RECOGNITION OF FOREIGN PARTICLES BY THE PROTOCHORDATE Botrylloides leachii.

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"If mammals are the "Rolls-Royce or Mercedes-Benz" model of elaborate immune responsiveness, the "jeep" and "bicycle" models are now discernible among invertebrates."

Hildemann, 1974.
(in Uhlenbruck, 1974).

ABSTRACT

This thesis is concerned with the mechanisms by which haemocytes from the ascidian Botrylloides leachii recognise foreign particles. The problem was addressed by investigating the functions of two haemagglutinins (HA-1 & HA-2) and a third non-agglutinating protein (LBP-3) all of which occur in B. leachii haemolymph.

The possibility that other species of ascidian may also possess molecules similar to the B. leachii agglutinins was examined using haemolymph from 22 different species belonging to the major families of ascidians. The divalent cation requirements and the agglutinating specificities (sugar inhibition) of samples with a high titre were determined. Column chromatography indicated that haemolymph samples from 4 different ascidians had molecules with similar size and specificity to the HA-1. One of these, a sample from Botryllus sp., also contained an "HA-2 like" molecule. The Botryllus sp. agglutinins were affinity purified and compared to the HA-1 & HA-2 agglutinins. The Botryllus sp. "HA-1 like" molecule was slightly smaller than the B. leachii HA-1. These two molecules exhibited identical sugar binding specificities and divalent cation requirements, but no antigenic cross-reactivity was observed. A Preliminary SDS-PAGE analysis suggested that the Botryllus sp. agglutinin was structurally quite distinct from the HA-1 agglutinin and LBP-3. The B. leachii HA-2 molecule and the Botryllus sp. sheep erythrocyte agglutinin were antigenically cross-reactive and identical in size and subunit structure, but they differed slightly in binding site specificity.

The possibility that the B. leachii agglutinins were opsonins was investigated both in vivo and in vitro. It was found that injecting colonies with erythrocytes bearing HA-2 receptors produced a small but significant increase in the HA-2 agglutinating titre of haemolymph. Moreover, this increase was specific. There was no indication of an anamnestic response. The HA-1 titre of haemolymph appeared to some extent to be dependent on environmental stimuli since colonies relocated to a new environment had titres significantly different from those of colonies remaining at the original location. The effect of environmental stimuli was not considered to be the only explanation; the possibility that genetic differences between colonies may also affect the level of the HA-1 agglutinin in haemolymph is noted.

A medium suitable for the in vitro culture of B. leachii haemocytes was developed. B. leachii haemocytes were found to bind and phagocytose untreated sheep erythrocytes. Binding was mediated by the HA-2 agglutinin, as shown by the inhibition of adhesion by lactose (which is specific for the HA-2 agglutinin) and an anti-(HA-2) IgG preparation. Immunoflourescence studies indicated that HA-2 molecules were secreted by the haemocytes; these secreted molecules then coated unsensitised erythrocytes causing them to bind haemocytes. No HA-2 agglutinin could be detected on the surface of the haemocytes in the absence of erythrocytes but receptors for this molecule were detected. These results suggested that the HA-2 agglutinin could function as a recognition molecule for particles bearing the appropriate carbohydrate moieties on their surface. At least one of the other two related proteins (HA-1 and LBP-3) also existed on the surface of haemocytes. The function(s) of these molecules is not known.

The above findings are discussed in relation to the two major requirements of a defence system: efficient phagocytosis and the control of self reactivity.

The possibility that a molecule with an opsonic function in a protochordate may be structurally related to immunoglobulin was investigated by testing the ability of the HA-2 agglutinin to act as an opsonin for the recognition of particles by phagocytic cells from a vertebrate. B. leachii haemolymph was found to mediate the adhesion and subsequent phagocytosis of sheep erythrocytes by mouse macrophages; the factor responsible was shown to be the HA-2 agglutinin. The HA-1 molecule did not bind to macrophages. Both erythrocytes and macrophages could be sensitised for adhesion with the agglutinin and in both instances sensitisation could be reversed with lactose. Hence adhesion appeared to be caused by the agglutinin cross-linking similar lactose-like determinants on the surfaces of both types of cell. There was no evidence to suggest any structural homology with immunoglobulin. This result is discussed in view of information on the structure of the HA-2 molecule and current ideas on the evolution of immunoglobulin.

STATEMENT.

I state that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

Deirdre R. Coombe

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ABBREVIATIONS USED IN THIS THESIS.

BCCM	*Botrylloides* cell culture medium.
BSA	Bovine serum albumin.
BSS	*Botrylloides* salt solution.
EDTA	Ethylenediaminetetra-acetic acid di-sodium salt.
EGTA	Ethyleneglycol-bis (ß-amino-ethyl ether) N, N-tetra-acetic acid.
GpRBC	Guinea pig erythrocytes.
HA-1	Haemagglutinin One (from *B. leachii* haemolymph).
HA-2	Haemagglutinin Two (from *B. leachii* haemolymph).
HAU	Haemagglutinating Unit.
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
IgG	Immunoglobulin G.
LBP-3	Lactose Binding Protein Three (from *B. leachii* haemolymph).
mol. wt.	Molecular Weight.
O.D.	Optical Density.
PBS	Phosphate buffered saline.
Saline	0.154 M NaCl.
SDS	Sodium dodecylsulphate.
SDS-PAGE	SDS polyacrylamide gel electrophoresis.
SRBC	Sheep erythrocytes.
Tris	Tris(hydroxymethyl)amino-methane.
TSA	Tris buffered saline.

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Chapter 1.

INTRODUCTION

1.1 Introduction to the problem

The original incentive for this work was an interest in the life history of a colonial ascidian, Botrylloides leachii (Fig 1.1), found subtidally, encrusting pier pilings and rocky outcrops around the South Australian coast.

At certain times of the year large numbers of one or two zooid colonies are found over small areas of pier piling. As growth occurs select colonies within a cluster fuse to form a single larger individual. Other colonies form regions of intimate contact with conspecifics but at no time is there any union of test matrices (Fig 1.1). Contact with other species usually results in the complete overgrowth of that animal. Thus it appears that these animals are able to recognise both individuals of their own species and also to distinguish conspecifics from other animals.

This ability is not restricted to B. leachii as other encrusting species of colonial ascidians exhibit a similar phenomenon. Reports in the literature support the prevalence of this type of behaviour among encrusting colonial ascidians (Oka & Usui, 1944; Freeman, 1970; Mukai & Watanabe, 1974; Tanaka, 1975) although histological studies of fusion have been confined to the Botryllinae.

