in only one isolated study has an opsonic effect of a purified agglutinin been undoubtedly proven, and this was in the mussel *Mytilus edulis* in *in vitro* phagocytosis of yeast (*Saccharomyces cerevisiae*) particles (Renwrantz and Stahmer, 1983). Opsonization of schistosome sporocysts by purified snail agglutinins has never been demonstrated. Probably this is due to the fact that few snail humoral factors have been purified to homogeneity. Opsonization of yeast, bacteria and of sporocysts have been shown using whole haemolymph or whole plasma, but the nature of the opsonization agent(s) is undetermined.

For specific binding of agglutinins or other such humoral factors to larval surfaces to be proven, it will probably be necessary to fractionate schistosome teguments and purify candidate receptor molecules. If these molecules could then be immobilised on sepharose beads, they could then be used in opsonisation assays of phagocytosis by haemocyte monolayers. I have deliberately referred to "larval schistosomes" instead of "sporocysts" in this last statement, as I believe the miracidial stages require inclusion in such studies. Very little attention appears to have been given to miracidia in the various published studies (namely *Biomphalaria glabrata-S. mansoni* and *Lymnaea stagnalis-Tricobilharzia ocellata* systems). Clearly, a miracidium is essentially a mother sporocyst with a ciliated "coat", but the possession of ciliated plates, and particularly the process in which they are shed are very important factors in initiating defence functions (activation).

Schistosome miracidia retain their ciliated plates on penetration of the

intermediate host, but some related species, such as *Fasciola hepatica* in *Lymnaea spp.*, shed their ciliated plates as the parasite penetrates the snail body tissues (Wright, 1971). The process of schistosome penetration and shedding of ciliated plates takes several hours. In the present study, changes in haemolymph composition were observed 1 hour post-infection in bulinid snails. It therefore seems likely that the snail defence reactions were already underway before the sporocyst tegument was exposed in its entirety. It was attempted to determine whether it was the physical damage inflicted by the invading miracidium as opposed to specific miracidial surface determinants that causes activation of defence mechanisms, by mimicking penetration. This was achieved by inflicting localised wounding of snail tissue by needle-point incisions. With snails as small as *Bulinus spp.* it was not practical to wound snails with any degree of certainty as to the depth of the wounding, and much of the haemolymph was exuded and therefore lost. Hence any comparison of defence activation by mechanical (piercing) and natural (infection) means is speculative. However, as the discarded ciliated plates are recognised and eventually encapsulated in both susceptible and resistant snails, then recognition of the miracidial surface during penetration cannot be disregarded as an important effector reaction, and hence miracidia and ciliated plates alone should be included in *in vitro* studies.

The activation of snail defence mechanisms was studied in a series of experiments involving activation either by injection of foreign substances, wounding or infection with either echinostomes or schistosomes. The initial study utilising anaesthesia of snails prior to injection of saline showed an elevation of plasma protein levels as analysed by SDS PAGE (Figure 3∂). However, the effects of anaesthesia and the mechanical wounding resulting from injection may have altered the responsiveness of the snails to the nonself material, in this case PBSA. However,

it was clear that there had been an alteration in haemolymph protein levels following the injection procedure, and this was assumed to reflect activation of defence mechanisms. This assumption formed the premise on which the subsequent wounding and infection experiments were based.

The wounding study with *B. africanus* 1583 showed that changes in haemolymph protein levels were altered on mechanical damage to the body wall. The initial decrease at 3 hours post-wounding (Figure 31) could reflect a loss of haemolymph from the wound, as well as a removal of proteins from the haemolymph to the injured tissue site. The increase in protein level at 48 hours post-wounding is significantly higher than the resting level, and this may reflect a production of new or a release of stored defence factors into the haemolymph, or alternatively a general increase in production or release of all proteins. The graph for variation in haemoglobin levels (Figure 32) shows an identical trend over the 96 hour period to that of the total protein level. This indicates that a general production of proteins is more likely occurring than a production of specific proteins. Few discernible differences on SDS PAGE analysis were evident in haemolymph banding patterns samples at the various time intervals post-wounding compared to resting levels (Figure 33), although at 3 and 24 hours post-wounding there was noticeably less of the higher M_r major bands present, which were probably haemoglobins.

An infection study using susceptible and resistant snail species was conducted in order to investigate defence activation at the various stages of parasite development within the snail host. There was clearly an elevation in plasma protein levels on miracidial penetration of both susceptible *B. natalensis* 272 and resistant *B. ugandae* 478, as recorded 3 hours post-challenge. During sporocyst multiplication (18 days) in *B. natalensis* 272, the total protein level was 30% higher than the resting level, but

the origin of proteins present in the haemolymph during this time requires determination. On the assumption that was made, that changes in the haemolymph protein levels were due solely to activation of snail defence systems, then it is difficult at this particular stage of parasite development to preclude the release of parasite secretory/excretory products into the haemolymph, and for the removal of proteins from the haemolymph by the parasite for nutritional purposes. These events would both serve to alter the composition of the haemolymph, irrespective of snail defence functioning. At the stage of cercarial shedding, the elevation in haemolymph protein levels observed in *B. natalensis* 272 might reflect damage to the body wall occuring during exit of parasites from the host. This would evoke wounding responses as were demonstrated in the wounding study. This elevation also demonstrates that the snail is still capable of defence activation (elevation in protein levels) despite having being parasitized for the preceding four weeks. In the non-shedding *B. natalensis* 272 specimens there was no elevation in haemolymph protein levels. It is unclear whether this was due to these specimens being uninfected, or if they were infected, whether they would show the same elevation when shedding eventually occured, as those specimens which were shedding cercariae by day 30 post-infection.

In the final infection experiment, the total haemolymph protein levels were investigated over the initial 3 days post-challenge in both *S. margrebowiei*-susceptible *B. natalensis* 272 and *S. margrebowiei*-resistant *B. umbilicatus* 1555. With the *B. umbilicatus* 1555 specimens, the protein levels had returned to the resting level after 48 hours, following an initial increase at 3 hours post-challenge, and a subsequent decrease at 24 hours post-challenge. The initial increase might be explained by both (a) presence of parasite proteins in the haemolymph and (b) production or release of snail proteins, some of which may have been defence factors. The decrease in

protein levels at 24 hours post-challenge might be explained by both (a) removal of parasite-derived proteins from the haemolymph as a result of phagocytosis by haemocytes, or (b) a removal of snail proteins, and in particular defence factors, out of the haemolymph and onto parasite surfaces within tissues. With susceptible *B. natalensis* 272 the haemolymph protein levels were also observed to fluctuate over the 72 hour period, with successive rises and falls at 3 and 48, and 24 and 72 hours post-infection respectively. While the variations over the first 24 hours can be explained in the same way as that for *B. umbilicatus* 1555 over this time period, the subsequent variations over 48-72 hours post-infection, and the greater initial elevation at 3 hours post-infection, cannot. The great initial elevation may be due to a greater release of parasite material into the haemolymph, and in particular the shedding ciliated plates. Alternatively, this could be attributed to breakdown of snail proteins, and in particular of defence factors, due to the activity of parasite secretory/excretory products. This latter explanation might further explain the second increase in protein levels at 48 hours post-infection.

The results of these wounding and infection studies indicate that activation of defence activities, as indicated by production or release of haemolymph proteins, is not due to nonspecific wounding responses. If this were the case, then the elevations observed on infection with larval schistosomes should have been of the same magnitude as those elicited by damage of the body wall by piercing, but this was clearly not the case. The correlation of return of protein levels to the resting level in resistant *B. umbilicatus* 1555 and wounded *B. africanus* 1583 after 48 hours also indicated that it is during this short time period following parasite penetration and therefore defence activation, that compatibility or incompatibility of the parasite with respect to the snail host is determined.

When the larval stages of *S. margrebowiei* were incubated with *B. nasutus* 1214 cell-free haemolymph, the miracidia and not the sporocyst adsorbed out the agglutinin from the haemolymph. This suggests that the agglutinin is binding specifically to the miracidium. When one considers that *S. margrebowiei* is incompatible with *B. nasutus* 1214, then this "specific recognition" *in vitro* of the miracida is interesting. When *B. nasutus* 1214 specimens were infected with *S. margrebowiei* miracidia and haemolymph was collected 3 hours post-infection, the level of agglutinating activity was reduced. Given the *in vitro* binding of the agglutinin to miracidia, one can postulate that the amount of agglutinin in the haemolymph had been reduced due to its binding onto the invading miracidial surfaces within snail tissues. Availability of a crossreactive **africanus** group antiserum to 135k protein to use in staining of frozen sections of infected specimens would have enabled substantiation of this hypothesis. Consider this information in the light of the study conducted by Kechemir and Combes (1982). Specimens of *B. truncatus* (susceptible) were infected with *S. haematobium* (2-50 miracidia per snail). Sporocysts were dissected out in grafts and transplanted into *Planorbarius metidjensis* (resistant). Remarkably the parasites developed here as they would have in the susceptible species, although the extent of cercarial production was reduced. The conclusion was made that if a miracidium were able to transform to a sporocyst and escape an encapsulating response in a host in which it is usually destroyed, then it would be able to undergo a quite normal development. There are several possible explanations for these data however - (i) transplanted *S. haematobium* sporocyst tegument receptors were occupied by *B. truncatus* recognition molecules which either did not permit binding of, and recognition, by *P. metidjensis* humoral factors, or which

exposed a haemocyte binding site only recognizable by *B. truncatus* and not *P. metidjensis* haemocytes ; (ii) the physical wounding effects of transplantation and anaesthesia suppressed normal defence reactions during the critical time interval before the *S. haematobium* sporocysts became established; (iii) by the time the sporocysts were transplanted into the new host, they were sufficiently mature to have developed a tegumental surface which precluded the binding of recognition factors; (iv) transplanted *S. margrebowiei* sporocysts were not recognised by the defence effectors of *P. metidjensis*, there being a necessity for miracidial determinants in order for an effective defence activation to occur.

In the present study then, the *B. nasutus* 1214 agglutinin is implied as a recognition factor for *S. margrebowiei* miracidia. When *B. nasutus* 1214 plasma was incubated with other *Schistosoma spp.* larvae, there was no loss of agglutinating activity. Several studies on the structure of schistosome teguments have indicated differences in their compositions in different species (Simpson & Smithers, 1980; Simpson,Correa-Olivera, Smithers & Sher, 1983; MacGregor, Stott & Kusel, 1985; Hayunga & Sumner, 1986; Boswell, Yoshino & Dunn, 1987; Dunn & Yoshino, 1988). Indeed, it has been reported that there are stage-specific carbohydrate determinants within a single species (Table 21), presumably reflecting adaptations to particular stages of development. Thus it is likely that the carbohydrate specificities of the snail agglutinins and the miracidial (or sporocyst) teguments vary among species. If recognition of miracidia is the critical stage in compatibility/incompatibility of snail-schistosome combinations, then non-recognition due to differential carbohydrate complements may be the basis of successful or non-successful snail defence activities.

If the recognition of miracidia is the important event, then why are ciliated plates encapsulated in both susceptible and resistant snail species, but **sporocysts** and not

TABLE 21 COMPARISON OF SCHISTOSOME LARVAL SURFACE CARBOHYDRATES USING LECTINS

LECTIN	SPECIFICITY	*S. margrebowiei* [a]		*T.ocellata* [b]		*S. mansoni* [a]		*S .mansoni* [c]		*S.mansoni* [d]
		M	S	M	S	M	S	M	S	S
Anguilla anguilla (EEL AGGLUTININ)	α -L fucose	-	-	ND	ND	+	-	+	ND	ND
Arachis hypogeae (PEANUT AGGLUTININ)	β -D-gal(1-3)-D-galNAc	+	-	+	+	-	-	ND	ND	+
Canavalia ensiformis (CONCANAVALIN A)	α -D-mannose, α -D-glucose	-	+	+	+	+	+	+	+	+
Dolichos biflorus (HORSE GRAM)	α -D-galNAc	-	-	+	-	+	-	+	+	ND
Erythrina cristagalli (CORAL TREE)	β -D-gal(1-4)-D-glcNAc	ND	ND	+	-	ND	ND	ND	ND	ND
Glycine max (SOYBEAN AGGLUTININ)	D-galNAc	ND	ND	+	-	ND	ND	ND	ND	ND
Ricinus communis (CASTOR BEAN)	β–D-gal	+	-	+	-	-	-	ND	ND	ND
Tetragonlobus purpureas (ASPARAGUS PEA)	α -L-fucose	-	-	+	-	-	-	ND	ND	+
Triticum vulgaris (WHEAT GERM AGGLUTININ)	(D-glcNAc)2	-	+	+	+	-	-	ND	ND	+

KEY: MIRACIDIA M, SPOROCYST S , POSITIVE + , NEGATIVE - , NOT DETERMINED ND

[a] Daniel, 1990; [b] Gerhardus, personal communication; [c] Yoshino *et al*, 1977; [d] Zelck & Becker, 1990

miracidia are observed to be destroyed in resistant species? Until schistosome surface components are completely characterised, there can be no definitive answer to this question. However, a number of possible explanations exist-(i) the miracidium is encountered by haemocytes in resistant snails, but by the time there are sufficient numbers of recruited haemocytes to arrest migration of the larva and facilitate encapsulation, the shedding of the plates is completed, and these haemocytes remain bound to the plates. However, localised haemocyte activity becomes directed from the plates to the naked sporocyst, and this might require the presence of agglutinin-bound "miracidial" molecules still being present on the sporocyst surface. In susceptible species the mobilisation of haemocytes may be slower, or the shedding of the plates faster, allowing the sporocyst to migrate out of the locality of haemocyte activity, thereby escaping attack; (ii) the sporocyst may produce substances which mask or mimic (see Introduction), thereby preventing successful binding of agglutinins or other recognition factors. In resistant snails, the binding of the agglutinin (or other recognition factor) may be so dynamic and with such avidity as to enable a successful haemocyte recruitment before such masking or mimicking can occur; (iii) the steric interactions of the agglutinin and parasite surface may differ for different schistosome species. If the agglutinin has two binding sites, one for the parasite surface and one for the haemocyte membrane, then orientation of these sites might decide whether haemocytes are able to become close enough to bind. In an incompatible combination one can postulate that the agglutinin binds to the parasite, and the haemocyte binding site is freely available for apposition of the haemocyte (Figure 43a). In a compatible combination of snail and schistosome, the recognition factor is able to recognise the same residues as before on the parasite surface but orientation of binding is altered, making the haemocyte binding site unavailable

FIGURE 43 HYPOTHETICAL MODE OF ACTION OF RECOGNITION FACTORS IN COMPATIBLE AND INCOMPATIBLE SNAILS.

(a) RECOGNITION OF NONSELF - recognition factor effectively binds to a receptor on the parasite surface, and the haemocyte binding site is orientated such that binding is possible.

(b) STERIC HINDRANCE - the recognition factor is able to bind to the receoptor on the parasite surface, but the orientation of this interaction precludes the availability of binding of the haemocyte-binding site. Thus although the snail has produced the appropriate recognition molecule, and it successfully binds to nonself, the second stage, recruitment of haemocytes, is not permitted.

(c) BLOCKAGE OF BINDING SITES - excretory/secretory products are produced by the parasite, thus although the recognition factor has bound to the parasite surface, the haemocyte binding site is unavailable for binding of the haemocyte.