Histological studies of the fusion reaction in Botryllus primigenus Oka. suggested that recognition takes place, not at the matrix surface but via the terminal vessels (Katow & Watanabe, 1980). The ampullae, terminal swellings of blood vessels, penetrate the test matrix of the opposite colony and pair with the ampullae of that animal. The paired ampullae fuse and an exchange of haemolymph occurs. Fusion is complete when blood vessels in the contact area resemble those of the surrounding colony. A non-fusion reaction is

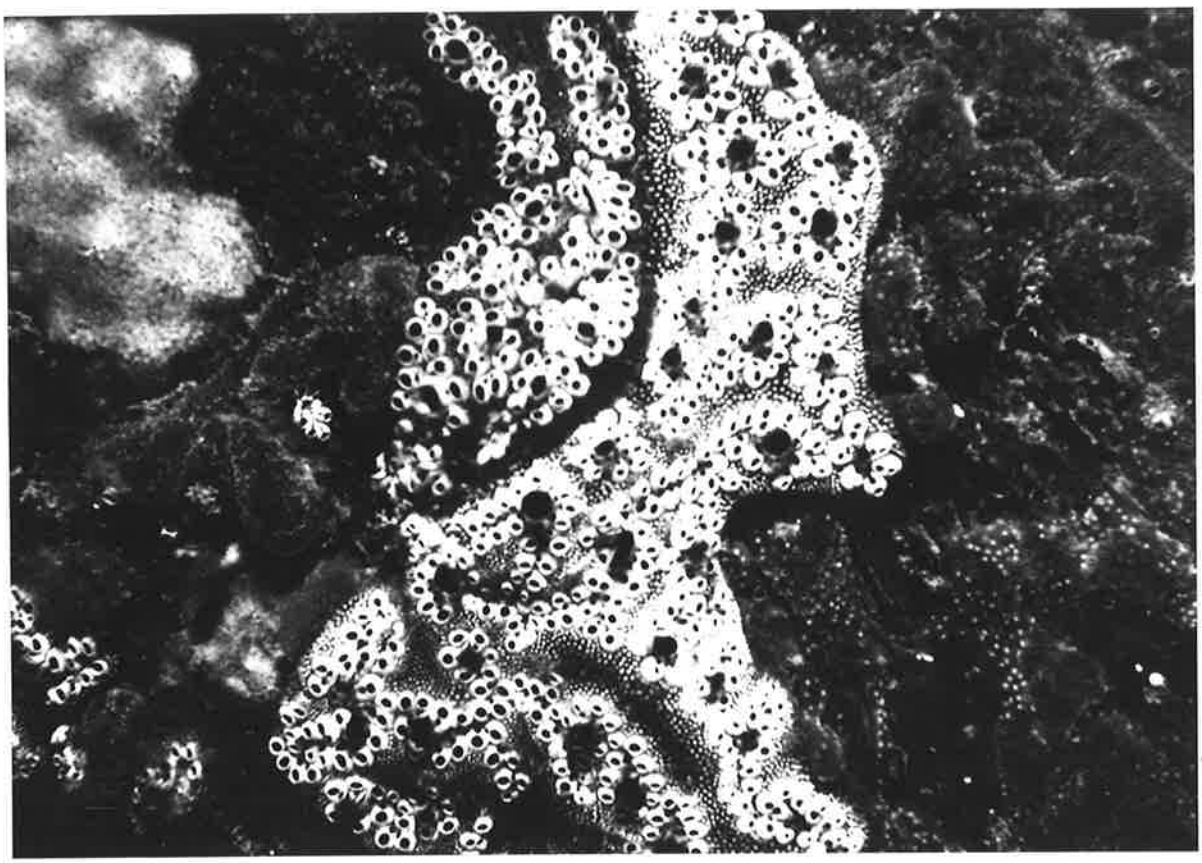


FIGURE 1.1 Botrylloides leachii colonies growing on a pier piling at Edithburgh. An interface is visible between the two colonies indicating that fusion has not occurred.

manifested by the degeneration of the test cells surrounding the penetrated ampullae. The ampullae contract such that haemolymph flow stops in the distal regions, thus separating the degenerating area from the rest of the colony.

A further feature of the life history of B. leachii is its ability to aestivate during unfavourable periods. In South Australia this occurs over the summer months. During aestivation the colony regresses. The breakdown in adult zooids is accompanied by an increase in the development of the circulatory system, so that the colony is reduced to a test matrix ramified with blood vessels. B. leachii colonies in the Venetian Lagoon (north west coast of Italy) behave similarly (Brunetti, 1976) and histological work with these animals has revealed that the degenerating zooids were phagocytosed by a vastly enlarged population of amoeboid cells (Burighel, et al., 1976).

B. leachii thus appeared to have the ability not only to recognise certain con-specifics but also to detect and eliminate vast numbers of degenerating self cells. This species therefore seemed to be an interesting organism in which to investigate the defence reactions of ascidians. The possibility that the fusion/non-fusion reaction of ascidians is a primitive version of certain elements of the vertebrate immune response, notably transplantation reactions and the killing of virus-infected cells by thymus-derived (T) lymphocytes, has been considered in theories proposed for the evolution of vertebrate immunity (Burnet, 1971, & 1974; Mäkelä et al., 1976; Lafferty & Woolnough, 1977). However, the notion that there may be molecules functioning as recognition factors within ascidians and that these factors may be genetically related to those of the vertebrate immune system has not been tested experimentally. Indeed very little is known about the ascidian immune system. Hence the aim of this work was to

investigate cell recognition in ascidians, concentrating on B. leachii, with a view to gaining a better understanding of both the defence response of this ascidian and the evolution of the vertebrate macrophage-antibody system.

1.2 Development of a theoretical framework

When looking at invertebrate defence systems it is necessary to define "immunity" in the broad sense. "Immunity", in invertebrates, does not necessarily require the involvement of specific memory with accelerated rejection of second set allografts and a marked increase in the concentration of a specific humoral component upon challenge as demonstrated by vertebrates. On the contrary an animal may be said to possess an "immune system" if it is capable of recognising and mounting a protective response, be it specific or otherwise, against foreign particles like viruses, bacteria or metazoan parasites.

A discussion of invertebrate defence mechanisms must consider both the diversity exhibited by this group of animals and their phylogenetic linkages. Although some present day invertebrates may utilise the protective mechanisms used by their ancestors, many have probably inherited systems which differ from the original through modifications which have been acquired over time according to the particular life history of each animal. Animals like the horse-shoe crab, Limulus, and the cockroach, Periplaneta, for example, are possibly separated by as much if not more evolutionary time than the fishes and mammals, and observations applicable to one will not necessarily apply to the other. In addition the possibility of convergent evolution should not be overlooked. Lectins, for example, have probably evolved many times during invertebrate evolution, making suggestions of homology in the absence of genetic data very tenuous.

Nevertheless, there does appear to be a certain conformity in the sorts of immune mechanisms utilised by different invertebrates, e.g. the uniform presence of phagocytic cells, thus allowing the construction of a theoretical framework around which these mechanisms can be discussed.

When the evolution of metazoans is considered it is clear that a method by which cells of one clone could either recognise all other cells as foreign or alternatively recognise cells of like genetic constitution as part of self must have been a prerequisite for the development of multicellular animals. Self/non-self recognition would seem to have been essential to prevent the formation of chimeras, to ensure cohesion and collaboration between cells and to prevent invasion by pathogens. Such recognition systems would also need to have the capacity to adapt to changing cell structures as the differentiation of specific cell types occurred.

The simplest mechanism for self/non-self discrimination is one where "self" is specifically recognised. Self recognition need not require an array of molecules directed at a variety of non-self determinants; a complementary pattern of effectors and receptors that function across the cell interface is sufficient. A defence process would be activated by the failure of one cell to recognise a self determinant because either the surface of the self cell has been altered by age or infection, or the cell (particle) is foreign.