KEY
H = haemocyte
P = parasite
ES = excretory/secretory products

FIGURE 43A RECOGNITION OF NONSELF

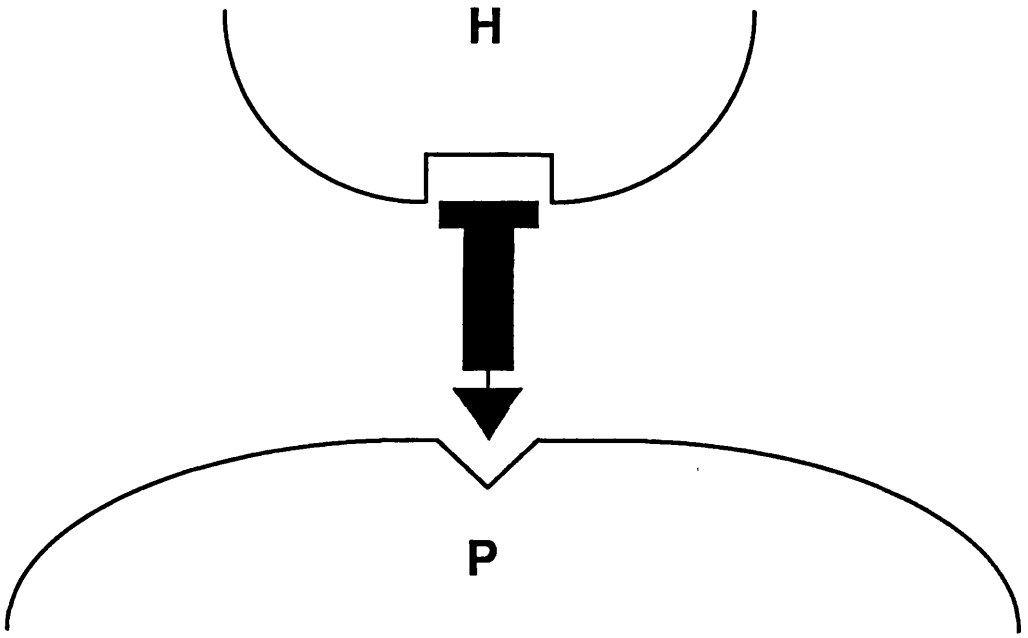


FIGURE 43B STERIC HINDRANCE

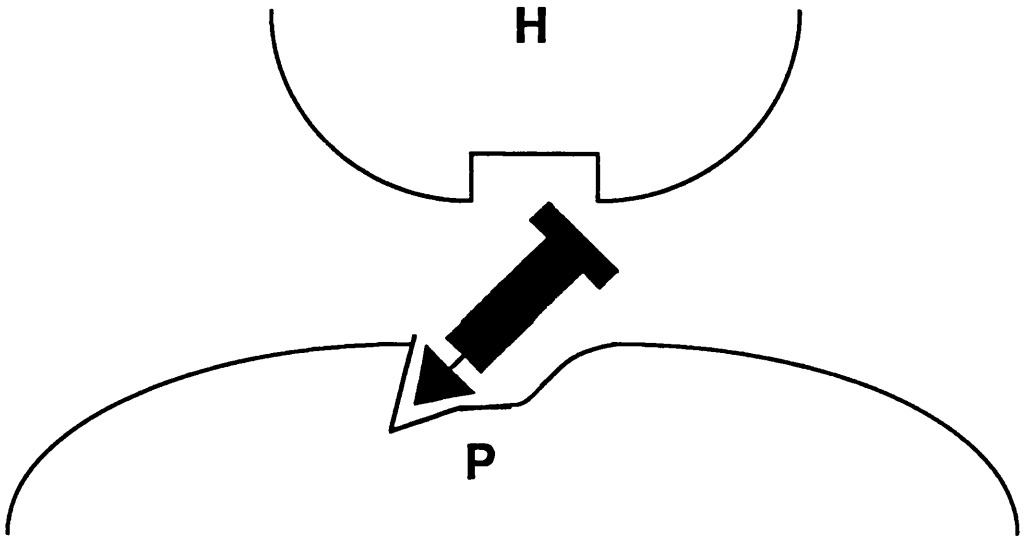
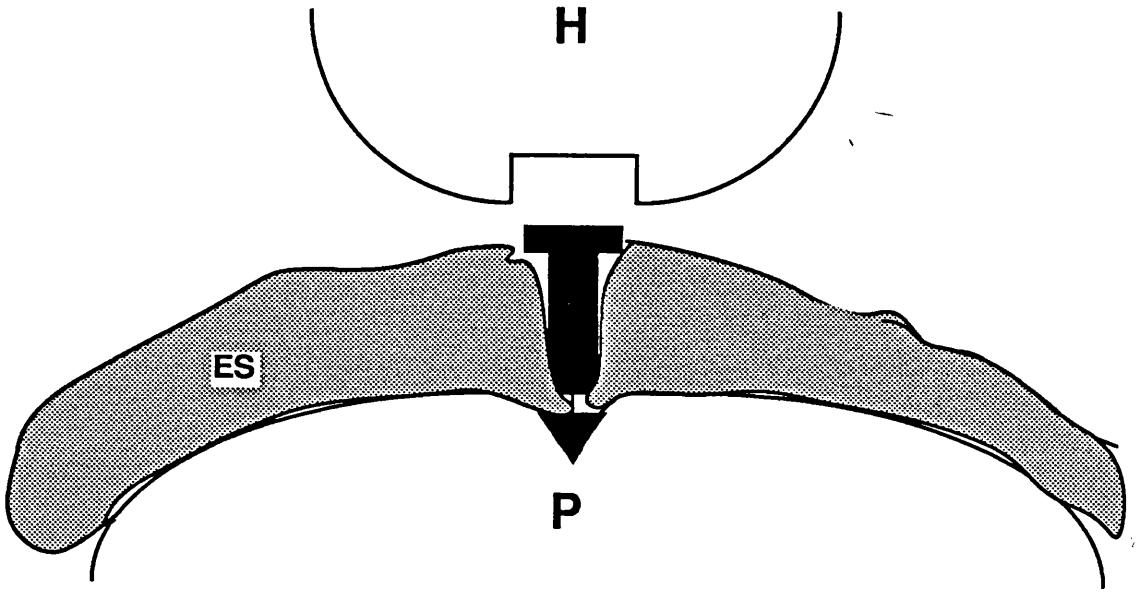


FIGURE 43C BLOCKAGE OF BINDING SITES



(Figure 43b). Alternatively, production of excretory/secretory products may preclude successful binding by the haemocyte to the recognition factor which has bound sterically optimally to the parasite surface (Figure 43c); (iv) the parasite produces substances which neutralise the recognition factors before they reach their target. The results of *in vitro* binding studies could then be explained by non-activation of both haemocytes and parasites i.e. *in vitro* and *in vivo* studies are not comparable. In all these possible cases, the ciliated plates are discriminated as being foreign or are just nonspecifically encountered and sequentially encapsulated.

HAEMOCYTES

Circulating haemocytes are the major effector cell type in snail defence reactions (Dikkeboom *et al*, 1988). A comparative study of haemocytes from six different snail species was made, in order to determine similarities or differences in haemocyte functioning between the species.

The number of circulating haemocytes, if one assumes that only circulating haemocytes were recovered, varied among the snail species. Partially this variation may be due to abiotic factors (Wolmarans & Yssel, 1988) such as ambient temperature (Pauley, Krassner & Chapman, 1971) or natural infections (van der Knaap *et al*, 1987), but if one assumes that these factors did not apply then it is assumed to reflect true differences between the species (Sminia, 1981). There is no correlation between habitat type and haemocyte number, the aquatic species *L. stagnalis* and *Biomphalaria glabrata* having the highest and lowest cell counts respectively. The surrounding milieu in which terrestrial and aquatic snails reside will undoubtedly affect the composition of haemolymph, presenting different quantities of organic and inorganic substances, but it appears that these have no quantitative

effects on haemocyte numbers. One must presume that a low number of circulating haemocytes as observed in *Biomphalaria glabrata* is sufficient to warrant an effective surveillance and defence against opportunistic infections. However, the observed cell counts may also be explained by the employed experimental procedure, with respect to both the methodology of haemolymph collection and the nature of the saline used for all cell work. The haemolymph collection method employed for terrestrial snails was one of shell puncture. The resultant exudation of haemolymph usually resulted in the inclusion of shell fragments in the collected sample, and of loss of haemolymph (and therefore haemocytes) over the shell surface itself. The foot retraction method employed with the aquatic species was far easier, and despite the inclusion of mucus on some occasions, this methodology of haemolymph collection was very efficient. The efficacy of both of these procedures on haemolymph collection could only be assessed by employing the alternative methodology. However, while the shell puncture collection method could be applied to the aquatic snail species, the foot retraction technique was unsuccessful with the terrestrial snails due to a massive production of mucus by these animals. Thus the given cell counts must be regarded as **indicators** of cell abundance, and not as exact representations of the real *in vivo* situations.

The composition of the buffer (SSS) in which haemolymph was collected may also have contributed to the haemocyte counts. The buffer has been designed for *L. stagnalis* haemocytes in that it is osmotically optimal for haemocytes of this species. Haemolymph from the other aquatic species and the three terrestrial species may have different physiological compositions, especially with regard to osmolarity. Thus the SSS may have caused lysis of haemocytes (or certain sub-populations) in some species tested (e.g. *H. aspersa*), resulting in low cell counts (possibly only reflecting

the presence of a single sub-population with the ability to withstand the affects of the buffer). In this respect, the cell counts can again be regarded as only indicative and not absolute values, of *in vivo* haemocyte densities. One would require a separate buffer designed for each individual species, which is physiologically optimal for haemocytes of each species, to overcome this limitation. However, this study was designed as a comparative one, and hence a uniformity of conditions, whether optimal or not is necessitated, and hence only a single buffer (SSS) was used.

Differences were observed in the morphologies and cell sizes of the glass adhering haemocytes (incubated in SSS) of different snail species. Again, this may be an effect of using SSS, the behaviour of the cells being affected by the imposed physiological conditions. However, the cells of *Biomphalaria glabrata* and *H. aspersa* displayed the same morphology as in the salines usually employed for the haemocytes of these species (reviewed by Sminia, 1981). Moreover, the haemocytes of all snail species were still functional, zymosan particles being phagocytosed in SSS; thus the observed morphologies are likely to be true features of the haemocytes of the different snail species. Interestingly, the haemocytes of the land snails were generally larger, and more elongate when adhered to glass than those of the aquatic species. The size of the cells may correlate with the size of the animals when fully-grown; for example, the largest haemocytes were observed from *A. achatina*, and adult shell diameters of this species can reach up to 25cm. Whether the observed glass-adherent haemocytes monolayers reflect all haemolymph circulating haemocyte types was not determined, however. Thus non-adherent types may have different morphologies, and in some species where the morphology of glass adherent types was constant, this may reflect a single sub-population. However, the monolayers were observed to contain at least two morphologically-distinct types in most species, with both rounded and

spreading types in evidence. Whether these represented distinct populations was investigated using surface markers. The lectin staining of haemocytes did not yield much information that has not been reported elsewhere, in that the lectins employed were not clearly differential of haemocyte subpopulations. Rather, all cells tended to have some degree of surface staining, although haemocytes of certain species were obviously stained with a greater avidity than were others. Interestingly, *L. stagnalis* haemocytes were not recognised by con A, while all other snail haemocyte populations were "seen" with this lectin, but *L. stagnalis* haemocytes were strongly stained with WGA. The importance of this difference, if any, has yet to be determined. This study has also indicated fundamental differences between the haemocytes of *A. fulica* and *A. achatina*, the former giving a stronger staining compared to the latter, with both con A and WGA. Specifically-designed surface markers are therfore necessary if haemocyte subpopulations are to be convincingly distinguished.

For this purpose, haemocyte monolayers were challenged with a variety of immunoreagents. These comprised monoclonal antibodies raised to *L. stagnalis* and *Biomphalaria glabrata* respectively, and two antisera, one raised against *A. achatina* haemocytes and the other against *B. truncatus* 1381 135k protein. The monoclonals have been specifically designed to distinguish haemocyte subpopulations. Thus one would have expected some degree of specificity of the monoclonal staining profiles. One can never be certain when using monoclonal antibodies in a cross-reactivity study such as this one, whether any observed staining is due to the presence of the same epitope to which the monoclonal was raised, or whether the antibody is just recognizing a particular conformation to which it binds. However, for the purposes of this discussion, staining of haemocytes with monoclonals will be

interpreted as indicating the presence of similar epitopes. As it transpired, each immunoreagent was observed to recognise epitopes on a variety of haemocyte types of all six snail species utilised in the study. Thus the "granule-staining" LS8 monoclonal recognised haemocyte surface determinants of other snail species, and while YII differentiated surface markers of *Biomphalaria glabrata* and *B. natalensis* 272 haemocyte subpopulations, the same marker recognised granules of *L. stagnalis* and *A. fulica* and did not recognise any determinants on *A. achatina* and *H. aspersa* haemocytes. Interestingly, there were no distinctive trends to the staining of terrestrial or aquatic species haemocytes.

It is clear that the specificity of haemocyte markers must be viewed with caution, and the characterization of such markers requires further attention. If haemocyte markers are eventually to be utilised in distinction of functional subpopulations of haemocytes, they need to be clearly defined. Fractionation of haemocyte membranes and purification of specific receptors will be a prerequisite in construction of such markers, as using mixed populations of whole haemocytes has not been successful to this end.

Phagocytosis of zymosan particles was common among the tested haemocytes, and in certain snail species this was observed to be more efficient than in others (e.g. *A. achatina* and *L. stagnalis* haemocytes ingested multiple zymosan particles). Differences in the percentage of phagocytosing cells occurred for different snail species, with the two Achatinidae species having the greatest numbers of phagocytosing cells (Table 19). Such differences may reflect experimental conditions, being more optimal or less favourable for the cells depending on the snail species. However, as the SSS buffer is presumed to be most favourable for *L. stagnalis* haemocytes, one would expect this species to show the highest percentage of

phagocytosing cells. As this was not the case, one can postulate that either (i) the *Achatina spp.* haemocytes had been phagocytically activated more than the *L. stagnalis* haemocytes (a function of the stimulus), or (ii) different subpopulations of *Achatina spp.* haemocytes were capable of phagocytosis, but only a certain subpopulation of *L. stagnalis* haemocytes were phagocytic. It is clear that haemocytes of all species were able to recognize zymosan as being foreign, but possibly the stimulation sensitivity caused by zymosan varies for haemocytes from different species. It is impossible to deduce from the conducted experimentation whether the haemocytes of different snail species employ the same mechanisms of non-self discrimination. The use of phagocytic stimuli other than yeast, such as latex beads or bacteria, might result in a higher percentage of phagocytosing cells for species such as *B. natalensis 272* which showed low activity.

Activity of endogenous peroxidase was observed in haemocytes of *L. stagnalis* and the bulinid species, concordant with previous reports (Sminia, 1980; Sminia, van der Knaap, Boerrigter-Barendsen, 1982). *H. aspersa* and *A. achatina* haemocytes were also positive, but surprisingly no activity could be detected in haemocytes of *A. fulica*. In contrast to reports on the presence of peroxidase in haemocytes of *Biomphalaria glabrata* (Sminia, 1980; Sminia *et al*, 1982; Granath & Yoshino, 1983), the results in the present study agree with those of McKerrow *et al* (1985) in that haemocytes of this species were negative in the peroxidase assay. This discrepancy is not due to strain differences, as the former two studies utilised the same strain as the present one. Rather this variation may be explained by the sensitivity of the assay. In fixed haemocytes, a positive reaction in this assay is probably due to mitochondrial respiratory enzymes only (Sminia *et al*, 1982). The positive staining in living cells, however, also indicates the presence of lysosomal enzymes. In living haemocytes,

DAB would be able to enter the lysosomal system through endocytosis to react with peroxidase, whereas the mitochondria of these intact cells should be inaccessible to this substrate, thus excluding interaction with mitochondrial enzymes. A stronger reaction was observed with living cells than in fixed cells. The fixative used may have caused (partial) enzyme inactivation. Moreover, the positive reaction in living cells may indicate that snail haemocytes display pinocytosis; following pinocytotic uptake of substrates from the assay medium, the cells transport it to the lysosomal system. There the substrate can react with lysosomal enzymes among which is peroxidase.

The superoxide assay (SOD inhibitable NBT reduction upon phagocytic stimulation with zymosan) performed in SSS was positive for haemocytes of all snail species tested. Previously haemocytes of *Biomphalaria glabrata*, *H. aspersa* and *L. stagnalis* have been reported to reduce NBT (Shozawa, 1986; Dikkeboom *et al*, 1988). In this study, not all phagocytosing haemocytes produced superoxide. Even within positive haemocytes superoxide production did not always take place in all phagosomes. This could indicate that ROI production is not always activated during phagocytosis of non-self particles, or that there has to be a sufficient build-up of formazan deposition before visual detection is possible, and in "negative-staining" phagogosomes insufficient ROIs had been produced to give a visible reaction. ROI production was also assayed for with SOD sensitive LDCL. Unfortunately a lack of snail material allowed testing of only *A. achatina* and *A. fulica* haemocytes; *L. stagnalis* was used as a positive control. Upon phagocytic stimulation haemocytes of *A. achatina* displayed clear SOD sensitive LDCL, a weak signal (twice the resting value) being detected from *A. fulica* haemocytes. Despite a strong NBT reaction relative to *L. stagnalis*, the *Achatina spp.* displayed a low LDCL signal when compared to *L. stagnalis*. LDCL is indicative for ROI activity, but it remains unclear

which agent(s) are responsible for the LDCL signal; very probably peroxidase activity contributes to the LDCL signal (Wishkowsky, 1988). Therefore it is striking that *A. fulica* haemocytes, containing no detectable levels of peroxidase, yield a low LDCL, whereas haemocytes of *A. achatina*, clearly retaining peroxidase, show a significant LDCL activity. As in *L. stagnalis* SOD inhibition of LDCL still affirms ROI involvement. *L. stagnalis* haemocytes may produce more of the factors responsible for LDCL than the *Achatina spp.* do, resulting in a higher LDCL response. For *H. aspersa* haemocytes LDCL activity has already been described (Dikkeboom *et al*, 1988). LDCL activities have not yet been shown for haemoglobin-containing snails, possibly because these pigments interfere with the assay.

POINTS OF PERSPECTIVE

The vertebrate immune system is well characterised. It is known to involve a variety of humoral and cellular components in a multiplicity of separate, concurrent or sequential responses. Antibody is the eventual humoral "nonself" recognition factor, but production of antibody requires prior interactions which may involve other humoral factors. Many invertebrate researchers have made speculations as to the potential homology of plasma and cellular effectors involved in vertebrate immune and invertebrate defence systems. Indeed, homologies have been reported from a variety of studies.

Preliminary observations suggest that crustacean haemocytes bear integrins on their surfaces (Johansson & Söderhall, 1989). In higher vertebrates, integrins mediate many direct cell-cell and cell-matrix adhesion phenomena during embryogenesis, morphogenesis and other processes including those of the immune and nervous systems. The interactions mediated by integrin cell adhesion molecules or extracellular matrix ligands are typically transient, characteristic of dynamic cellular interactions occurring between migratory cells. These proteins might also transduce intracellular signals and thereby influence the proliferative, migratory or other outcomes arising from cellular interactions (Hynes, 1987). Insect integrins have been identified in *Drosophila* that mediate cell-cell and cell-extracellular matrix interactions during embryogenesis (Séméревira, Naidet, Krejci & Gratecos, 1989). Another family of cell surface molecules is the immunoglobulin (Ig) superfamily. Based on a fundamental "Ig-domain" structure, members of this family have undergone sequence diversification and most contain multiple domains. Members of

the Ig superfamily are well characterized in the mammalian immune system where they serve as surface recognition molecules, acting as receptors for other cell surface ligands, antigens and growth factors. It has been suggested that that the Ig superfamily evolved from Ig-like molecules originally involved in primitive cell-cell interactions (Williams, 1987). At present members of this family have been described in only a handful of invertebrates (Williams, Tse & Gagnon, 1988; Harrelson & Goodman, 1988; Seeger, Haffley & Kaufman, 1988). A family of vertebrate lectins, the "pentraxins" comprise pentameric discs composed of individual sub-units, and include C-reactive protein (Maudsley, Baltz, Munn, Buttress, Herbert, Feinstein & Pepys, 1987). Their calcium-dependent binding is directed towards either foreign particles (e.g. C-reactive protein) or self (e.g. serum amyloid protein). Pentraxins are well conserved molecules among vertebrates, and have been isolated from a variety of species (Maudsley *et al*, 1987). "Limulin", the agglutinin from *Limulus polyphemus*, has significant sequence homology with members of the pentraxin family (Robey & Liu, 1981; Maudsley *et al*, 1987), as have the "tridacins", isolated from tridacna clams (Uhlenbruck, Solter & Janssen, 1983). It is possible that other invertebrate agglutinins will also be demonstrated to have structural similarities with vertebrate pentraxins. The calcium requirement for binding might suggest that homology with crustacean and insectan agglutinins be first addressed, as haemagglutinins of species of these orders also require the presence of calcium for their activities (Table 5).

Many vertebrate researchers are of the opinion that invertebrate defence functions should not be equated with vertebrate immune functions, the level of complexity of the former being conceivably unattainable of that of the latter, given the size of invertebrate genomes (Klein, 1989). However, both vertebrate and invertebrate defence systems warrant some comparison, as both are involved in

recognition and removal of non-self, and both rely on humoral components to achieve this end. Phagocytosis, encapsulation and nodule formation are invertebrate effector responses, and these parallel vertebrate cellular phagocytosis and encapsulation (e.g. granuloma formation in schistosome infection), although they are not equivalent. For example, in granuloma formation, layers of collagen are deposited in vertebrates, and cellular recruitment continues to form extensive multiple-layered capsules. Invertebrate encapsulation responses can involve multiple layers also, but the involvement of other factors is undetermined. The nature of the cellular effectors in invertebrate defence reactions is still a much-debated issue. The existence of different haemocytic subpopulations has been shown *in vitro* both with respect to morphology and biochemistry, but the reported diversity is not borne out by all phyla studied. *In vitro* functional studies (such as killing of schistosome sporocysts by molluscan haemocytes) have yielded much speculation as to haemocytic function and functional diversity of subpopulations, and this has been likened to vertebrate functional diversity of morphologically and biochemically distinct cellular populations (e.g. leucocytes). However, in many of these studies that have been conducted on invertebrates, experimental design leads one to wonder as to how relevant to the *in vivo* situation are such inferred functions.

Cellular responses are usually supported by humoral factor involvement, and many studies with invertebrates have speculated as to the importance of humoral factors in recognition. The existence of lectins within the body fluids of many invertebrate species has lead to a widespread study of lectin function, particularly in parasite-vector interactions. Lectins have been postulated to be the invertebrate equivalents of antibodies. While the enormous diversity of antibody molecules has not been demonstrated with invertebrate lectins, the generation of multiple

specificities based on a single structure can not be excluded for invertebrate lectins. Indeed, this dissertation has alluded to such a possibility in discussion of a common glycoprotein (135k) in haemolymph of *Bulinus spp.* If these 135k molecules are indeed recognition factors, as a large part of this thesis suggests they indeed are, then variations in their recognition functions in different snail species might be achieved in the same way as the diversity of vertebrate antibody receptor specificity, at the primary protein structure level due to DNA rearrangement. Thus in the same way as immunoglobulin in vertebrates, and variable surface glycoproteins in *Trypanosoma spp.* diversities are achieved, one cannot exclude the possibility that various specificities of snail recognition factors could not be produced in a similar fashion.

The techniques for experimental manipulation of nucleic acids have now reached the level of sensitivity where it should be possible to investigate such speculations. Haemolymph proteins and haemocytes could be compared among invertebrate phyla, and also between invertebrates and vertebrates. Homology of cytoskeletal elements, nervous system components and, tentatively, of immunological factors has already been demonstrated both between invertebrate species and between vertebrates and invertebrates, and thus it seems likely that evolutionary links in non-self recognition and effector components will also be demonstrated in the future. Such homologies will only indicate structural, and not necessarily functional similarities in such comparative studies. Antibody and agglutinin functions in vertebrates and invertebrates respectively are unlikely to be proved identical, but they may be based on a common theme, which during the course of evolution has diverged.

It is likely that the most primitive of recognition factors were derived from cellular components, being integral parts of surface membranes, which detached and began to circulate in the body fluids. Many invertebrate lectins are reported to

be haemocyte-derived, and the vertebrate lymphokine families are a good example of cell-produced humoral glycoproteins. Multiple binding specifities could then have been achieved either by association of single-specificity molecules to generate a single, composite molecule with multiple sites, or diversification and retention of a variety of single specificity molecules. The existence of single lectin species with complex carbohydrate specificities (such as the *B. nasutus* 1214 agglutinin) contrasts with other invertebrate species, notably the chordates, in which multiple lectins are present in body fluids. Vertebrate antibody diversity would seem to suggest a trend for the latter situation, if there are such functional evolutionary parallels between these humoral factors.

There still exists little experimental evidence to support a role of non-self recognition for invertebrate lectins. However, in this thesis a body of evidence supports a recognition role for the 135k proteins in recognition of larval schistosomes. The mechanisms of recognition (if any) of invertebrate lectins remains speculative. It is likely that their mode of activity will involve multiple receptor functioning. It is possible that there may be more than one hapten binding site, and that such sites may be either protein or carbohydrate in nature. If the carbohydrate specificity involves a simple sugar (e.g. galactose) then binding to a range of targets on the surface of parasites will be possible, as such simple sugars will be components of many glycoconjugates. Lectin recognition molecules almost certainly will have a haemocyte-binding site, possibly at the spatial opposite end of the molecule to the hapten-binding site(s). One would expect a fixed specificity for this haemocyte-binding site, although different haemocyte subpopulations may require different types of fixed-specificity binding sites. It is also plausible that a haemocyte binding site only becomes "available" for binding following some structural modification of the

molecule following binding of the hapten site(s) to nonself. The requirement for such a conformational change on "activation" might explain why these recognition factors do not normally occupy haemocyte receptors in the resting state. Such a mechanism occurs in vertebrate processes, so it does not seem unreasonable that invertebrate processes might involve similar mechanisms.

Whatever the mode of action of recognition molecules, it is clear that in compatible snail-schistosome combinations such processes do not result in the elimination of the parasites. The results of infection studies reported in this thesis suggest that the miracidium, and not the sporocyst, is the crucial stage in the determination of the outcome of the interaction. The *B. nasutus* 1214 agglutinin binds to the miracidial, and not sporocyst stages of *S. margrebowiei*, and the changes in plasma protein levels (if they do reflect activation of snail defence processes) occurred in both *S. margrebowiei*-susceptible *B. natalensis* 272 and *S. margrebowiei*-resistant *B. umbilicatus* 1555 after 3 hours post-infection. SDS PAGE analysis of haemolymph from the respective snail species showed an increase in the number of banding proteins for *B. natalensis* 272 after 3 hours post-infection, but with *B. umbilicatus* 1555 this was seen after 48 hours post-infection. These observations are intriguing, as there appear to be differential responses to infection by resistant and susceptible snail species.

The results of the cryostat section staining indicated that the susceptible *B. natalensis* 272 135k protein bound to the *S. margrebowiei* miracidia, thus a lack of appropriate recognition molecules, or an inability for these molecules to bind to nonself does not seem to be the basis of susceptibility. I propose that persistence of the infection is decided by the parasite, and in particular, by the execution of the miracidium-sporocyst transformation. The timing of this event may be the decisive

factor. In susceptible snails, shedding of the ciliated plates and formation of the sporocyst tegument may occur rapidly, i.e. within the first 6 hours post-infection. While the snail haemolymph recognition factors bind to the discarded plates and direct haemocyte encapsulation responses, the sporocyst might migrate out of the immediate vicinity of this cellular activity, releasing defence modulatory substances and acquiring host molecules (masking) which will serve to protect the parasite from further recognition events. The acquisition of host molecules is a very realistic evasion strategy, as it is utilised by immature schistosomula following penetration of the vertebrate host, in order to prevent immunorecognition (Clegg *et al*, 1971; Bout *et al*, 1974). Utilisation of such an evasion strategy need not necessarily be confined to the adult parasite stages, and thus sporocyst populations may have a critical time period in which they must acquire a "protective coat" in order to survive. After this time, the snail defence mechanisms will be unable to perceive their foreigness, and parasite development is facilitated. In resistant snails, the shedding of ciliated plates, sporocyst migration, acquisition of host molecules and production of modulatory substances may be more prolonged, particularly the initial transformation process. This may be due to physiological constraints of the parasite or to a rapid recognition and encapsulation response by the snail host. The later appearance (48 hours) of "extra" protein bands in haemolymph from resistant snails as opposed to susceptible snails (3 hours) may be an indicator of a delayed process on the part of the parasite, such as shedding of plates, or production of modulatory substances (e.g. excretory-secretory products) within the incompatible host. It appears that whatever the final outcome, i.e. parasite destruction or survival, the crucial snail defences are initiated on penetration and are elicited within the first 72 hours post-infection. Support for this hypothesis will require further investigation.

CONCLUSION

It is apparent that invertebrate defence functions requires further study. In order for any advancement to occur, I feel it will be necessary for researchers to address the subject in a comparative way. Many of the studies conducted so far, especially with respect to snail-schistosome interactions, have involved the use of single species of host and parasite, which have become extremely inbred. The present dissertation has involved the use of over 20 species of the same genus of snail, and in studying infection by schistosomes, an extensive time course was employed. The effect of alternative defence stimuli other than schistosome invasion was addressed using a simple wounding technique. A comparative study of haemocyte populations from different snail species highlighted the problems of defining "single specificities" of monoclonal antibodies. More importantly, the purification of an agglutinin, despite experimental difficulty, was achieved.