The basic function of any immune system can be considered to be the removal of infectious agents and debris, since cells specialised for phagocytosis occur universally in multicellular animals. In order to function effectively, the phagocytes must be able to distinguish unwanted matter from living host tissue. In a primitive invertebrate with no coelom or circulatory system, recognition must occur via the cell surface. Self tolerance may be effected by the phagocyte

receiving a "don't kill" signal upon contact with a self-cell while the attachment of other particles to the phagocyte induced phagocytosis non-specifically. Only with the evolution of a coelom and a circulatory system would the development of molecules which could function as opsonins in a manner analagous to antibody, i.e. coating particular particles so that they are efficiently phagocytosed, be of advantage to the invertebrate. To have been selectively advantageous, the early opsonins presumably must have recognised a wide range of potential pathogens; they were probably directed against determinants found commonly on cell surfaces. If, as seems likely, these determinants also occurred on the surface of self cells, the opsonins would coat self cells and self-tolerance would have to be maintained through the operation of a two signal recognition system. The phagocyte could be stimulated to ingest any particle to which it became linked via opsonins (signal 1) unless it simultaneously received a "don't kill" signal (signal 2) through the occupancy of its self recognition receptors. It is conceivable that an increase in opsonin specificity could reduce the problem of self-reactivity and increase the strength of the bond between the phagocyte and the unwanted particle. However this would reduce the variety of foreign particles which could be recognised by the animal unless an increased number of different opsonins was produced. Clearly the advantages of specificity must to some extent out weigh the cost of this increased metabolic load for such a system to be selected.

1.3 Evidence pertaining to the model

I would like now to examine the literature pertaining to invertebrate immunity using the presented theory as a basis for discussion.

1.3.1 The recognition of Self

A classic example of cell recognition, though not necessarily relevant to immunity, is provided by sponge cell re-aggregation. If a sponge is dissociated into individual cells, given the appropriate conditions the cells will re-aggregate to form a functioning sponge. It is possible that mixtures of sponge cells from different species re-aggregate in a species-specific manner i.e. recognition of self from non-self may occur. McClay (1971) studied the interaction of radioactively labelled cells from five different sponge species. He found that although non-specific adhesions occurred early in the aggregation process, these were neither as strong nor as stable as those between cells of the same species. Consequently over an extended period of time all heterospecific aggregates were eliminated leaving a functioning sponge constructed of homo-specific cells. Within the freshwater sponge, Ephydata fluviatilis, adhesion between cells from different individuals is strain specific. Compatibility apparently exists within a strain but not across strains (Van de Vyver, 1975).

The nature of sponge cell re-aggregation indicates that the union of like cells may result from the interaction of "self receptors" on the surface of the sponge cells. A variety of studies have indicated that re-aggregation is facilitated by molecules, called "aggregation factors", which are released into the culture medium upon dissociation of the sponge. The aggregation factors examined were either proteins (Müller & Zahn, 1973) or glycoproteins (Henkart et al., 1973; Van de Vyver, 1975). They apparently function by cross-linking particular carbohydrate receptors on sponge cell surfaces (Kuhns et al., 1974; Müller & Müller, 1980). The specificity of binding is such that, with a few exceptions (MacLennon & Dodd, 1967), only cell surface receptors from the species or strain from which the aggregation

factor is derived are recognised (Humphreys, 1963; Moscona, 1963; McClay, 1974; Van de Vyver, 1975). In those species where aggregation factors have been shown to be cross-reactive, it is possible that they may be recognising a carbohydrate on the foreign cell which mimics the aggregation receptor on "self cells". The degree of cross-reactivity may be an indicator of phylogenetic relatedness, since some aggregation molecules and receptors could possibly have arisen by divergence from an original molecule and receptor system. This will remain as speculation until the binding sites and exact chemical structure of both receptors and aggregation factors are well characterised.

Grafting experiments have also indicated that cell recognition occurs in sponges; Callyspongia diffusa for example, was consistently found to reject allografts but not isografts (Hildemann et al., 1979; Hildemann et al., 1980; Bigger et al., 1981). Experiments with a species of Hymeniacidon have shown a close correlation between graft rejection and incompatible cell aggregation (Curtis, 1979), but whether the same mechanism is involved in both processes is not known.

Two experimental systems involving grafting (or fusion) that present evidence to support the recognition of self are Theodor's work with gorgonians and the genetic analysis of fusion/non-fusion compatibility of Botryllus colonies.

Theodor (1976) carried out an extensive series of grafts with explants of gorgonians and concluded that these animals reject all xenografts and most allografts (0.7% of allografts fused) but accept autografts, which consistently fused. The interaction of two antagonistic explants (killer & target) resulted in the complete lysis of one explant (target). Experiments using metabolic inhibitors (Theodor, 1970) and the uptake of C^{14}-labelled amino acids (Theodor & Senelar, 1975) have indicated that lysis is an active process and

requires a metabolically active target. If too many target cells are destroyed (greater than 75%), or metabolism is inhibited by actinomycin-D the target will not lyse. Rejection was believed to have involved the transfer of a lethal factor from the killer cells to the target explant: some target cells are killed and finally autolysis of the whole target explant occurs. As the initiation of killing was extremely rapid, a 20% decrease in the incorporation of C^{14}-labelled amino acids (the assay used to indicate cytotoxicity) in the target explant being recorded after only 5 minutes of contact with the killer explant (Theodor & Senelar, 1975), the lethal factor was presumed to have been preformed and contained in all cells. In the model presented (Acton, 1974; review: Burnet, 1976), the active site of the lethal factor was conceived to be non-specific in that both self and non-self cells could be killed. Self cells were presumed to contain an inhibitor which specifically inactivates only the lethal factor within self cells. Thus, it was speculated that if the lethal factor passed to a foreign incompatible explant (from the killer to the target) then this lethal factor would not be recognised by the inhibitor within the foreign explant and cell death, leading ultimately to autolysis of the incompatible explant, would occur. Lysis was found to require some living cells (Theodor & Carriere, 1975), hence it was suggested (Theodor & Senelar, 1975) that lysis may be the result of "release by the dead cells of substances inducing phagocytosis (?) and almost certainly a synthesising activity of other cells". The meaning of this statement was not clear.

Although the induction of lysis could be more complex than the model presented, the hypothesis that autolysis only occurred when the positive recognition of self was not possible is in accordance with the data presented. Hence one could postulate that cytoxicity caused a

change in cell membrane molecules, such that the activity of the phagocytes against self was no longer inhibited, and so led to autolysis.

From an analysis of the genetics of the fusion/non-fusion reaction in Botryllus primigenus it was concluded that the ability to fuse is controlled by a single genetic locus with multiple alleles (Oka & Watanabe, 1957; Oka, 1970). All colonies in the field are heterozygous with respect to this gene and can be represented as AB, CD etc.. Fusion occurs between colonies which appear to share at least one gene, e.g. AB will fuse with BC but CD is rejected. The results from recent studies with another Botryllus species are consistent with the idea that the recognition of the product of a "self" allele is necessary for colony fusion (Scofield et al., 1982a & b). The ability of one colony to fuse with another may be modified by a previous fusion. For example if BC has fused with CD previously then AB is now rejected and the severity of rejection was found to be dependent on the duration of the BC-CD fusion. These findings led Mukai (1967) and Tanaka (1973) to suggest that colony specific factors, either cellular or humoral, are contained in the haemolymph and the onset of the non-fusion reaction depends on the concentration of these factors.

As the animals examined are all encrusting colonial organisms, often found in habitats where competition for living space is fierce, the development of a self recognition system has possibly been favoured by selection as a means of maintaining individual integrity. Hence, it could be argued that we are observing the result of convergent evolution rather than evidence supporting the hypothesis that the recognition of "self" is common throughout the animal kingdom.