I feel that if other researchers addressed some of these points in their own studies, then our knowledge of invertebrate defence functions would be greatly increased. Without the use of purified humoral factors, defined haemocyte populations and varied activation stimuli, many studies will only contribute to advances in understanding of insular systems; species-specific interactions are not necessarily applicable to invertebrate systems as a whole. Only when definitive studies, such as Renwrantz and Mohr's (1978) first and only clearcut demonstration of agglutinin opsonization, have become more widespread will postulations as to functional parallels between invertebrate defence and vertebrate immune phenomena become possible. Technical advances, particularly in the field of molecular biology, have presented invertebrate and vertebrate researchers alike, with the tools and

methodologies to allow definition of physiological processes that have never before been encountered. It only remains for appropriate use of these methods to be made. With respect to this dissertation, the next obvious step is to identify and clone the gene encoding the 135k proteins in *Bulinus spp.* This will require generation of further antibodies, if λ-GT11 libraries are to be screened, or sequencing of the protein to enable consensus oligonucleotides to be used in PCR screening of genomic DNA. Homology of the 135k proteins within *Bulinus* members, and also between snail genera, could then be looked for. The carbohydrate component of the 135k molecule could also be established, which may yield evidence as to the nature of the binding sites on schistosome larval surfaces. The cell proliferation assay could be investigated further, using cell lines. One that is responsive to *H. pomatia* lectin would be a likely candidate. This would allow for a sensitive, dose-response assay for presence of 135k proteins which are non-agglutinating of vertebrate erythrocytes. Establishment of haemocyte cell lines is the real stumbling block for systems using such small animals as *Bulinus*. With a lack of available material, and only small numbers of haemocytes available at the best of times, all *in vitro* killing and recognition assays have not been able to be conducted. Establishment of cell lines would allow for such studies to be conducted. The infection study highlighted the importance of the first 72 hours post-infection, in schistosome infections of snails. There is great potential within the genus *Bulinus* for extensive studies of the effects of different parasite species within this time interval on defence functioning, and in particular, of the 135k glycoproteins present in all bulinid species.

REFERENCES.

ABEL, C.A., CAMPBELL, P.A., VANDERWALL, J. & HARTMAN, A.L. (1984). Studies on the structure and carbohydrate binding properties of lobster agglutinin 1 (LAg1), a sialic acid-binding lectin. In *"Recognition Proteins, Receptors and Probes : Invertebrates."* E.Cohen (ed.) pp.103-114. Alan R.Liss Inc., New York.

ABDUL-SALAM, J.M. & MICHELSON, E.H. (1980a). *Biomphalaria glabrata* amoebocytes: effect of *Schistosoma mansoni* infection on *in vitro* phagocytosis. *J.Invertebr.Pathol.* 35:241-248.

ABDUL-SALAM, J.M. & MICHELSON, E.H. (1980b). *Biomphalaria glabrata* amoebocytes: assay of factors influencing *in vitro* phagocytosis. *J.Invertbr.Pathol.* 36:52-59.

ACTON, R.T., BENNETT, J.C., EVANS, E.E. & SCHROENLOHER, R.E. (1969). Physical and chemical characterization of an oyster haemagglutinin. *J.Biol.Chem.* 15:4128-4135.

AFONSO, A.M.M., ARRIETA, M.R. & NEVES, A.G.A. (1976). Characterization of the haemoglobin of *Biomphalaria glabrata* as a glycoprotein. *Biochim.Biophys.Acta.* 439:77-81

ALMEIDA, A.P. & NEVES, A.G.A. (1974). The hemoglobin of *Biomphalaria glabrata*: chemical composition and some physiochemical properties. *Biochim.Biophy.Acta.* 371:140-146.

AMIRANTE, G.A. & BASSO, V. (1984). Analytical study of lectins in *Squilla mantis* L. (Crustacea, Stomatopoda) using monoclonal antibodies. *Dev.Comp.Imm.* 6:721-726.

AMIRANTE, G.A. & MAZZALAI, F.G. (1978). Synthesis and localization of haemagglutinins in haemocytes of the cockroach *Leucophaea maderae*. *Dev.Comp.Immunol.* 2:735-740.

ANDERSON, R.S. (1980). Hemolysins and haemagglutinins in the coelomic fluid of a polychaete annelid *Glycera dibranchiata*. *Biol.Bull.*159:259-268.

ANDERSON, R.S. & GOOD, R.A. (1975). Naturally occurring haemagglutinin in a tunicate *Halocynthia pyriformis*. *Biol.Bull.* 148:357-369.

ARIMOTO, R. & TRIPP, M.R. (1977). Characterization of a bacterial agglutinin in the haemolymph of the hard clam *Mercenaria mercenaria*. *J.Invertbr.Pathol.* 30:406-413.

BACHERE, E., CHAGOT, D. & GRIZEL, H. (1988). Separation of *Crassostrea gigas* haemocytes by density gradient centrifugation and counterflow centrifugal elutriation. *Dev.Comp.Immunol.* 12:549-555.

BALDO, B.A., SAWYER, W.H., STICK, R.V. & UHLENBRUCK, G. (1978). Purification and characterization of a galactan-reactive agglutinin from the clam *Tridacna maxima* (Roding) and a study of its combining site. *Biochem J.* 175:467-477.

BANG, F.B. (1962). Serological aspects of immunity in invertebrates. *Nature* 196:88-89.

BANG, F.B. (1967). Serological responses among invertebrates other than insects. *Fed.Proc., Fed.Amer.Soc.Exp.Biol.* 26:1680-1684.

BASCH, P.F. (1976). Intermediate host specificity in *Schistosoma mansoni*. *Exp.Parasitol.* 39:150-169.

BAYNE, C.J. (1973). Molluscan internal defence mechanisms "the fate of ^{14}C-labelled bacteria in the land snail *Helix pomatia* (L)." *J.Comp.Physiol.* 86:17-25.

BAYNE, C.J. (1980). Humoral factors in molluscan parasite immunity. In J.B.Solomon (ed.) : *"Aspects of Developmental and Comparative Immunology,"* p.115. Pergamon Press, Oxford.

BAYNE, C.J. (1982). Lectin-induced mitogenesis of cytotoxic amoebae *(Nucleria) isolated from Biomphalaria glabrata* (Mollusca:Gastropoda). *Dev.Comp.Immunol.* 6:369-373.

BAYNE, C.J. (1983). Molluscan immunobiology, In *"The Mollusca"* (A.S.M.Saleuddin & K.M.Wilbur, eds.), Vol.5.part 2,pp.407-486. Academic Press, New York.

BAYNE, C.J. & LOKER, E.S. (1988). Survival within the Snail Host. In *"The Biology of Schistosomes: From Genes To Latrines,"* (D.Rollinson and A.J.G.Simpson ed.), chap.11.pp.321-346, Academic Press, London.

BAYNE, C.J., BUCKLEY, P.M. & DeWAN, P. (1980a). Macrophage-like haemocytes of resistant *Biomphalaria glabrata* are cytotoxic for sporocysts of *Schistosoma mansoni in vitro. J.Parasitol.* 66:413-419.

BAYNE, C.J., BUCKLEY, P.M. & DeWAN, P. (1980b). *Schistosoma mansoni*: Cytotoxicity of haemocytes from susceptible snail hosts for sporocysts in plasma from resistant *Biomphalaria glabrata. Exp.Parasit.* 50:409-416.

BAYNE, C.J., LOKER, E.S. & YUI, M.A. (1986). Interactions between the plasma proteins of *Biomphalaria glabrata* (Gastropoda) and the sporocyst tegument of *Schistosoma mansoni* (Trematoda). *Parasitology.*92:653-664.

BAYNE, C.J., MOORE, M.N., CAREFOOT, T.H. & THOMPSON, R.J. (1979). Haemolymph functions in *Mytilus californicus* : the cytochemistry of haemocytes and their responses to foreign implants and haemolymph factors in phagocytosis. *J.Invertebr.Pathol.* 34:1-20.

BIRD, G.W.G. (1971). Erythrocyte polyagglutination. *Nouv.Rev.Fr.Haematol.* 11:885-887.

BISHAYEE, S. & DORAI, D.T. (1980). Isolation and characterization of a sialic acid-binding lectin (carcinoscorpin) from Indian horseshoe crab *Carcinoscorpius rotunda cauda*. *Biochim.Biophys.Acta* 623:89-97.

BOSWELL, C.A. & BAYNE, C.J. (1984). Isolation, characterization and functional assessment of a haemagglutinin from the plasma of *Biomphalaria glabrata*, intermediate host of *Schistosoma mansoni*. *Dev.Comp.Immunol.* 8:559-568.

BOSWELL, C.A. & BAYNE, C.J. (1985). *Schistosoma mansoni*: lectin-dependent cytotoxicity of haemocytes from susceptible host snails *Biomphalaria glabrata*. *Exp.Parasitol.* 60:113-138.

BOSWELL, C.A., YOSHINO, T.P. & DUNN, T.S. (1987). Analysis of tegumental surface proteins of *Schistosoma mansoni* primary sporocysts. *J.Parasitol.* 73:778-786.

BOUT, D., CAPRON, A., DUPAS, H. & CAPRON, M. (1974). Characterization of *Schistosoma mansoni* antigens. *Proceedings of the 3rd International Congress for Parasitology* 2:1146

BOYD, W.C. & BROWN, R. (1965). A specific agglutinin in the snail *Otala (Helix) lactea*. *Nature* 208:593-594.

BOYD, W.C., BROWN, R. & BOYD, L.G. (1966). Agglutinins for human erythrocytes in molluscs. *J.Immunol.* 96:301-303.

BRANDIN, E.R. & PISTOLE, T.G. (1983). Polyphemin: a teichoic acid - binding lectin from the horseshoe crab, *Limulus polyphemus*. *Biochem.Biophys.Res.Commun.* 113:611-617.

BREHELIN, M.M. (1972). Etude du mechaniseme de la coagulation de l'haemolymph d'un acridien, *Locusta migratoria migratoroides* (R. and F.) *Acrida* 1:167-175.

BRETTING, H. & RENWRANTZ, L. (1973). Investigations of invertebrates of the Mediterranean Sea with regard to contents of haemagglutinins. *Zeit.Immun.Forsch.* 145:242-254.

BRETTING, H., KALTHOFF, H. & FEHR, S. (1978). Studies on the relationship between lectins from *Axinella polyploides* agglutinating bacteria and human erythrocytes. *J.Invertebr.Pathol.* 32:151-157.

BROWN, D.S. (1980). *"Freshwater snails of Africa and their medical importance"*. Taylor and Francis, London.

BROWN, R., ALMOD VAR, L.R., BHATIA, H.M. & BOYD.W.C. (1968). Blood group specific agglutinins in invertebrates. *J.Immunol.* 100:214-216.

CAMUS, M.L. (1899). Recherches experimentales sur une agglutinine produite par la glande de l'albumen chez l'*Helix pomatia*. *Comp.Rend.Hebd.Sc.Acad.* 129:233-235.

CANTACUZENE, J. (1912). Sur certaines anticorps naturales observes chez *Euparurus prideauxii*. *C.R.Soc.Biol.* 73:663-665.

CANTACUZENE, J. (1918). Anticorps normaux et experimentaux chez quelques invertebres marins. *C.R.Soc.Biol.* 82:1087-1008.

CANTACUZENE, J. (1923). Le probleme de l'immunite chez les invertebres. *C.R.Seances Soc.Biol. (Paris) 75th anniversary (Suppl.)* 88:48-55.

CHAIN, B.M. & ANDERSON, R.S. (1983). Antibacterial activity of the coelomic fluid from the polychaete, *Glycera dibranchiata*. II.Partial purification and biochemical characterization of the active factor. *Biol.Bull.* 164:41-49.

CHENEY, D.P. (1971). A summary of invertebrate leucocyte morphology with em phasis on blood elements of the Manila clam, *Tapes semidecussata*. *Biol.Bull.* 140:353-358.

CHENG, T.C. (1981). Bivalves, in *"Invertebrate Blood Cells 1"*, N.A.Ratcliffe & A.F.Rowley (eds.), p.223 Academic Press, London.

CHENG, T.C. (1988). *In vivo* effects of heavy metals on cellular defence mechanisms of *Crassostrea virginica*: total and differential cell counts. *J.Invertebr.Pathol.* 51:207-210.

CHENG, T.C. & RIFKIN, E. (1970). Cellular reactions in marine molluscs in response to helminth parasitism. In *"A symposium on diseases of fish and shell fishes"*, S.F.Sniesko (ed.), p.443. Am.Fish.Soc.(Washington DC).

CHENG, T.C. & CALI, A. (1974). An electron microscope study of the fate of bacteria phagocytosed by granulocytes of *Crassostrea virginica*. *Contemp.Top.Immunobiol.* 4:25-31.

CHENG, T.C. & RODRICK, G.E. (1975). Lysozyme and other enzymes in the haemolymph of *Crassostrea virginica* and *Mercenaria mercenaria*. *Comp.Biochem.Physiol.* 52B:443-447.

CHENG, T.C. & AULD, K.R. (1977). Haemocytes of the pulmonate gastropod *Biomphalaria glabrata*. *J.Invertebr.Pathol.* 30:119-122.

CHENG, T.C. & DOWNS, J.C.U. (1988). Intracellular acid phosphatase and lysozyme levels in subpopulations of oyster, *Crassostrea virginica*, haemocytes. *J.Invertebr.Pathol.* 25:261-264.

CHENG, T.C., CHORNEY, M.J. & YOSHINO, T.P. (1977a). Lysozyme-like activity in the haemolymph of *Biomphalaria glabrata* challenged with bacteria. *J.Invertebr.Pathol.* 29:170-174.

CHENG, T.C., CHORNEY, M.J. & YOSHINO, T.P. (1977b). Lysozyme-like activity in *Biomphalaria glabrata* (Mollusca) parasitized by *Echinostoma lindoense* (Trematoda). *J.Invertebr.Pathol.* 31:57-62.

CHENG, T.C., RODRICK, G.E., FOLEY, D.A. & KOEHLER, S.A. (1975). Release of lysozyme from haemolymph cells of *Mercenaria mercenaria* during phagocytosis. *J.Invertebr.Pathol.* 25:261-265.

CHENG, T.C., LIE, K.J., HEYNEMAN, D. & RICHARDS, C.S. (1978). Elevation of aminopeptidase activity in *Biomphalaria glabrata* (Mollusca) parasitized by *Echinostoma lindoense* (Trematoda). *J.Invertebr.Pathol.* 31:57-62.

CHENG, T.C., HOWLAND, K.H., MORAN, H.J. & SULLIVAN, J.J. (1983). Studies on parasitic castration: aminopeptidase activity levels and protein concentrations in *Ilyanassa obsoleta* (Mollusca) parasitized by larval trematodes. *J.Invertebr.Pathol.* 42:42-50.

CHERNIN, E. (1970). Behavioural responses of miracidia to *Schistosoma mansoni* and other trematodes to substances emitted by snails. *J.Parasitol.* 56:287-296.

CLEGG, J.A., SMITHERS, S.R. & TERRY, R.J. (1971). Acquisition of human antigens by *Schistosoma mansoni* during cultivation *in vitro*. *Nature* 232:653-654.

CLEVELAND, D.W., FISCHER, S.G., KIRSCHINER, M.W. & LAEMMLI, U.K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl-sulfate and analysis by gel electrophoresis. *J.Biol.Chem.* 252:1102-1106.

COHEN, E., ROZENBERG, M. & MASSERO, E.J. (1974). Agglutinins of *Limulus polyphemus*. *Ann.New York Acad.Sci.* 234:28-35.

COHEN, E. & VASTA, G.R. (1982). Immunohematological significance of ubiquitous lectins. In E.L.Cooper & M.A.B.Brazier (eds.) : *"Developmental Immunology:Clinical Problems and Aging,"* p.99. Academic Press, New York.

COHEN, E., ROBERTS, S.C., NORDLING, S. & UHLENBRUCK, G. (1972). Specificity of *Limulus polyphemus* agglutinins for erythrocyte receptors common to M and N antigenic determinants. *Vox.Sang.* 23:300-307.

COOPER, E.L., LEMMI, C.A.E. & MOORE, T.C. (1974). Agglutinins and cellular immunity in earthworms. *Ann.N.Y.Acad.Sci.* 234:34-50.

COOPER, E.L., STEIN, E.A. & WOJDANI, A. (1984). Recognition receptors in annelids. In *"Recognition Proteins, Receptors and Probes: Invertebrates."* E.Cohen (ed.) p43-54. Alan R.Liss Inc., New York.

COOPER-WILLIS, C.A. (1979). Changes in acid phosphatase levels in the haemocytes and haemolymph of *Patella vulgata* after challenge with bacteria. *Comp.Biochem.Physiol.* 63A:627-631.

CORNICK, J.W. & STEWART, J.E. (1968). Pathogenicity of *Gaffkya homari* for the crab *Cancer irroratus*. *J.Fish Res.Bd.Can.* 25:795-799.

CORNICK, J.W. & STEWART, J.E. (1973). Partial characterization of a natural agglutinin in the haemolymph of the lobster *Homarus americanus*. *J.Invertbr.Pathol.* 21:255-262.

CRABTREE, J.E. & WILSON, R.A. (1986). *Schistosoma mansoni:* an ultrastructural examination of pulmonary migration. *Parasitology* 92:343-354.

CRIDLAND, C.C. (1955). The experimental infection of several species of African freshwater snails with *Schistosoma mansoni* and *Schistosoma haematobium. J.Trop.Med.Hyg.* 58:1-11.

CUSHING, J.E. (1967). Invertebrates, immunology and evolution. *Fed.Proc.Fed.Amer.Soc.Exp.Biol.* 26:1666-1689.

CUSHING, J.E., EVANS, E.E. & EVANS, E.L. (1971). Induced bactericidal responses of abalones. *J.Invertebr.Pathol.* 17:446-448.

DALES, R.P. (1982). Haemagglutinins and haemolysins in the body fluids of *Neoamphitrite figulus* and *Arenicola marina* (Annelida:Polychaeta). *Comp.Biochem.Physiol.* 73A:663-667.

DAMIAN, R.T. (1987). Molecular mimicry revisited. *Parasitol.Today* 3:263-266.

DANIEL, B.E. (1990). Ph.D. thesis. University of London. Title of thesis KH

DIKKEBOOM, R., TIJNAGEL, J.M.G.H. & Van der KNAAP, W.P.W. (1988). Monoclonal antibody recognized hemocyte subpopulations in juvenile and adult *Lymnaea stagnalis*: functional characteristics and lectin binding. *Dev.Comp.Immunol.* 12:17-32.

DIKKEBOOM, R., Van der KNAAP, W.P.W., MEULEMAN, E.A. & SMINIA, T. (1984). Differences between the blood cells of juvenile and adult specimens of the pond snail *Lymnaea stagnalis. Cell Tiss.Res.* 238:43-47.

DIKKEBOOM, R., MULDER, E.C., TIJNAGEL, J.M.G.H. & SMINIA, T. (1985)(a) Phagocytic blood cells of the pond snail *Lymnaea stagnalis* generate active forms of oxygen. *Eur.J.Cell.Biol.Suppl.* 11:12-17.

DIKKEBOOM, R., Van der KNAAP, W.P.W., MEULEMAN, E.A. & SMINIA, T. (1985)(b) A comparative study on the internal defence system of juvenile and adult *Lymnaea stagnalis*. *Immunol.* 55:547-553.

DIKKEBOOM, R., Van der KNAAP, W.P.W., MAASKANT, J.J. & De JONGE, A.R. (1985)(c) Different subpopulations of hemocytes in juvenile, adult and *Tricobilharzia ocellata*-infected *Lymnaea stagnalis*: a characterization using monoclonal antibodies. *Cell Tissue Res.* 71:815-824.

DIKKEBOOM, R., TIJNAGEL, J.M.G.H., MULDER, E.C. & Van der KNAAP, W.P.W. (1987). Haemocytes of the pond snail *Lymnaea stagnalis* generate reactive forms of oxygen. *J.Invertebr.Pathol.* 49:321-331.

DIKKEBOOM, R., BAYNE, J., Van der KNAAP, W.P.W. & TIJNAGEL, J.M.G.H. (1988)(a) Possible role of reactive forms of oxygen in *in vitro* killing of *Schistosoma mansoni* sporocysts by haemocytes of *Lymnaea stagnalis*. *Parasitol.Res.* 75:148-155

DIKKEBOOM, R., Van der KNAAP, W.P.W., Van der BOVENKAMP, W., TIJNAGEL, J.M.G.H. & BAYNE, C.J. (1988)(b) The production of toxic oxygen metabolites by haemocytes of different snail species. *Dev.Comp.Immunol.* 12:509-521.

DUNN, T.S. & YOSHINO, T.P. (1988). *Schistosoma mansoni*: Origin and expression of a tegumental surface antigen on the miracidium and primary sporocyst. *Exp.Parasitol.* 67:167-175.

EY, P.L. & JENKIN, C.R. (1982). Molecular basis of self/nonself discrimination in the invertebrata. In: N.Cohen & M.M.Siegel (eds.) *"The Reticuloendothelial System. A Comprehensive Treatise."* vol.3 "Phylogeny and Ontogeny." p.321. Plenum Press, New York and London.

FAYE, I. & WYATT, G.R. (1980). The synthesis of antibacterial proteins in isolated fat body from *Cecropia* silk-moth pupae. *Experientia* 36:1325-

FIGUEIREDO, E.A., GOMEZ, M.V., HENEINE, I.F., SANTOS, I.O. & HARGREAVES, F.B. (1973). The hemoglobin of *Biomphalaria glabrata* (Mollusca. Planorbidae). *Comp.Biochem.Physiol.* 44:481-491.

FISHER, W.S. (1986). Structure and functions of oyster hemocytes, in *"Immunity in Invertebrates"*, M.Brehelin (ed.), p25. Springer-Verlag, Berlin/Heidelberg, Germany,

FRYER, S.E. & BAYNE, C.J. (1989). Opsonization of yeast by the plasma of *Biomphalaria glabrata* (Gastropoda): a strain-specific, time-dependent process. *Parasit.Immunol.* 11:269-278.

FUKE, M.T. & SUGAI, T. (1972). Studies on the naturally occurring haemagglutinin in the coelomic fluid of an ascidian. *Biol.Bull.* 143:140-149.

GARTE, S.J. & RUSSELL, C.S. (1976). Isolation and characterization of a haemagglutinin from *Amphitrite ornata*, a polychaetous annelid. *Biochim.Biophys.Acta.* 439:368-379.

GEORGE, W.C. & FERGUSON, J.H. (1950). The blood of gastropod molluscs. *J.Morph.* 86:315-327.

GHIDALIA, W., LAMBIN, P. & FINE, J.M. (1975). Electrophoretic and immunologic studies of a haemagglutinin in the haemolymph of the decapod *Macropipus puber*. *J.Invertebr.Pathol.* 53:197-205.

GILLIAM, M. & JETER, W.S. (1970). Synthesis of agglutinating substances in adult honeybees against *Bacillus larvae*. *J.Invertebr.Pathol.* 16:69-70.

GOLD, E.R. & BALDING, P. (1975). In *"Receptor Specific Proteins. Plant and Animal Lectins"*. Elsevier, Amsterdam/New York.

GRABER, M. (1978). *Schistosoma margrebowiei* of cobs in Chad. *J.Helminthol.* 52:72-74.

GRANATH, W.O. Jnr. & YOSHINO, T.P. (1983). Lysosomal enzyme activities in susceptible and refractory strains of *Biomphalaria glabrata* during the course of infection with *Schistosoma mansoni*. *J.Parasitol.* 69:1018-1026.

GRANATH, W.O., SPRAY, F.J. & JUDD, R.C. (1987). Analysis of *Biomphalaria glabrata* (Gastropoda) haemolymph by SDS-PAGE, HPLC, and Immunoblotting. *J.Invertebr.Pathol.* 49:198-208.

HALL, J.L. & ROWLANDS, D.T. (1974a). Heterogeneity of lobster agglutinins. I. Purification and characterization. *Biochemistry* 13:821-827.

HALL, J.L. & ROWLANDS, D.T. (1974b). Heterogeneity of lobster agglutinins. II. Specificity of agglutinin-erythrocyte binding. *Biochemistry* 13:828-832.

HAMMARSTRÖM, S. & KABAT, E.A. (1969). Purification and characterization of a blood-group A reactive haemagglutinin from the snail *Helix pomatia* and a study of its combining site. *Biochemistry* 8:2696-2705.

HAMMARSTRÖM, S., WESTÖÖ, A. & BJÖRK, J. (1972). Subunit structure of *Helix pomatia* A haemagglutinin. *Scan.J.Immunol.* 1:295-309.

HAPNER, K.D. & JERMYN, M.A. (1981). Haemagglutinin activity in the haemolymph of *Teleogryllus commodus* (Walker). *Insect Biochem.* 11:287-295.

HARDY, S.W., FLETCHER, T.C. & OLAFSEN, J.A. (1977). Aspects of cellular and humoral defence mechanisms in the pacific oyster *Crassostrea gigas*. In *"Dev.Immunobiology"* (J.B.Solomon & J.D.Horton eds.) pp.59-66, Elsevier/North Holland, Amsterdam.

HARRELSON, A.L. & GOODMAN, C.S. (1988). Growth cone guidance in insects: fascilin II is a member of the immunoglobulin superfamily. *Science* 242:700-708.

HARRIS, K.R. & CHENG, T.C. (1975). The encapsulation process of *Biomphalaria glabrata* experimentally infected with the metastrongylid *Angiostrongylus cantonensis*: enzyme histochemistry. *J.Invertebr.Pathol.* 26:367-374.

HAYUNGA, E.G. & SUMNER, M.P. (1986). Characterization of surface glycoproteins on *Schistosoma mansoni* adult worms by lectin affinity chromatography. *J.Parasitol.* 72:283-291.

HOCKLEY, D.J. & McLAREN, D.J. (1973). *Schistosoma mansoni:* changes in the outer membrane of the tegument during development from cercaria to adult worm. *Int.J.Parasitol.* 3:13-21.

HOWLAND, K.H. & CHENG, T.C. (1982). Identification of bacterial chemoattractants for oyster (*Crassostrea virginica*) hemocytes. *J.Invertebr.Pathol.* 39:123-132.

HYNES, R.O. (1987). Integrins: a family of cell surface receptors. *Cell* 48:549-554.

IBRAHIM, E.A.R., INGRAM, G.A. & MOLYNEUX, D.H. (1984). Haemagglutinins and parasite agglutinins in haemolymph and gut of *Glossina*. *Tropenmed. und Parasitologie* 35:151-156.

INGRAM, G.A., EAST, J. & MOLYNEUX, D.H. (1983). Agglutinins of *Trypanosoma, Leishmania* and *Crithidia* in insect haemolymph. *Dev.Comp.Immunol.* 7:649-652.

INGRAM, G.A., EAST, J. & MOLYNEUX, D.H. (1984). Naturally occurring agglutinins against trypanosomatid flagellates in the haemolymph of insects. *Parasitology.* 89:435-451.

JANDL, J.H. & SIMMONS, R.L. (1957). The agglutination and sensitization of red cells by metallic cations:interactions between multivalent metals and the red cell membrane. *Brit.J.Haematol.* 3:19-24.

JEONG, K.H., LIE, K.J. & HEYNEMAN, D. (1983). The ultrastructure of the amoebocyte-producing organ in *Biomphalaria glabrata*. *Dev.Comp.Immunol.* 7:217-228.

JEONG, K.H., SUSSMAN, S., ROSEN, S.D., LIE, K.J. & HEYNEMAN, D. (1981). Distribution and variation of haemagglutinating activity in the haemolymph of *Biomphalaria glabrata*. *J.Invertebr.Pathol.* 28:256-263.

JENKIN, C.P. & ROWLEY, D. (1970). Immunity in invertebrates. The purification of a haemagglutinin to rat and rabbit erythrocytes from the haemolymph of the murray mussel *Velesunio ambigus*. *Aust.J.Exp.Biol.Med.Sci.* 48:129-137.

JOHANSSON, M.W. & SÖDERHALL, K. (1989). A peptide containing the cell adhesion sequence RGD can mediate degranulation and cell adhesion of crayfish granular haemocytes *in vitro*. *Insect Biochem.* 19:573-575.

JOHNSON, H.M. (1964). Human blood group A_1 specific agglutinin of the Butter Clam *Saxidomus giganteus*. *Science* 146:548-549.

JOKY, A., MATRICON-GONDRAN, M. & BENEX, J. (1985). Response of the amoebocyte-producing organ in sensitized *Biomphalaria glabrata* after exposure to *Echinostoma caproni* miracidia. *J.Invertebr.Pathol.* 45:28-33.

JURENKA, R., MANFRIED, K. & HAPNER, K.D. (1982). Haemagglutinin activity in Acrididae (grasshopper) haemolymph. *J.Insect Physiol.* 28:177-181.

KAMIYA, H., MURAMOTO, K. & GOTO, R. (1987). Isolation and characterization of agglutinins from the hemolymph of an acorn barnacle. *Megabalanus volcano*. *Dev.Comp.Immunol.* 11:297-307.

KAMIYA, H., MURAMOTO, K., GOTO, R., SAKAI, M. & YAMAZAKI, M. (1989). Antibacterial protein(s) in the secretion of a sea hare *Aplysia juliana*. *Dev.Comp.Immunol.* 13:450-454.

KAPPUS, H. (1985). Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. In *"Oxidative Stress"*, H.Sies (ed.), p.273. Academic Press, London.

KÉCHEMIR, N. & COMBES, C. (1982). Développement du Trématode *Schistosoma haematobium* après transplantation microchirurgicale chez le Gastéropode *Planorbarius metidjensis*. *C.R.Acad.Sci.Paris* 295:505-508.

KINOTI, G.K. (1971). The epidemiology of *Schistosoma haematobium* infection on the Kano Plain of Kenya. *Trans.Royal Soc.Trop.Med.Hyg.* 65:637-645.

KLEIN, J. (1989). Are invertebrates capable of anticipatory immune responses? *S Can.J.Immunol.* 29:499-505.

KOHLER, G. & MILSTEIN, C. (1975). Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* 256:495-497.

KOLE, M. (1979). Electron microscopical observations on defence mechanisms against daughter sporocysts in a prosobranch gastropod. *Zentralbl.Bakteriol.Hyg.* Abt.1.Ref.263, 206 (Abstract).

KOMANO, H., MIZUNO, D. & NATORI, S. (1980). Purification of lectin induced in the haemolymph of *Sarcophaga peregrina* larvae on injury. *J.Biol.Chem.* 255:2919-2924.

KOMANO, H., MIZUNO, D. & NATORI, S. (1981). A possible mechanism of induction of insect lectin. *J.Biol.Chem.* 256:7087-7089.