The fact that vertebrates also exhibit self recognition should not be ignored. Recent work has indicated that co-operative

interactions between cells such as the killing of virus-infected cells by thymus-derived (T) lymphocytes and T cell help in the activation of bursa-equivalent, bone marrow-derived (B) lymphocytes require the interacting cells to share at least one allele of the major histocompatibility complex (Zinkernagel & Doherty, 1975; Sprent, 1978). In fact Streilein (1979) has suggested that the recognition of self determinants appears to be essential to promote and regulate specific immune responses. It seems unlikely that an attribute that appears to be essential for the normal function of sponges, gorgonians, colonial tunicates and vertebrates should have arisen independently in each instance. A more probable hypothesis is that the ability to specifically recognise "self" has been retained throughout evolution although the molecules through which "self" is perceived may have changed.

1.3.2 The importance of phagocytosis in invertebrate defence.

Phagocytic or amoeboid cells are of great importance throughout the animal kingdom. In some animals, particularly primitive invertebrates, they have a major role in nutrition. In the sponges, for example, digestion is completely intracellular; wandering amoebocytes digest and transport food products throughout the animal. In other animals, such as corals and some flatworms, digestion is both intra- and extra-cellular. In corals, enzymes secreted into the gastrovascular cavity reduce the prey to a suspension of small food particles which are then engulfed by fixed phagocytes in the endoderm lining the cavity. In a similar manner, food fragments are engulfed by phagocytic cells in the lining of the gastrovascular cavity of turbellarians and digestion is completed intracellularly (Barnes, 1980). As invertebrates become more complex, extracellular digestion

assumes greater importance and the part played by amoebocytes in nutrition declines. In other invertebrates, phagocytes are important during differentiation. Metamorphosis in insects, for example, is accompanied by an increase in the number of circulating haemocytes. It appears that these cells are required to remove dead and auto-lysing tissue, for haemocytes in the blowfly (Caliphera) pupa have been observed packed with portions of disintegrated tissue (Wigglesworth, 1954).

Observations on the involvement of amoeboid cells in the atrophy of the larval organs of Synaptae led Metchnikoff to hypothesize that phagocytic cells may play a major role in the defence against disease (Metchnikoff, 1905). Accordingly, he introduced rose spines beneath the skin of bipinnaria larvae of a starfish and found that within a short time, the spines were surrounded by amoeboid cells. Similarly, spores from a parasitic fungus were rapidly surrounded by leukocytes when introduced into the body cavity of a Daphnia and in many cases large numbers of spores were ingested and destroyed. In another experiment, a culture of anthrax bacilli was injected into the larvae of the rhinoceros beetle (Orctes nasicornis) and the haemolymph examined the following day. The injected bacilli were found not in the plasma, but internalised by leucocytes. From experiments of this nature, Metchnikoff (1905) developed the idea that amoeboid cells, involved in intracellular digestion in many primitive invertebrates, had been retained throughout the evolution of more advanced animals as they were capable of digesting a variety of inert particles and micro-organisms and so contributed to the defence of the host.

Although Metchnikoff's ideas of the importance of phagocytic cells in the defence of the host against pathogens have long been recognised for vertebrates, the possibility that invertebrates may also

utilise such cells in host defense was largely ignored. However, in recent years Metchnikoff's early work with invertebrates has been extended and the role of phagocytes in invertebrate immunity has been re-examined.

The classical method of investigating the phagocytic ability of invertebrates has been to introduce a variety of particulate materials into a living animal and record either the rate at which various particles are removed from the circulation or, using histological methods, their anatomical distribution. The conclusion derived from an examination of a number of these studies is that amoeboid cells are utilised in the removal of foreign particles by representatives from a wide range of invertebrate phyla encompasing both coelomate and acoelomate animals (Table 1.1). Whether the material is phagocytosed or encapsulated by amoeboid cells appears to be dependent on the size of the particle. Salt (1970) in a discussion on phagocytosis in insects suggested that particles larger than 10 μm in diameter were not engulfed by insect cells but usually became either encapsulated or the object of nodule formation.

In acoelomate invertebrates, the defence response may well be confined to that provided by wandering amoebocytes. This mechanism has apparently proved quite efficient. In sponges, for example, wandering amoebocytes have been shown to engulf injected particles, then migrate through the mesoglea to the excurrent canals. In this manner, the mesoglea could be cleared of india ink within 24 hours of injection, although the clearance of erythrocytes and carmine was shown to take longer, 48 hours and 96 hours respectively (Cheng et al., 1968a & b).

In the coelomate animals, phagocytes may be concentrated and fixed within reticular tissues as well as circulating in the haemolymph, thus permitting a rapid uptake of foreign material. For

TABLE 1.1 The invertebrate phyla in which it has been recorded that amoeboid cells remove foreign material.

Animal Phylum	Particle or substance Injected	Response*		Reference
		Phag.	Encap.	
PORIFERA	India ink, carmine	+		Cheng et al., 1968a
	erythroctyes,	+		Cheng et al., 1968b
	Trematode redia, cercaria		+	Cheng et al., 1968b
ANNELIDA	India ink, carmine,	+		
	iron particles, erythrocytes	+		Cammeron, 1932.
	foreign spermatozoa	+		
SIPUNCULIDA	latex beads, bacteria	+		Dybas, 1981
MOLLUSCA	carmine	+		Reade & Reade, 1972
	India ink	+		Pauley & Krassner, 1972
	erythrocytes, yeast, bacteria	+		Tripp, 1961
	Thorium dioxide	+		Brown, 1967
ARTHROPODA CRUSTACEA	bacteria, carmine	+		Smith & Ratcliffe, 1980; Fontaine & Lightner, 1974
	bacteria	+		McKay & Jenkin, 1970a
INSECTA	latex beads	+	+	Lackie, 1976;
	iron saccharide	+		Brehélin & Hoffmann, 1980
	araldite implants		+	Schmit & Ratcliffe, 1978
	Bacillus thuringiensis,	+		Zachory et al., 1981
	India ink, carmine	+		
	erythrocytes, bacteria	+		Cammeron, 1934
ECHINODERMATA	Bovine serum albumin	+		Hilgard & Phillips, 1968
	Bovine gamma globulin	+		
	Sea urchin cells (into a sea star)	+		Reinisch & Bang, 1971
TUNICATA	carmine	+		Fulton, 1920
	glass fragments		+	Anderson, 1971
	trypan blue	+		
	thorium dioxide	+		Brown & Davies, 1971

* Phagocytosis or encapsulation.

example, when carbon was injected into the ventral sinus of the crayfish Cherax destructor (formerly Parachaeraps bicarinatus), cells lining the walls of small vessels within the digestive diverticula were seen to have taken up carbon as rapidly as 1 minute after injection and by 3 hours all of these cells were densely packed with material (Reade, 1968). This is not an isolated example, as a number of studies have indicated that within this group, fixed phagocytes commonly located in the gills, heart or hepatopancreas are of considerable importance in the removal of particulate material (Cornick & Stewart, 1968; McKay & Jenkin, 1970a; Fontaine & Lightner, 1974; Smith & Ratcliff, 1980).

Within the Mollusca, however, the relative importance of fixed and circulating phagocytic cells seems to vary with the organism studied. Reade (1968) reported that carbon was engulfed by cells of the small vessels in the digestive gland and Bayne (1974) accepted this conclusion as an explanation of the astonishingly rapid rate of clearance from the haemolymph and the accumulation of bacteria in the digestive gland that he observed. A more recent study concerned with the clearance of erythrocytes by the snail Helix pomatia has indicated that the rapid accumulation of erythrocytes in the digestive gland is due to the adhesion of these cells to the surface of cells lining the vascular sinuses, rather than internalisation. Erythrocytes accumulating in the kidney and foot muscle were similarly found only to adhere to the cells lining the sinuses of these tissues (Renwrantz et al., 1981). It was demonstrated that yeast cells are cleared from the circulation of H. pomatia by 90 minutes after injection, yet the yeast-laden phagocytes were not detected until much later, the number of phagocytes containing yeast cells reaching a maximum of 23 - 25% between 40 and 50 minutes after injection (Renwrantz et al., 1981). These results were interpreted to mean that adherent foreign cells are

phagocytosed some time later by circulating phagocytes. In the chiton Liolophura gaimardi fixed phagocytes abundant in highly vascularized connective tissue, particularly in the gills and foot, were reported to play a major role in the phagocytosis of particles; haemocytes were calculated to have ingested only 2% of the ingested dose of the bacterium Staphlococcus aureus (Crichton et al., 1973). In contrast, circulating phagocytes were exclusively responsible for the uptake of carbon particles introduced into the adductor muscle of the clam, Tridacna maxima (Reade & Reade, 1972).

Whatever the extent of involvement of circulating or fixed phagocytes, it is apparent that phagocytosis is an important component of the defence response of invertebrates. The effective action of phagocytic cells must, however, reside in their capacity to distinguish unwanted matter, such as aged cells or foreign material, from living host tissue. How in the absence of antibody is this accomplished?

1.3.3 Discrimination by phagocytic cells: The possibility of cell surface receptors

In vertebrates, foreign particles may interact either directly with the phagocytes (Rabinovitch, 1970; Weir & Ögmundsdöttir, 1980; Cabilly & Gabilly, 1981a & b) or through an opsonic mediator (Jenkin & Benacerraf, 1960; Jenkin & Rowley, 1961). Opsonins in vertebrates are primarily IgG and IgM antibodies (Rabinovitch, 1970) or the third component of complement (C3; Shurin & Stossel, 1978; Bar-Shavit et al., 1979; Newman et al., 1980). These molecules "coat" the foreign particle which then becomes bound to the surface of the phagocyte via C3 or Fc receptors. Although all available evidence indicates that invertebrates lack lymphocytes and that they cannot make antibody, it is nonetheless possible that recognition by invertebrate phagocytes may

be accomplished by analogous mechanisms, through the involvement of opsonins and/or cell surface receptors.

I would like to consider the discriminatory mechanism of invertebrate phagocytes by looking initially at the behaviour of amoebae. As free living phagocytes, amoebae would presumably require few, if any, of the restrictions on phagocytosis that are necessary for cells living within a multicellular organism. They do however exhibit considerable discriminatory ability. They do not, for example, ingest either their own or another amoeba's pseudopodia (Reynolds, 1924), hence indicating a rudimentary capacity for "self" recognition. They may also feed selectively and some species can detect quite small differences between prey items. For example, when Amoeba proteus were fed equal numbers of two flagellate species they preferred Chilomonas sp. over Monas sp. (Mast & Hahnert, 1935). Although little is known about the molecular basis of the selectivity it has been suggested that recognition involved the interaction of complementary molecules at the cell surface (review: Jenkin, 1976).

Invertebrate phagocytes have also been shown to ingest particles selectively. Coelomocytes from two species of sea urchin, Strongylocentrotus purpuratus and Strongylocentrotus franciscanus, were shown to actively phagocytose a number of gram-positive bacteria whereas gram-negative species were seldom ingested (Johnson, 1969). Similarly, haemocytes from the shore crab, Carcinus maenas, when cultured in vitro, were shown to ingest Moraxella sp., a gram negative bacterium, at a much higher rate than that found for two gram positive species. Sheep erythrocytes, on the other hand, were not ingested at all (Smith & Ratcliffe, 1978). Echinoid phagocytes were also unable to ingest normal sheep erythrocytes when cultured in vitro although erythrocytes treated with glutaraldehyde, tannin or IgM and complement were phagocytosed (Kaplan & Bertheussen, 1977).

Although not able to differentiate between cellular and humoral contributions to recognition, in vivo experiments similarly indicated that injected particles were selectively cleared from the haemolymph. The sea hare, Aplysia californica, was able to remove completely four different marine bacteria but was unable to clear completely a terrestrial bacterium (Pauley et al., 1971b). A study with the blue crab, Callinectes sapidus, indicated that the clearance rates for different viral particles were not correlated with the size of the particle, hence indicating that clearance was not simply the filtration of particles from the haemolymph. Further support for some selective capability was the finding that different particles became concentrated in different organs. Xenogeneic proteins and poliovirus particles were cleared to the central axis of the gills, where as bacteriophages were concentrated in the hepatopancreas (McCumber & Clem, 1977).

Cells from certain echinoid and ascidian species have been found capable of recognising quite subtle differences in cell surface configuration. Echinoid cells when cultured in vitro recognised allogeneic and xenogeneic cells as demonstrated by a cytotoxic reaction (Bertheussen, 1979). Of the allogeneic pairs tested 70% showed cytotoxity while the frequency of reactivity increased to 90% when cells from one species were mixed with cells from either of two other echinoid species. There was no evidence of cytoxicity within cultures of cells from one individual. Coelomic cells from a number of species of solitary ascidians were also found to exhibit a cellular reaction when mixed in vitro either in allogeneic or xenogeneic combinations (Fuke, 1980). The reaction was triggered by direct cellular contact and resulted in the lysis of both cells. Like the echinoid cells, not all allogeneic combinations of ascidian cells were cytotoxic. Both authors have attempted a discussion in terms of histocompatibility

theories. However, until breeding studies produce evidence of genetic relationship, conclusions regarding the histocompatibility loci are pure conjecture. Nevertheless, this does not imply that these animals are not capable of recognising self.

In contrast to the allogeneic recognition described above, experiments with earthworms have indicated that spermatozoa from allogeneic individuals are not phagocytosed when injected into the coelomic cavity. Mammalian spermatozoa and spermatozoa from other earthworms however, were ingested (Cammeron, 1932). A series of studies with sipunculids, (review: Cushing & Boraker, 1975) also found that animals failed to encapsulate homologous eggs, despite the fact that eggs do not normally occur in the male coelom, while eggs from a sea urchin and another species of sipunculid were encapsulated. The failure to recognise allogeneic eggs as foreign was used as a basis for further experiments in an investigation of the mechanism of recognition. They found that if eggs damaged by staining, heating or sonification were introduced into the coelom of male worms, then these eggs were rapidly encapsulated. Frozen eggs, despite appearing dead, were not encapsulated. Of further interest was the discovery that eggs and sperm shared at least two antigenic determinants. Whether recognition of these shared determinants inhibited encapsulation is not known, but it is clear that recognition depended on the normal configuration of the egg surface.

Two general conclusions can be drawn from the studies discussed. Firstly, ingestion is not the automatic consequence of contact between an invertebrate phagocyte and a foreign particle. Secondly, the selection of particles to be ingested appears to occur at the cell surface, probably through the adhesion of the particle to the phagocyte surface. It is possible to explain the selective processes

reported by invoking one or more of the following recognition mechanisms:

a) selection could be the result of adhesion through a relatively specific phagocyte surface receptor and a complementary molecule on the foreign particle,

b) foreign particles could adhere to phagocyte receptors possessing a range of carbohydrate specificities, or

c) the physical characteristics of certain particles (e.g. surface charge, or hydrophobicity) may cause them to adhere to the phagocyte.