KOMANO, H., NOZANA, R., MIZUNO, D. & NATORI, S. (1983). Measurement of *Sarcophaga peregrina* on injury of the body wall. *J.Biol.Chem.* 258:2143-2147.

KRUPA, P.L., LEWIS, L.M. & Del VECCHIO, P. (1977). *Schistosoma haematobium* in *Bulinus guernei: electron* microscopy of haemocyte-sporocyst interactions. *J.Invertebr.Pathol.* 30:35-45.

KRISHNAMOORTHY, R., CAHOUR, A., ELION, J., HARTMAN , L. & LABIE, D. (1983). Monosaccharides bound to hemoglobins in normal and diabetic individuals. *Eur.J.Biochem.* 132:345-350.

KUSEL, J.R. (1970). Studies on the structure and hatching of the eggs of *Schistosoma mansoni. Parasitology* 60:79-88.

KÜSTER, H.C. (1841). Limnaeacea. In *"Systematisches Conchylien - Cabinet von Martini und Chemnitz"*, 2nd Ed., 1 (17) part 32.

LACKIE, A.M. (1981). The specificity of the serum agglutinins of *Periplaneta americana* and *Schistocerca gregaria* and its relationship to the insect's immune response. *J.Insect Physiol.* 27:139-143.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277:680-685.

LANDSTEINER, K. (1947). In *"The Specificity of Serological Reactions,"* Harvard University Press, Cambridge, Massachussets.

LASSEGUES, M., ROCH, P. & VALEMBOIS, P. (1989). Antibacterial activity of *Eisenia fetida andrei* coelomic fluid:evidence, induction, and animal protection. *J.Invertebr.Pathol.* 53:1-6.

LE ROUX, P.L. (1933). A preliminary note on *Bilharzia margrebowiei,* a new parasite of ruminants and possibly man in Northern Rhodesia. *J.Helminthol.* 11:57-58.

LE ROUX, P.L. (1955). A new mammalian schistosome (*S. leiperi spp.*nov.) from herbivora in North Rhodesia. *Trans.Royal Soc.Trop.Med.Hyg.* 49:293-294.

LI, C.P., PRESCOTT, B. & JOHNES, W.G. (1962). Antiviral activity of a fraction of Abalone juice. *Proc.Soc.Exp.Biol.Med.* 109:534-536.

LI, M.F. & FLEMMING, C. (1967). Haemagglutinins from oyster hemolymph. *Can.J.Zool.* 45:1225-1230.

LIE, K.J. (1982). Swellengrebel lecture. Survival of *Schistosoma mansoni* and other trematode larvae in the snail *Biomphalaria glabrata.* A discussion of the interference theory. *Trop.Geogr.Med.* 34:111-122.

LIE, K.J., HEYNEMAN, D. & YAU, P. (1975). The origin of amoebocytes in *Biomphalaria glabrata. J.Parasitol.* 61:574-576.

LIE, K.J., HEYNEMAN, D. & JEONG, K.H. (1976). Studies on resistance in snails.4.Induction of ventricular capsules and changes in the amoebocyte-producing organ during sensitization of *Biomphalaria glabrata* snails. *J.Parasitol.* 62:286-291.

LIE, K.J., HEYNEMAN, D. & RICHARDS, C.S. (1977). Studies on resistance in snails : interference by nonirradiated Echinostome larvae with natural resistance to *Schistosoma mansoni* in *Biomphalaria glabrata. J.Invertebr.Pathol.* 29:118-125.

LIE, K.J., JEONG, K.H. & HEYNEMAN, D. (1982). Further characterization of acquired resistance in *Biomphalaria glabrata. J.Parasitol.* 68:529-531.

LIN, J.H., FANG, W.S., HUANG, R.T. & CHEN, S.W. (1974). Trematode control by dual infection with suitable and unsuitable miracidia in the same snail. *Taiwan J.Vet.Med.Anim.Husb.* 25:55-62.

LO, C.T., BURCH, J.B. & SCHUTTE, C.H.J. (1970). Infection of diploid *Bulinus spp.* with *Schistosoma haematobium. Malac.Rev.* 3:121-126.

LOKER, E.S. & BAYNE, C.J. (1982). *In vitro* encounters between *Schistosoma mansoni* primary sporocysts and haemolymph components of susceptible and resistant strains of *Biomphalaria glabrata. Ann.J.Trop.Med.Hyg.* 31:999-1005.

LOKER, E.S., BAYNE, C.J. & YUI, M.A. (1986). *Echinostoma paraensi*: hemocytes of *Biomphalaria glabrata* as targets of echinostome mediated interference with host snail resistance to *Schistosoma mansoni*. *Exp.Parasitol.* 62:149-154.

LOKER, E.S., BOSTON, M.E. & BAYNE, C.J. (1989). Differential adherence of M line *Biomphalaria glabrata* haemocytes to *Schistosoma mansoni* and *Echinostoma paraensi* larvae and experimental manipulation of haemocyte binding. *J.Invertebr.Pathol.* 54:260-268.

LoVERDE, P.T., GHERSON, J. & RICHARDS, C.S. (1982). Reaction against self in an invertebrate? *J.Invertebr.Pathol.* 39:247-249.

MacGREGOR, A.N., STOTT, D.I. & KUSEL, J.R. (1985). Lectin binding to glycoproteins in the surface membrane of *Schistosoma mansoni*. *Mol.Biochem.Parasitol.* 16:163-172.

MacKAY, D.& JENKIN, C.R. (1970). Immunity in the invertebrates. The role of serum factors in phagocytosis of erythrocytes by haemocytes of the freshwater crayfish (*Parachaeraps bicarinatus*). *Aust.J.Exp.Biol.Med.Sci.* 48:139-150.

MALEK, E.A. & CHENG, T.C. (1974). In *"Medical and Economic Malacology"*. pp.398. Academic Press, New York & London.

MANDAHL - BARTH, G. (1965). The species of *Bulinus*, intermediate hosts of *Schistosoma*. *Bull.Wld.Hlth.Org.* 33:33-34.

MARCHALONIS, J.J. (1980). Molecular interactions and recognition specificity of surface receptors. *Contemp.Top.Immunobiol.* 9:255-260.

MARCHALONIS, J.J & EDELMAN, G.M. (1968). Isolation and characterization of a haemagglutinin from *Limulus polyphemus*. *J.Mol.Biol.* 32:453-465.

MARKWELL, M.A.K., HAAS, S.M., BIEBER, L.L. & TOLBERT, N.E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal.Biochem.* 87:206-210.

MARTENS, E. (1879). Neritina. In *"Systematisches Conchylien Cabinet von Martini und Cheminitz*, 2nd ed., H.C.Kuster & W.Kobelt (eds.) Bauer & Raspe, Nuremberg.

MASON, P.R. & FRIPP, P.J. (1977). The reactions of *Schistosoma mansoni* miracidia to light. *J.Parasitol.* 10:686-689.

MAUCHAMP, B. (1982). Purification of an N-acetyl-D-glucosamine specific lectin (P.B.A.) from epidermal cell membranes of *Pieris brassicae* L. *Biochimie* 64:1001-1008.

MAUDSLEY, S., BALTZ, M.L., MUNN, E.A., BUTTRESS, N., HERBERT, J., FEINSTEIN, A. & PEPYS, M.B. (1987). Isolation and characterisation of goat C-reactive protein. *Biochim.Biophys.Acta* 924:75-80.

MAURICE, J. (1985). Tropical Disease Research. *Africa Health.* 7:19-22.

McCLELLAND, W.F. (1967). Production of *Schistosoma haematobium* and *Schistosoma mansoni* cercariae in Tanzania. *Exp.Parasitol.* 20:205-218.

McDADE, J.E. & TRIPP, M.R. (1967). Mechanism of agglutination of red blood cells by oyster haemolymph. *J.Invertebr.Pathol.* 9:523-530.

McKENZIE, A.N.J. (1988). Ph.D thesis. University of London.

McKERROW, J.H., JEONG, K.H. & BECKSTEAD, J.H. (1985). Enzyme histochemical comparison of *Biomphalaria glabrata* amoebocytes with human granuloma macrophages. *J.Leukocyte Biol.* 37:341-347.

MENEGHINI, R. (1988). Genotoxicity of active oxygen species in mammalian cells. *Mutat.Res.* 195:215-218.

MEULEMAN, E.A., BAYNE, C.J. & van der KNAAP, W.P.W. (1986). Immunological aspects of snail-trematode interactions. In *"Proceedings of the Third Congress International Society for Developmental and Comparative Immunology."* E.L.Cooper (ed.), Alan Liss Inc., New York.

MICHELSON, E. (1958). A method for the relaxation and immobilisation of pulmonate snails. *Trans.Am.Micr.Soc.* 77:316-319.

MICHELSON, E.H. (1975). In, *"Invertebrate Immunity: Mechanisms of Invertebrate Vector-Parasite Relations."* K.Maramarosch & R.E.Shope (eds.) pp.181-195. Academic Press, New York, San Francisco, London.

MICHELSON, E.H. & DUBOIS, L. (1977). Agglutinins and lysins in the family Planorbidae: a survey of haemolymph, egg masses, and albumen gland extracts. *Biol.Bull.* 153:219-227.

MIGLIETTA, A., CANICATTI', C. & COOPER, E.L. (1989). *In vitro* clotting reaction in *Holothuria poli.* In "Invertebrate Immunobiology Symposium III" booklet, Lecce.

MILLER, R.L. & CANNON, J.D. (1984). *Limax flavus* agglutinin- properties of a sialic acid specific lectin and its biomedical application. In *"Recognition Proteins, Receptors and Probes: Invertebrates"* E.Cohen (ed.) p31-41. Alan R.Liss Inc., New York.

MINNICK, M.F., RUPP, R.A. & SPENCE, K.D. (1986). A bacterial-induced lectin which triggers hemocyte coagulation in *Manduca sexta. Biochem.Biophy.Res.Comm.* 137:729-735.

MITRA, D. & SARKAR, M. (1988). A galactose-specific agglutinin from the haemolymph of the snail *Achatina fulica:* Purification and characterization. *Dev.Comp.Immunol.* 12:33-52.

MITRA, D., SARKAR, M. & ALLEN, A.K. (1987). Further characterization of the cold agglutinin from the snail *Achatina fulica. BiochemJ.* 242:331-338.

MOHANDAS, A. & CHENG, T.C. (1985). Release pattern of aminopeptidase from *Biomphalaria glabrata* haemocytes subjected to high-level bacterial challenge. *J.Invertebr.Pathol.* 45:298-303.

MORI, K., MURAYAMA, K., KANNO, N., NAKAMURA, M., OHIRA, E., KATO, Y. & NOMURA, T. (1984). Occurrence and characterization of the defense factors in the japanese oyster *Crassostrea gigas. Tohoku J.Agr.Res.* 35:55-59.

MORRISSEY, J.H. (1981). Silverstain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal.Biochem.* 117:307-310.

NAKAMURA, N., MORI, K., INOOKA, S. & NOMURA, T. (1985). *In vitro* production of hydrogen peroxide by amoebocytes of the scallop *Patinopecten yessoensis* (Jay). *Dev.Comp.Immunol.* 9:407-412.

NOGUCHI, H. (1903). On the multiplicity of the serum agglutinins of cold blooded animals. *Zentrabl.Bakt.Abt.1:Orig.Reihe.* 34A:286-287.

NOWAK, T.P. & BARONDES, S.H. (1975). Agglutinin from *Limulus polyphemus.* Purification with formalinized horse erythrocytes as the affinity absorbent. *Biochem.Biophys.Acta* 393:115-122.

O'SHANNESSY,D.J., VOORSTAD, P.J. & QUARLES, R.H. (1987). Quantitation of glycoproteins on electroblots using the biotin-streptavadin complex. *Anal.Biochem.* 163:204-209.

PALLADINO, M.A., RANGES, G.E., SCHEID, M.P. & OETTGEN, H.F. (1983). Suppression of T cell cytotoxicity by nude mouse spleen cells: reversal by monosaccharides and interleukin 2. *J.Immunol.* 130:2200-2203.

PAN, C.T. (1965). Studies on the host-parasite relationship between *Schistosoma mansoni* and the snail *Australorbis glabratus*. *Am.J.Trop.Med.Hyg.* 14:931-976.

PARISH, C.R. (1977). Simple model for self/non-self discrimination in invertebrates. *Nature* 267:711-713.

PARRINELLO, N. & PATRICOLO, E. (1975). Erythrocyte agglutinins in the blood of certain ascidians. *Experienta* 31:1092-1094.

PARRINELLO, N., RINDONE, D. & CANICATTI, C. (1981). Studies on the natural hemolytic system of the annelid worm *Spirographis spallanzanii*. *Dev.Comp.Immunol.* 5:33-37.

PAULEY, G.B. (1971). Bacterial clearance in the marine gastropod mollusk, *Aplysia californica* (Cooper). *Proc.Nat.Shellfish.Ass.* 61:11-12.

PAULEY, G.B. (1974). Comparison of a natural agglutinin in the hemolymph of the blue crab, *Callinectes sapidus*, with agglutinins of other vertebrates. In *"Comtemporary Topics in Immunology"*. (E.L.Cooper ed), Vol.4, pp.241-260. Plenum Press, New York.

PAULEY, G.B., GRANGER, G.A. & KRASSNER, S.M. (1971). Characterization of a natural agglutinin in the hemolymph of the California Sea Hare, *Aplysia californica*. *J.Invertebr.Pathol.* 18:207-210.

PAULEY, G.B., KRASSNER, S.M. & CHAPMAN, F.A. (1971). Bacterial clearance in the Californian Sea Hare *Aplysia californica*. *J.Invertebr.Pathol.* 18:227-239.

PEFEROEN, M., STYNEN, D. & De LOOF, A. (1982). A re-examination of the protein pattern of the haemolymph of *Leptinotarsa decemlineata*, with specific reference to the vitellogenins and diapause proteins. *Comp.Biochem.Physiol.* 72B:345-351.

PENDLAND, J.C. & BOUCIAS, D.G. (1986). Characteristics of a galactose-binding haemagglutinin (lectin) from hemolymph of *Spodoptera exigua* larvae. *Dev.Comp.Immunol.* 10:477-487.

PEREIRA, M.E.A., ANDRADE, A.F.B. & RIBEIRO, J.M.C. (1981). Lectins of distinct specificity in *Rhodnius prolixus* interact selectively with *Trypanosoma cruzi*. *Science* 211:597-600.

PETERS, W., KOLB, H. & KOLB-BACHOFEN, V. (1983). Evidence for a sugar receptor (lectin) in the peritrophic membrane of the blowfly, *Calliphora erythrocephala* Mg.(Diptera). *J.Insect Physiol.* 29:275-280.

PISTOLE, T.G. (1979). Bacterial agglutinins from *Limulus polyphemus*. An overview. E.Cohen (ed.) *Biomedical applications of the horseshoe crab (Limulidae)* p.547. Alan R.Liss Inc., New York.

PITCHFORD, R.J. (1976). Preliminary observations on the distribution, definitive host and possible relation with other schistosomes of *Schistosoma margrebowiei* Le Roux, 1933 and *Schistosoma leiperi* Le Roux, 1955. *J.Helminthol.* 50:111-123.

POPIEL, I., IRVING, D.L. & BASCH, P.F. (1985). Wound healing in the trematode *Schistosoma. Tissue and Cell* 17:69-72.