Adhesion by any of these means may or may not involve a serum opsonin. All three are operative in the interactions of vertebrate cells with foreign particles (Jenkin & Bencerraf, 1960; Rabinovitch, 1970; van Oss & Gillman, 1972; Capo, et al., 1979; Weir & Ögmundsdöttir, 1980; Glass et al., 1981).

Experiments designed to investigate the nature of the interaction between invertebrate phagocytes and foreign particles are rare. Hilgard's group, working with in vitro cultures of coelomocytes from the sea urchin, Strongylocentrotus purpuratus, addressed the question of whether cell surface receptors were involved in the uptake of certain proteins. Uptake was assessed as the amount of cell-associated radio-labelled protein, hence with these data it is not possible to determine whether "uptake" was due to ingestion (pinocytosis or phagocytosis) or adherence. They attempted to inhibit the uptake of a C^{14}-labelled protein by other related, unlabelled proteins. The uptake of labelled bovine serum albumin (BSA-C^{14}) was inhibited by the addition of unlabelled BSA but not chicken serum albumin (CSA) or human serum albumin (HSA). Similarly, the uptake of CSA-C^{14} was inhibited by CSA but not HSA or BSA (Hilgard et al.,

1974). The uptake of HSA-C^{14} was somewhat different. If HSA-C^{14} was present at a concentration of 80 µg/ml then its uptake was not inhibited by 80 µg/ml of BSA or HSA. However if the concentration of HSA-C^{14} was raised to 320 µg/ml and 320 µg/ml of BSA or HSA was added then both proteins inhibited the uptake of HSA-C^{14} (Hilgard, 1970). These results were interpreted to mean that the same receptor was responsible for the uptake of BSA and HSA, although this receptor has greater affinity for BSA. This receptor was also said to be distinct from the CSA receptor. Further *in vitro* experiments (Hilgard, 1970) and an *in vivo* clearance study (Hilgard *et al.*, 1967) with BSA-C^{14} and labelled bovine gamma globulin (BGG-C^{14}) similarly showed that BGG-C^{14} uptake could be inhibited by BSA and *vice versa*. These results were again taken to indicate that receptors of different binding specificities were involved in the uptake of BGG and BSA.

Two *in vivo* clearance studies performed on other invertebrates gave similar results. The removal of both injected labelled BSA and HSA from the haemolymph of the chiton *Liophura gaimardi* was inhibited by the injection of a high dose of unlabelled HSA. However, the removal of *Poneroplax*-haemocyanin (haemocyanin from another chiton) was not affected by HSA (Critchton & Lafferty, 1975). These data, the authors said, "can only mean that distinct recognition units are involved in the elimination of these two proteins". Results from clearance experiments with the crayfish *Procambarus clarkii* were also interpreted to indicate that this animal had recognition molecules for at least three groups of foreign proteins: albumins, gamma globulins and haemocyanins (Sloan *et al.*, 1975).

Although the interpretation that specific molecular receptors were involved in the uptake of these proteins may be correct, there are alternative explanations. As charge or hydrophobicity of proteins is

known to influence their adhesion to surfaces (van Oss & Singer, 1966), it is quite possible that the patterns of uptake observed were due to differences in the physical properties of these molecules. The adherence of a very hydrophobic molecule, for example, may not be affected by the presence of another molecule which binds through electrostatic attractions and vice versa. As these studies give no data to indicate that the proteins were internalised, the possibility of adherence cannot be ignored. Similarly, studies like that performed by Scott (1971), where cockroach phagocytes were treated with a variety of chemicals in an effort to inhibit the binding of erythrocytes, cannot without considerably more detailed and rigorous experimentation differentiate between non-specific sticking and adhesion through a receptor, since alteration of the physical properties of the haemocyte surface could well have the same effect on erythrocyte adhesion as the removal of a receptor.

Although specific surface receptors may well be essential for some biological processes (e.g. sperm egg interaction in sea urchins: Frazier & Glaser (review), 1979; and the uptake of glycoproteins by liver cells: Ashwell & Morell, 1974), it is conceivable that selective phagocytosis of foreign particles may be accomplished non-specifically through the "recognition" of physical differences. If, on the other hand, binding occurs via receptors, the specificity of the latter may be the result of interactions with carbohydrate determinants. Haemocytes from some invertebrates do appear to possess sugar-specific cell surface receptors. A study of the cytotoxic response of haemocytes from the mollusc, Megathura crenulata, and the echinoderm, Pisaster gigantus, towards mammalian erythrocytes revealed that certain sugars could block killing. Since the blocking efficiency of the sugars examined varied with both the type of erythrocytes used as

targets and the derivation of the effector cells (i.e. whether from the echinoderm or the mollusc), it appeared that recognition could be through sugar-specific molecules on the surface of the invertebrate haemocytes (Decker et al., 1981). The best evidence for the uptake of particles via a sugar-specific cell surface receptor, in the absence of an opsonin, comes from a study by Renwrantz et al. (1981) with the snail Helix pomatia. The clearance of yeast cells was concluded to be independent of an opsonin, as repeated injections of yeast cells did not alter the rate at which a subsequent dose of cells was cleared. However, when yeast cells were injected in a concentrated solution (0.2 M) of either N-acetyl galactosamine or N-acetyl glucosamine, their clearance was signifcantly retarded ($P < 0.02$) whereas fucose, at the same concentration, had no effect. These data strongly suggest that a sugar-specific cell surface receptor was involved in the removal of yeast cells from the haemolymph.

It should be apparent from this discussion that phagocytes from a wide variety of invertebrates ingest foreign particles selectively and that recognition appears to occur via adhesion to the phagocytic cell. There is no evidence to date of any highly specific cell bound receptor, comparable to cytophilic antibody, on phagocytes from an invertebrate, although phagocytes from some invertebrates do seem to have sugar-specific (lectin-like) receptors. In one instance (the study with Helix pomatia, Renwrantz et al., 1981) such a receptor appeared to be involved in ingestion but whether this is of general occurrence is not known.

1.3.4 Recognition by phagocytic cells: The involvement of serum opsonins

It is readily apparent from studies done with vertebrates that opsonins greatly increase the efficiency of the phagocytic system (Jenkin & Benacerraf, 1960; Berken & Benacerraf, 1969). Although invertebrates do not produce antibodies, at least not in amounts detectable in the body fluids, it might nevertheless be advantageous for those animals possessing a circulatory system to make molecules which could fulfil a similar, if more limited, function as opsonins. Indeed, a number of investigations have indicated that opsonins do occur in the haemolymph of some invertebrates. Amoebocytes from the horseshoe crab Limulus polyphemus, for example, exhibit no significant bactericidal effect in the absence of serum, but in the presence of serum they kill Escherichia coli but not another bacterium, Micrococcus luteus (Pistole & Britko, 1978). Sheep erythrocytes sensitised with serum from the lobster, Homarus americanus, formed rosettes with a larger proportion of lobster haemocytes than that obtained with unsensitised erythrocytes (59% compared to 30%). The number of haemocytes containing phagocytosed sensitised erythrocytes was also double that obtained with unsensitised erythrocytes (2% and 1% respectively) (Paterson & Stewart, 1974). The susceptibility of chicken erythrocytes to ingestion by haemocytes from the sea hare was enhanced by pretreatment with serum (Pauley et al, 1971a). Similarly, phagocytosis of human erythrocytes by haemocytes from the octopus Eledone cirrosa occurred only after the erythrocytes had been exposed to Eledone serum (Stuart, 1968). A study with earthworm coelomocytes revealed a differential requirement for opsonins. Only cells categorized as neutrophils exhibited enhanced phagocytosis of yeast that had been exposed to coelomic fluid; the phagocytic acivity of all

other cells was not altered by the presence or absence of coelomic fluid (Stein & Cooper, 1981). Prowse & Tait (1969) working with Helix aspersa amoebocytes suggested that there may be two opsonins in the haemolymph of this animal, one that binds formalinised sheep erythrocytes and another for formalinised yeast cells. Phagocytosis was shown to occur in normal serum or in serum adsorbed with the heterologous particle, but no significant phagocytosis of either yeast or erythrocytes occurred in serum adsorbed with the homologous particle.