PRINGLE, G., OTIENO, L.H. & CHIMTAWI, M.B. (1971). Notes on the morphlogy, susceptibility to *Schistosoma haematobium* and genetic relationships of *Bulinus (Physopsis) globosus* and *Bulinus(Physopsis)nasutus* from north-eastern Tanzania. *Ann.Trop.Med.Parasitol.* 65:211-219.

PROKOP, O., UHLENBRUCK, G. & KOHLER, W. (1968). A new source of antibody-like substances having anti-blood group specificity: A discussion of the specificity of *Helix* agglutinins. *Vox.Sang.* 14:321-333.

PROWSE, R.H. & TAIT, N.N. (1969). *In vitro* phagocytosis by amoebocytes from the haemolymph of *Helix aspersa* (Muller). Evidence for opsonic factor(s) in serum. *Immunology* 17:437-443.

QU, X., STEINER, H., ENGSTROM, A., BENNICH, H. & BOMAN, H.G. (1982). Insect Immunity: Isolation and structure of cecropins B and D from pupae of the Chinese oak silk moth, *Antheria pernyi*. *Eur.J.Biochem.* 127:219-224.

RAVINDRANATH, M.H., HIGA, H.H., COOPER, E.L. & PAULSON, J.C. (1985). Purification and characterization of an O-acetylsialic acid-specific lectin from a marine crab *Cancer antennarius*. *J.Biol.Chem.* 260:8850-8856.

READE, P.C. (1968). Phagocytosis in invertebrates. *Aust.J.Exp.Biol.Med.Sci.* 46:219-229.

RENTON, P.H. & HANCOCK, J.A. (1957). Antibody like effects of colloidal silica. *Vox.Sang.* 2:117-118.

RENWRANTZ, L. (1979). Eine Untersuchung molekularer und zellulaeren Bestandteile der Haemolymphe von *Helix pomatia* unter besonderer Beruecksichtigung immunobiologisch aktiver Komponenten. *Zool.Jahrb.Physiol.* 83:283-333.

RENWRANTZ, L. & BERLINER, U. (1978). A galactose specific agglutinin, a blood-group H active polysaccharide, and a trypsin inhibitor in albumin glands and eggs of *Arianta arbustorum* (Helicidae). *J.Invertebr.Pathol.* 31:171-179.

RENWRANTZ, L. & MOHR, W. (1978). Opsonizing effect of serum and albumin gland extracts on the elimination of human erythrocytes from the circulation of *Helix pomatia*. *J.Invertebr.Pathol.* 31:164-170.

RENWRANTZ, L. & STAHMER, A. (1983). Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from *Mytilus edulis*. *J.Comp.Physiol.* 149:535-546.

RICHARDS, C.S. (1975a). Genetic studies on variation in infectivity of *Schistosoma mansoni*. *Int.J.Parasitol.* 61:233-241.

RICHARDS, C.S. (1975b). Genetic factors in susceptibility of *Biomphalaria glabrata* for different strains of *Schistosoma mansoni*. *Parasitology.* 70:231-241.

RICHARDS, C.S. (1984). Influence of snail age on genetic variations in susceptiblity of *Biomphalaria glabrata* for infection with *Schistosoma mansoni*. *Malacologia.* 25:493-502.

ROBEY, F.A. & LIU, T.Y. (1981). Limulin: a C-reactive protein from *Limulus polyphemus*. *J.Biol.Chem.* 265:969-971.

ROCHE, A.C. & MONSIGNY, M. (1974). "Purification and properties of limulin: A lectin (agglutinin) from hemolymph of *Limulus polyphemus*". *Biochim.Biophys.Acta.* 371:242-246.

ROCHE, A.C. & MONSIGNY, M. (1979). Limulin (*Limulus polyphemus* lectin). Isolation, physicochemical properties, sugar specificity and mitogenic activity. In *"Biomedical applications of the horseshoe crab (Limulidae)."* E.Cohen, J.Levin, J.J.Marchalonis, T.G.Pistole, R.A.Prendergast, C.Shuster Jnr. & S.W.Watson (eds.) Alan R. Liss Inc., New York.

RÖGENER, W., RENWRANTZ, L. & UHLENBRUCK, G. (1985). Isolation and characterization of a lectin from the haemolymph of the cephalopod *Octopus vulgaris* (Lam.) inhibited by α-D-lactose and N-acetyl-lactosamine. *Dev.Comp.Immunol.* 9:605-616.

ROLLINSON, D. & SOUTHGATE, V.R. (1985). Schistosomes and snail populations: genetic variability and parasite transmission. In *"Ecology and Genetics of Host- Parasite Interactions."* (D.Rollinson and R.M.Anderson, eds.), pp.91-109. Academic Press, London.

ROLLINSON, D. & SOUTHGATE, V.R. (1987). The genus *Schistosoma*: a taxonomic appraisal. In "The Biology of Schistosomes: From Genes To Latrines", (D.Rollinson and A.J.G.Simpson eds.) chap.1. pp7-47, Academic Press, London.

RONDELAND, D. & BARTHE, D. (1981). The development of the amoebocyte-producing organ in *Lymnaea trunculata* (Müller) by *Fasciola hepatica* (L). *Z.Parasitenkd.* 65:331-335.

ROSTAM-ABADI, H. & PISTOLE, T.G. (1982). Lipopolysaccharide-binding lectin from the horseshoe crab, *Limulus polyphemus,* with specificity for 2-keto-3-deoxyoctonate (KDO). *Dev.Comp.Immunol.* 6:209-213.

ROWLEY, A.F., RATCLIFFE, N.A., LEONARD, C.M., RICHARDS, E.H. & RENWRANTZ, L. (1986). Humoral recognition factors in insects, with particular reference to agglutinins and the prophenoloxidase system. In A.P.Gupta (ed.) *"Hemocytic and Humoral Immunity in Arthropods"*, pp.381-406. Wiley-Interscience, New York.

RUSSEL, C.S., RODRIQUES, J. & LAI, P.S. (1983). Haemagglutinating activity in *Nereis coelomic* fluid. *Comp.Biochem.Physiol.* 75A:57-64.

SAMMONS, D.W., ADAMS, L.D. & NISHIZAWA, E.E. (1981). Ultrasensitive silver-based color staining of polypeptides in polyacrylamide gels. *Electrophoresis* 2:135-141.

SARKAR, M., BACHHAWAT, B.K. & MANDAL, C. (1984). A new cold agglutinin from *Achatina fulica* snails. *Arch.Biochem.Biophys.* 233:286-289.

SCHLUTER, S.F., EY, P.L., KEOUGH, D.R. & JENKIN, C.R. (1981). Identification of two carbohydrate-specific erythrocyte agglutinins in the haemolymph of the protochordate *Botrylloides leachii. Immunology* 42:241-250.

SCHMID, L.S. (1975). Chemotaxis of haemocytes from the snail *Viviparus malleatus. J.Invertebr.Pathol.* 25:125-131.

SCHOENBERG, D.A. & CHENG, T.C. (1980a). Lectin-binding specificities of haemocytes from two strains of *Biomphalaria glabrata* as demonstrated by microadsorption assays. *Dev.Comp.Immunol.* 4:617-628.

SCHOENBERG, D.A. & CHENG, T.C. (1980b). Phagocytic funnel-like pseudopodia in lectin-treated gastropod haemocytes. *J.Invertebr.Pathol.* 36:141-143.

SCHOENBERG, D.A. & CHENG, T.C. (1981). The behaviour of *Biomphalaria glabrata* (Gastropoda:Pulmonata) haemocytes following exposure to lectins. *Trans.Am.Microsc.Soc.* 100:345-350.

SCHOENBERG, D.A. & CHENG, T.C. (1982). Concanavalin-A mediated phagocytosis of yeast by *Biomphalaria glabrata* haemocytes *in vitro:* effects of temperature and lectin concentration. *J.Invertbr.Pathol.* 39:314-322.

SCHUTTE, C.H.J. (1975). Studies on the South African strain of *Schistosoma mansoni.* Part 3. Notes on certain host-parasite relationships between intra-molluscan larvae and intermediate host. *South African J.of Science.* 71:8-20.

SCHWANBEK, A., BECKER, W. & RUPPRECHT, H. (1986). Quantification of parasite development in the host-parasite system *Biomphalaria glabrata* and *Schistosoma mansoni. Z.Parasitenknd.* 72:365-371.

SCOTT, M.T. (1971). A naturally occurring haemagglutinin in the haemolymph of the American cockroach. *Archives de Zoologie et generale* 112:73-80.

SCOTT, M.T. (1972). Partial characterization of the haemagglutinating activity in haemolymph of the American cockroach (*Periplaneta americana*). *J.Invertebr.Pathol.* 19:66-71.

SEEGER, M.A., HAFFLEY, L. & KAUFMAN, T.C. (1988). Characterization of *amalgam*: a member of the immunoglobulin superfamily from *Drosophila*. *Cell* 55:589-600.

SÉMÉREVIRA, M., NAIDET, C., KREJCI, E. & GRATECOS, D. (1989). Towards the molecular biology of cell adhesion in *Drosophila*. *Trends Genet.* 5:24-27.

SHIMIZU, S., ITO, M. & NIWA, M. (1977). Lectins in the haemolymph of Japanese horseshoe crab *Tachypleus tridentatus*. *Biochim.Biophys.Acta.* 500:71-74.

SHISHIKURA, F. & SEKIGUCHI, K. (1983). Agglutinins in the horseshoe crab haemolymph: Purification of a potent agglutinin of horse erythrocytes from the haemolymph of *Tachypleus tridentatus*, the Japanese horseshoe crab. *J.Biol.Chem.* 93:1539-1546.

SHOZAWA, A. (1986). A reducing factor produced by haemocytes of *Biomphalaria glabrata* and its role in the host defence. *Dev.Comp.Immunol.* 10:636.

SHOZAWA, A. (1989). Aggregation of phagocytes in the apple snail, *Pomacea canaliculata* (Gastropoda:Prosobranchiata). *Dev.Comp.Immunol.* 13:410.

SHOZAWA, A., SUTO, C. & KUMADA, N. (1987). Internal defence factors in the apple snail, *Pomacea canaliculata*. *Zool.Sci.* 4:1006-1008.

SIMPSON, A.J.G. & SMITHERS, S.R. (1980). Characterization of the exposed carbohydrates on the surface membranes of adult *Schistosoma mansoni* by analysis of lectin binding. *Parasitology* 81:1-15.

SIMPSON, A.J.G., CORREA-OLIVERA, R., SMITHERS, S.R. & SHER, A. (1983). The exposed carbohydrates of schistosomula of *Schistosoma mansoni* and their modification during maturation *in vivo*. *Mol.Biochem.Parasitol.* 8:191-196.

SMINIA, T.(1972). Structure and function of blood and connective tissue cells of the freshwater pulmonate *Lymnaea stagnalis* studied by electron microscopy and enzyme histochemistry. *Cell Tissue Res.* 130:497-526.

SMINIA, T. (1980). Phagocytic cells in Mollusca. In *"Aspects of Developmental and Comparative Immunology.I."* (J.B.Solomon, ed),pp.125- 132. Pergamon, Oxford/New York.

SMINIA, T. (1981). Gastropods. In *"Invertebrate Blood Cells I".* chap.7 p.191-232. N.A.Ratcliffe & A.F.Rowley eds., Academic Press, London.

SMINIA, T. & BOER, H.H. (1973). Haemocyanin production in pore cells of the freshwater snail *Lymnaea stagnalis*. *Z.Zellforsch.* 145:443-445.

SMINIA, T. & BARENDSEN, L. (1980). A comparative morphological and enzyme histochemical study on blood cells of the freshwater snails *Lymnaea stagnalis, Biomphalaria glabrata,* and *Bulinus truncatus*. *J.Morphol.* 165:31-39.

SMINIA, T. & Van der KNAAP, W.P.W. (1986). Immunorecognition in Invertebrates with Special Reference to Molluscs. In *"Immunity in Invertebrates,"* M.Brehelin (ed.) p.112. Springer-Verlag, Berlin/Heidelberg.

SMINIA, T., PIETERSMA, K. & SCHEERBOOM, J.E.M. (1973). Histological and ultrastructural observations on wound healing in the freshwater pulmonate *Lymnaea stagnalis*. *Cell Tiss.Res.* 141:561-573.

SMINIA, T., Van der KNAAP, W.P.W. & EDLENBOSCH, P. (1979). The role of serum factors in phagocytosis of foreign particles by blood cells of the freshwater snail *Lymnaea stagnalis. Dev.Comp.Immunol.* 3:37-44.

SMINIA, T., Van der KNAAP, W.P.W. & KROESE, F.G.M. (1979). Fixed phagocytes in the freshwater snail *Lymnaea stagnalis. Cell Tiss.Res.* 196:545-548.

SMINIA, T., Van der KNAAP, W.P.W. & BOERRIGTER-BARENDSEN, L.H. (1982). Peroxidase-positive blood cells in snails. *J.ReticuloendothelSoc.* 31:399-404.

SMINIA, T., Van der KNAAP, W.P.W. & VAN ASSELT, L.A. (1983). Blood cell types and blood cell formation in gastropod molluscs. *Dev.Comp.Immunol.* 7:665-676.

SMITH, A.C. (1981). Sex-specific agglutination of fish red blood cells by sea urchin coelomic fluid. *Dev.Comp.Immunol.* 2:357-360.

SMITH, A.C. (1984). Sex-specific agglutination of human erythrocytes by body fluids from marine invertebrates and fishes: a preliminary study. In *"Recognition Proteins, Receptors and Probes: Invertebrates."* E.Cohen (ed.) pp.97-102. Alan R.Liss Inc., New York.

SMYTH, J.D. & HALTON, D.W. (1983). In *"The Physiology of Trematodes"* (2nd.ed.) p.1-446. Cambridge University Press, London.

SOUTHGATE, V.R. & KNOWLES, R.J. (1977). On *Schistosoma margrebowiei* Le Roux, 1933: the morphology of the egg, miracidium and cercaria, the compatibility with species of *Bulinus*, and development in *Mesocricetus auratus*. *Z.Parasitenknd.* 54:233-250.

SOUTHGATE, V.R. & ROLLINSON, D. (1987). Natural history of transmission and schistosome interactions. In *"The Biology of Schistosomes; From Genes to Latrines"*, (D.Rollinson & A.J.G.Simpson eds.), pp.347-378. Academic Press, London.

SPLITTER, G.A. & EVERLITH, K.M. (1982). Suppression of bovine T- and B-lymphocyte responses by feutin, a bovine glycoprotein. *Cell Immunol.* 70:205-209.

STEBBINS, M.R. & HAPNER, K.D. (1985). Preparation and properties of haemagglutinin from haemolymph of Acrididae (grasshoppers). *Insect Biochem.* 15:451-462.

STEIN, P.C. & BASCH, P.F. (1979). Purification and binding properties of a haemagglutinin from *Biomphalaria glabrata. J.Invertebr.Pathol.* 33:10-18.

STEIN, E.A. & COOPER, E.L. (1982). Agglutinins as receptor molecules: a phylogenetic approach. In E.L.Cooper, M.A.B.Brazier (eds), *"Developmental Immunology : Clinical Problems and Aging,"* p.85, Academic Press, New York.