Although it has been widely accepted that haemolymph has opsonic activity, very little is known about the molecular nature of the opsonic factors or the interaction between the foreign particles, the opsonic factors and the phagocytic cells. Numerous studies have indicated the presence of naturally occurring agglutinins in the haemolymph of representatives from a variety of invertebrate phyla (Table 1.2) and as early as 1966 it was suggested that these molecules might function in recognition (Boyden, 1966). As agglutinins bind and cross-link various cells and bacteria, it is not unreasonable to expect that they could also cross-link foreign particles to the surface of phagocytes, thereby stimulating ingestion. However, the evidence that agglutinins have opsonic properties is, with a few exceptions, largely circumstantial.

One of the earliest attempts to correlate haemagglutinating activity with an opsonic function was performed by Tripp (1966) using the oyster, Crassostrea virginica. The in vitro phagocytosis of rabbit erythrocytes was enhanced if the erythrocytes were pretreated with coelomic fluid which contained agglutinins for these erythrocytes. Similarly, RS-005 bacteria were phagocytosed more rapidly by haemocytes from the clam Mercenaria mercenaria in the presence of clam haemolymph (Arimoto & Tripp, 1977). Phagocytosis in haemolymph adsorbed with

TABLE 1.2 Examples of invertebrates shown to possess haemagglutinins.

Species	Source of Agglutinin	Reference
PORIFERA		
Axinella sp.	extract	Gold *et al.*, 1974
COELENTERATA		
Leptogorgia virgulata	extract	Lesniak & Liu, 1982
ANNELIDA		
Glycera dibranchiata	coelomic fluid	Anderson, 1980
Lumbricus terrestris	coelomic fluid	Cooper *et al.*, 1974
MOLLUSCA		
Aplysia californica	haemolymph	Pauley *et al.*, 1971a
Helix pomatia	albumin gland	Hammarström, 1974; Ishiyama, *et al.*, 1974
Euhadra callizona amaliae	albumin gland	Ishiyama, *et al.*, 1974
Velesunio ambiguus	haemolymph	McKay *et al.*, 1969
ARTHROPODA		
Limulus polyphemus	haemolymph	Finstad *et al.*, 1974; Cohen *et al.*, 1974
Birgus latro	haemolymph	Cohen *et al.*, 1974
Parachaeraps bicarinatus	haemolymph	McKay *et al.*, 1969
Homarus americanus	haemolymph	Cornick & Stewart, 1973 Hall & Rowlands, 1974
Melanoplus sanguinipes	haemolymph	Jurenka *et al.*, 1982
ECHINODERMATA		
Anthocidaris crassipina	coelomic fluid	Ryoyama, 1974
Pseudocentrotus depressus	coelomic fluid	Ryoyama, 1974
Hemicentrotus pulcherrimus	coelomic fluid	Ryoyama, 1974
Asteria forbsi	haemolymph	Finstad *et al.*, 1972
PROTOCHORDATA		
Halocynthia pyriformis	haemolymph	Anderson & Good, 1975
Halocynthia hilgendorfi	haemolymph	Fuke & Sugai, 1972
Styela plicata	haemolymph	Fuke & Sugai, 1972

RS-005 was not significantly different from that in seawater. Two other species of bacteria, not agglutinated by clam haemolymph, were phagocytosed to the same degree regardless of the presence or absence of haemolymph. As RS-005 was agglutinated by the haemolymph, the authors suggested that the agglutinin was also the opsonin. It is possible, however, both in this and the previous study, that molecules other than the agglutinin became bound to the ingested particle and one of these could have an opsonic function.

Phagocytosis of yeast, bacteria and erythrocytes from the gastropod Otala lactea was studied by Anderson and Good (1976). The results obtained with sheep erythrocytes are the most interesting. Sheep erythrocytes treated with formalin were phagocytosed to a greater extent than fresh cells, although in neither case did haemolymph alter the uptake of the erythrocytes. Evidence obtained by others, they said, indicated that the haemolymph of this species did not contain any haemagglutinins; extracts from the albumin gland however, did. Accordingly, the effect of albumin gland extract on phagocytosis was investigated. Although both untreated and formalinised erythrocytes were agglutinated by the extract, only the phagocytosis of formalinised cells was enhanced in the presence of the agglutinin. It is difficult to see the role of an opsonin in the albumin gland for in vivo clearance unless the opsonin had been secreted by the gland and was present in the haemolymph in low doses, or on the surface of fixed and circulating haemocytes. As some formalinised erythrocytes were phagocytosed in the absence of the agglutinin, it is possible that the opsonin may indeed occur on the surface of the haemocytes.

Work with the freshwater gastropod Lymnaea stagnalis suggested that haemolymph factors which bound to both bacteria and erythrocytes may also be on the surface of amoebocytes (van der Knaap, et al.,

1981a). Rabbit antisera were prepared against E. coli, Staphlococcus saprophyticus and rabbit erythrocytes, all of which had been incubated with haemolymph from L. stagnalis and extensively washed before injection. Both the cell membranes and the cytoplasm of L. stagnalis amoebocytes stained with the three antisera while control amoebocytes, incubated with normal rabbit serum or with rabbit antisera directed against the unsensitised bacteria or erythrocytes, remained negative. However it is not clear whether the molecules which bound to these cells were opsonic. Although bacteria of both species could be cleared from the haemolymph, the rate of clearance was not increased if the cells had been first sensitised with serum (van der Knaap et al., 1981b). Apparently no attempt was made to reduce the concentration of the bacterial binding factors in the haemolymph prior to injecting the bacteria, and it is possible that unsensitised bacteria became opsonised with the factor upon entering the snail. If this was the case, then previous treatment with serum may not increase significantly the rate of clearance. If clearance had been examined in animals injected with a blockading dose of bacteria (e.g. Tyson & Jenkin, 1973), then the presence of a serum opsonin may have been detected. An earlier in vitro study with phagocytes from this species recorded serum opsonins for yeast and sheep erythrocytes (Sminia et al., 1979). However, it is not known whether these molecules are the factors that bind to bacteria or rabbit erythrocytes.

As was already indicated by the Otala lactea study (Anderson & Good, 1976), exposure of particles to serum agglutinins may not always enhance the rate of uptake of these particles. The rate of ingestion of rabbit erythrocytes by phagocytes from the ascidian Styela plicata was also not affected by coelomic fluid, even though the fluid contained an agglutinin for these erythrocytes (Fuke & Sugai, 1972).