STIBBS, H.H., OWCZARZAK, A., BAYNE, C.J. & DeWAN, P. (1979). Schistosome sporocyst-killing amoebae isolated from *Biomphalaria glabrata. J.Invertebr.Pathol.* 33:159-170.

STONE, J.D. (1946). Lipid haemagglutinins. *Aust.J.Exp.Biol.Med.* 24:197-202.

STUART, A.E. (1968). The reticulo-endothelial apparatus of the lesser octopus, *Eledone cirrosa. J.Pathol.Bacteriol.* 96:401-412.

STUTMAN, O., DIEN, P., WILSUN, R.E. & LATTUNE, E.C. (1980). Natural cytotoxic cells against solid tumors in mice: blocking of cytotoxicity by D-mannose. *Proc.Natl.Acad.Sci.USA* 77:2895-2898.

STYNEN, D., VANSTEENWEGEN, K. & De LOOF, R. (1985). Anti-galactose lectins in the haemolymph of *Sarcophaga bullata* and three other calliphorid flies. *Comp.Biochem. and Physiol.* 81B:171-175.

SUZUKI, T. & NATORI, S. (1983). Identification of a protein having haemagglutinating activity in the haemolymph of the silkworm *Bombyx mori*. *J.Biochem*. 93:583-587.

SULLIVAN, J.T. & RICHARDS, C.S. (1981). *Schistosoma mansoni*, NIH-SM-PR-2 strain, in susceptible and non-susceptible stocks of *Biomphalaria glabrata:* comparative histology. *J.Parasitol*. 67:702-708.

SULLIVAN, J.T., CHENG, T.C. & HOWLAND, K.H. (1986). Responses of the pericardial wall of *Biomphalaria glabrata* (Mollusca) subjected to challenge. *J.Invertebr.Pathol*. 44:114-119.

SVEDBERG, T. (1933). Sedimentation constants, molecular weights and isoelectric points of the respiratory proteins. *J.Biol.Chem*. 103:311-325.

TAKAHASHI, T., MORI, K. & NOMURA, T. (1986). Occurrence and characterization of lysozyme in the marine bivalves. *Bull.Jpn.Soc.Sci.Fish*. 52:863-868.

TAKAHASHI, H., KOMANO, H., KAWAGUCHI, N., KITAMURAI, N., NAKANISHI, S. & NATORI, S. (1985). Cloning and sequencing of cDNA of *Sarcophaga peregrina* humoral lectin induced on injury of the body wall. *J.Biol.Chem*. 260:12228-12233.

TAYLOR, M.G., NELSON, G.S. & SMITH, M. (1973). Studies on heterologous immunity in schistosomiasis. *Bull.Wld.Hth.Org*. 49:65

TEICHBERG, V.I., SILMAN, I., BEITSCH, D.O. & RESHEFF, G. (1975). A β-D-galactoside binding protein from electric organ tissue of *Electrophorus electricus*. *Proc.Natl.Acad.Sci.USA* 72:1383-1387.

TOMASKA, L.D. & PARISH, C.R. (1981). Inhibition of secondary IgG responses by N-acetyl-D-glucosamine. *Eur.J.Immunol*. 11:181-188.

TRIPP, M.R. (1966). Haemagglutinin in the blood of the oyster *Crassostrea virginica*. *J.Invertebr.Pathol.* 8:478-484.

TSIVION, Y. & SHARON, N. (1981). Lipid-mediated haemagglutination. *Biochem.Biophys.Acta.* 642:336-339.

TYLER, A. (1946). Natural heteroagglutinins in the body fluids and seminal fluids of various invertebrates. *Biol.Bull.Woods Hole* . 90:213-219.

TYLER, A. & METZ, B. (1943). Natural heteroagglutinins in the serum of the spiny lobster, *Panilurus interruptus*. I. Taxonomic range of activity, electrophoretic and immunizing properties. *J.Exp.Zool.* 100:387-397.

TYSON, C.J., McKAY, D. & JENKIN, C.R. (1974). Recognition of foreigness in the freshwater crayfish, *Parachaeraps bicarinatus*. In E.Cooper (ed): *"Contemporary Topics in Immunobiology"*, Vol.4, p.159. Plenum Press, New York.

UHLENBRUCK, G. & PROKOP, O. (1966). An agglutinin from *Helix pomatia* which reacts with terminal N-acetyl-D-galactosamine. *Vox.Sang.* 11:519-526.

UHLENBRUCK, G., SOLTER, J. & JANSSEN, E. (1983). Neue Reaktionsmechanismen des C-Reaktiven Protein (CRP) und verwandter Protein. *J.Clin.Biochem.* 19:1201-1210.

UMETSU, K., KOSAKA, S. & SUZUKI, T. (1984). Purification and characterization of a lectin from the beetle *Allomyrina dichotoma*. *J.Biochem.* 95:239-245.

Van der KNAAP, W.P.W., MEULEMAN, E.A. & SMINIA, T. (1987). Alterations in the internal defence system of the pond snail *Lymnaea stagnalis* induced by infection with the schistosome *Trichobilharzia ocellata*. *Parasitol.Res.* 73:57-65.

Van der KNAAP, W.P.W., LOKER, E.S. & MEULEMAN, E.A. (1990). Immune mechanisms in trematode-snail interactions. *Para.Today* 6:175-182.

Van der KNAAP, W.P.W., BOERRIGTER-BARENDSEN, L.H., Van der HOEVEN, D.S.P. & SMINIA, T. (1981). Immunocytochemical demonstration of a humoral defence factor in blood cells (amoebocytes) of the pond snail, *Lymnaea stagnalis. Cell Tiss.Res.* 219:291-296.

Van DER KNAAP, W.P.W., SMINIA, T., KROESE, F.G.M. & DIKKEBOOM, R. (1981). Elimination of bacteria from the circulation of the pond snail *Lymnaea stagnalis*. *Dev.Comp.Immunol.* 5:21-32.

Van DER KNAAP, W.P.W., DODERER, A., BOERRIGTER-BARENDSEN, L.H. & SMINIA, T. (1982). Some properties of an agglutinin in the haemolymph of the pond snail *Lymnaea stagnalis*. *Biol.Bull.* 162:404-412.

Van der KNAAP, W.P.W., TENSEN, C.P., KROESE, F.G.M. & BORREIGTER-BARENDSEN, L.H. (1982). Adaptive defense reactions against bacteria in the pond snail *Lymnaea stagnalis. Dev.Comp.Immunol.* 6:775-780.

Van der KNAAP, W.P.W., SMINIA, T., SCHUTTE, R. & BORRIGTER-BARENDSEN, L.H. (1983). Cytophilic receptors for foreignness and some factors which influence phagocytosis by invertebrate leucocytes: *in vitro* phagocytosis by amoebocytes of the snail *Lymnaea stagnalis. Immunology* 48:377-382.

VASTA, G.R. & COHEN, E. (1982). The specificity of *Centruroides sculpturatus* Ewing (Arizona lethal scorpion) agglutinins. *Dev.Comp.Immunol.* 6:219.-230.

VASTA, G.R. & COHEN, E. (1983). Naturally occuring haemagglutinins in the haemolymph of the scorpion *Paruroctonus mesaensis* Stahnke. *Experementia* 39:721-722.

VASTA, G.R. & COHEN, E. (1984a). Sialic acid-binding lectins in the "whip scorpion" *Mastigoproctus giganteus* serum. *J.Invert.Pathol.* 43:226-233.

VASTA, G.R. & COHEN, E. (1984b). Characterization of the carbohydrate specificity of serum lectins from the scorpion *Hadrurus arizonensis* Stahnke. *Comp.Biochem.Physiol.* 77B:721-727.

VASTA, G.R. & COHEN, E. (1984c). Serum lectins from the scorpion *Vaejovis spinigerus* Wood bind sialic acids. *Experientia* 40:601-606.

VASTA, G.R. & MARCHALONIS, J.J. (1984). Distribution, specificity and macromolecular properties of tunicate plasma lectins. In *"Recognition Proteins, Receptors: Invertebrates"* E.Cohen (ed.) p125-141. Alan R.Liss Inc., New York.

VASTA, G.R., WARR, G.W. & MARCHALONIS, J.J. (1982). Tunicate lectins: distribution and specificity. *Comp.Biochem.Physiol.* 73:887-900.

VASTA, G.R., WARR, G.W. & MARCHALONIS, J.J. (1983). Serological characterization of humoral lectins from the freshwater prawn *Macrobrachium rosenbergii*. *Dev.Comp.Immunol.* 7:13-19.

VASTA, G.R., SULLIVAN, J.T., CHENG, T.C., MARCHALONIS, J.J. & WARR, G.W. (1982). A cell membrane-associated lectin of the oyster hemocyte. *J.Invertbr.Pathol.* 40:367-378.

WARR, G.W., VASTA, G.R., LEDFORD, B.E. & MARCHALONIS, J.J. (1983). Evidence for the existence of a diverse family of immunoglobulin-related recognition molecules. *J.Immunogenet.* 10:17-22.

WATSON, J.M. & AZIM, M.A. (1949). Comparative efficiency of various methods of infecting mice with *Schistosoma mansoni*. *Ann.Trop.Med.Hyg.* 19:292-298.

WEBBE, G. (1962). Population studies of intermediate hosts in relation to transmission of Bilharziasis in East Africa. In *"Ciba Foundation Symposium on Bilharziasis"*. G.E.Wolstenhome & M.O'Connor (Eds.) p.7-22. Churchill, London.

WEBBE, G. (1965). Transmission of bilharziasis. 1. Some essential aspects of snail population dynamics and their study. *Bull.Wld.Hlth.Org.* 33:147-148.

WEBBE, G. & MSANGI, A.S. (1958). Observations on three species of *Bulinus* on the East Coast of Africa. *Ann.Trop.Med.Parasitol.* 52:302-314.

WEINHEIMER, P.F. (1971). Ph.D thesis. University of Alabama.

WEIR, D.M. (1980). Surface carbohydrates and lectins in cellular recognition. *Immunol.Today* 1:45-49.

WEIR, D. & OGMUNDSDOTTIR, H.M. (1977). Non-specific recognition mechanisms by mononuclear phagocytes. *Clin.Exp.Immunol.* 30:323-330.

WILLIAMS, A.F. (1987). A year in the life of the immunoglobulin superfamily. *Immunol.Today* 8:293-303.

WILLIAMS, A.F., TSE,A.G.D. & GAGNON, J. (1988). Squid glycoproteins with structural similarities to Thy-1 and Ly-6 antigens. *Immunogen.* 27:265-272.

WISHKOVSKY, A. (1988). Chemiluminescence: an advanced tool for measuring phagocytosis. American fisheries society special publication 18, p.292-297. In *"Disease processes in marine bivalve molluscs"*, W.S.Fisher (ed.)

WOLMARANS, C.T. & YSSEL, E. (1988). *Biomphalaria glabrata*: influence of selected abiotic factors on leucocytosis. *J.Invertebr.Pathol.* 51:10-14.

WRIGHT, C.A. (1971). Physiological host-parasite relationships and pathology.In *"Flukes and Snails"*, p.110-134. George Allen & Union, London.

WRIGHT, C.A., SOUTHGATE, V.R. & HOWARD, G.W. (1979). Observations on the life-cycle of *Schistosoma margrebowiei* in Zambia. *J.Nat.Hist.* 13:499-506.

WRIGHT, R.K. & COOPER, E.L. (1981). Agglutinin producing hemocytes in the ascidian *Styela clava*. *Am.Zool.* 21:974-981.

YSSEL, E. & WOLMARANS, C.T. (1989). Factors influencing the leucocyte concentration of the freshwater snail *Bulinus africanus*. *J.Invertebr.Pathol.* 53:269-271.

YOKOSAWA, H., SAWADA, H., ABE, Y., NUMAKUNAI, T. & ISHII, S. (1982). Galactose-specific lectin in the haemolymph of solitary ascidian, *Halocynthia roretzi* : Isolation and characterization. *Biochem.Biophys.Res.Comm.* 107:451-452.

YOSHINO, T.P. (1981). Comparison of Concanavalin A-reactive determinants on haemocytes of two *Biomphalaria glabrata* stocks: receptor binding and redistribution. *Dev.Comp.Immunol.* 5:229-240.

YOSHINO, T.P. (1983). Lectins and antibodies as molecular probes of molluscan haemocyte surface membranes. *Dev.Comp.Immunol.* 7:641-644.

YOSHINO, T.P. (1988). Phospholipase C-like activity in phagocytic cells of the asian clam, *Corbicula fluminea*, and its possible role in cell-mediated cytolytic reactions. *J.Invertebr.Pathol.* 51:32-40.

YOSHINO, T.P. & BAYNE, C.J. (1983). Mimicry of snail host antigens by miracidia and primary sporocysts of *Schistosoma mansoni*. *Parasite Immunol.* 5:317-328.

YOSHINO, T.P. & GRANATH, W.O. Jnr. (1983). Identification of antigenically distinct hemocyte subpopulations in *Biomphalaria glabrata* (Gastropoda) using monoclonal antibodies to surface membrane markers. *Cell Tissue Res.* 232:553-561.

YOSHINO, T.P. & GRANATH, W.O. Jnr. (1985). Surface antigens of *Biomphalaria glabrata* (Gastropoda) hemocytes: functional heterogeneity in cell subpopulations recognised by a monoclonal antibody. *J.Invertebr.Pathol.* 45:174-186.

YOSHINO, T.P. & BAYNE, C.J. (1986). Surface membrane components of circulating invertebrate blood cells and their role in internal defence. In "*Immunity in Invertebrates*" (M.Brehlin, ed.), pp.12-24. Springer-Verlag, Berlin/Heidelberg.

YOSHINO, T.P. & BOSWELL, C.A. (1986). Antigen sharing between larval trematodes and their snail hosts: how real a phenomenon in immune evasion? *Zool.Soc.London* 56:18-36.

YOSHINO, T.P., CHENG, T.C. & RENWRANTZ, L.R. (1977). Lectin and human blood group determinants of *Schistosoma mansoni:* Alteration following *in vitro* transformation of miracidium to mother sporocysts. *J.Parasitol.* 63:818-824.

ZELCK, U. & BECKER, W. (1990). Lectin binding to cells of *Schistosoma mansoni* sporocysts and surrounding *Biomphalaria glabrata* tissue. *J.Invertebr.Pathol.* 55:93-99.

FLOWER, R.L.P., WILCOX, G.E. & PASS, D.A. (1985). Detection of two lectins in haemolymph from the oyster *Pinctada maxima*. *Aust.J.Exp.Biol.Med.Sci.* 63:703-707.

GILBERTSON, D.E. & ETGES, F.J. (1967). Haemagglutinins in the haemolymph of planorbid snails. *Am.Trop.Med.Parasitol.* 61:144-153.

OTTAVIANI, E. & TARUGI, P. (1986). Isolation and characterization of an agglutinin present in the haemolymph of the freshwater snail *Planorbarius corneus* (L.) *Comp.Biochem.Physiol.* 84B:295-297.

PEMBERTON, R.T. (1974). Anti-A and Anti-B of gastropod origin. *Ann.NY.Acad.Sci.* 234:95-121

WAGNER, M. (1982). Agglutination of bacteria by a sialic acid-specific lectin of the snail *Cepaea hortensis*. *Acta histochem.* 71:35-39.