Similarly, the haemolymph from Clitumnus extradentatus and Periplaneta americana contained sheep erythrocytes agglutinins, but the sensitisation of sheep erythrocytes with haemolymph from either of these two insects did not cause an increase in the number of erythrocytes ingested by their haemocytes (Rowley & Ratcliffe, 1980). In both instances, phagocytosis was quite high in the absence of haemolymph. It is impossible, without some information as to the specificity of both erythrocyte adhesion to the haemocytes and erythrocyte agglutination, to determine whether this is due to agglutinin associated with the haemocyte membrane or to another, unrelated recognition molecule. As a large number of agglutinins seem to be directed against sugars common on cell surfaces (review: Ey & Jenkin, 1982), it would not be surprising to find that haemocytes were coated with agglutinins. Nor would it be surprising if haemocytes were involved in the synthesis and secretion of agglutinins, as is suggested by studies with the lobster (Cornick & Stewart, 1973 & 1978) and particularly the cockroach (Amirante, 1976; Amirante & Mazzalai, 1978). In the latter study, fluorescein-labelled antibodies, directed against haemagglutinins from the cockroach, Leucophaea maderae, were used to label haemocytes cultured in vitro. Haemagglutinins were detected on the plasma membrane, in the cytoplasm and in the cytoplasmic vacuoles of two of the four classified cell types. After treatment with cycloheximide, an inhibitor of protein synthesis, the haemocytes no longer became labelled. However, labelling was restored if the treated haemocytes were incubated without cycloheximide for a further 24 hours, indicating that the haemagglutinins were being synthesised by at least some of the cells in the haemocyte culture (Amirante & Mazzalai,1978).

Such findings illustrate the need for careful interpretation of data. The finding that haemolymph is not required for phagocytosis does not necessarily mean that agglutinins are not involved, for agglutinin molecules present in the haemolymph may also be associated with cell surfaces and so act as receptors by which the phagocyte can bind to and ingest foreign particles. In addition, the possibility should be considered that haemolymph factors other than the agglutinins, proteolytic enzymes for example, could alter the surface of the foreign particle and so induce attachment and phagocytosis by non-specific means.

A study which refutes the suggestion of non-specific attachment of particles to haemocytes is one performed by Jenkin and colleagues with the crayfish Cherax destructor (formerly Parachaeraps bicarinatus). They showed that haemolymph, containing agglutinins, was necessary to obtain efficient phagocytosis of erythrocytes in vitro. The adsorption of haemolymph with sheep erythrocytes removed both the agglutinating and the opsonic activity for these cells. Nevertheless, the adsorbed haemolymph still agglutinated human erythrocytes and enhanced the phagocytosis of these cells (McKay & Jenkin, 1970b). Subsequent investigations indicated that the in vivo elimination of bacteria also depended on the presence of haemolymph factors (Tyson & Jenkin, 1973). They found that crayfish given a large primary dose of bacteria were effectively "blockaded", i.e., a second dose of bacteria, given soon after the crayfish had cleared the first dose, was eliminated much more slowly. In vertebrates blockaded in this manner, the rate of clearance can be restored if the second dose of bacteria is pretreated in vitro with specific antibody. Similar results were obtained with the crayfish; the blockade could be reversed by pretreating the second dose of bacteria with haemolymph. Bacteria

treated with haemolymph which had been adsorbed with this bacterial strain, were cleared at the same rate as untreated bacteria. It is noteworthy that if the primary dose of bacteria was treated with haemolymph before injection, the removal of haemolymph factors was minimised and a second dose of unsensitised bacteria was cleared at normal rates.

Additional experiments indicated that bacteria could be ingested and killed by the phagocytic cells in vitro, regardless of whether the bacteria had been pretreated with haemolymph (Tyson & Jenkin, 1974). However, haemocytes treated with trypsin could not ingest bacteria unless the bacteria or the haemocytes were incubated with haemolymph prior to experimentation. Trypsinised haemocytes, incubated in haemolymph adsorbed with the bacteria, were unable to ingest unopsonised bacteria. These results suggest that recognition factors for bacteria were both free in the haemolymph and bound to the phagocytic cell membrane. They also support the notion that the recognition factor(s) in the haemolymph and the cell-bound molecule(s) are identical. Concrete evidence that the recognition molecule is also an agglutinin has yet to be presented. Nevertheless, the most likely interpretation of the present data is that a specific recognition molecule(s) is involved in phagocytosis.

The only unambiguous evidence supporting the hypothesis that an agglutinin molecule can function as an opsonin in vivo is that presented by Renwrantz's group working with the snail Helix pomatia. They found that the rate of clearance of human type A and B erythrocytes could be increased if these cells were preincubated in haemolymph. Further evidence for the requirement of a serum opsonin in phagocytosis was provided by blockade experiments. Snails blockaded with unsensitised A erythrocytes cleared a second dose of

unsensitised A cells more slowly, but if the second dose of erythrocytes had been pretreated with haemolymph then the blockade was reversed (Renwrantz & Mohr, 1978). The clearance of radiolabelled human serum albumin could be similarly blockaded, and this suggested that serum opsonins were required for the removal of both A and B erythrocytes and serum albumin. Furthermore, it appeared that the same opsonin was involved in each case, since blockading snails with serum albumin lowered the clearance rate of a subsequent dose of A erythrocytes and similarly blockading with A erythrocytes reduced the clearance of B erythrocytes (Renwrantz et al., 1981).

A haemagglutinin detected in the haemolymph of H. pomatia using pronase-treated type A erythrocytes could be specifically inhibited by N-acetylgalactosamine and N-acetylglucosamine (Renwrantz, 1979). The possibility that the opsonic activity of haemolymph for type A-erythrocytes was due to this agglutinin was investigated by Harm & Renwrantz (1980). They reasoned that if both the clearance and the agglutination of erythrocytes could be inhibited by N-acetylglucosamine then this would support the suggestion that one molecule is responsible for both activities. Accordingly, snails were injected with erythrocytes suspended in either 0.2 M N-acetylglucosamine or 0.2 M fucose and the rates of clearance were compared to that of erythrocytes injected in saline. N-acetylglucosamine was found to significantly ($P < 0.005$) reduce the rate of clearance over that obtained with erythrocytes suspended in saline. The clearance of cells in a fucose solution was not inhibited. These data strongly support the suggestion that the haemolymph opsonin is also the agglutinin.

It had been previously demonstrated that a number of plant and animal lectins could mediate the attachment of human erythrocytes to H. pomatia haemocytes in vitro (Renwrantz & Cheng, 1977a & b).

Accordingly, the rate of clearance of erythrocytes that had been treated with one of a number of different agglutinins before injection into blockaded snails was measured. The results indicated that not all agglutinins were opsonic. Concanavalin A and a lectin from Axinella polypoides, despite being able to mediate the adherence of erythrocytes to the snail haemocytes in vitro, were unable to reverse the blockade (Renwrantz & Mohr, 1978; Harm & Renwrantz, 1980). In contrast, extracts of the albumin glands of H. pomatia and Cepaea nemoralis, (both of which exhibited agglutinating activity) reversed the blockade (Renwrantz & Mohr, 1978). The purified blood group A specific agglutinin from H. pomatia albumin gland extracts was also capable of reversing the blockade (Harm & Renwrantz, 1980). It is noteworthy that this agglutinin can be inhibited by N-acetylgalactosamine and N-acetylglucosamine (Uhlenbruck & Prokop, 1966; Prokop et al., 1968; Ishiyama & Uhlenbruck, 1972), the same sugars that inhibited the clearance of erythrocytes and yeast (reported earlier). It appears, therefore, that clearance of type A erythrocytes and yeast cells in this species occurs by adhesion to a specific cell surface receptor, which may be a cell-bound form of the agglutinin for type A erythrocytes.

1.4 Re-examination of the Model.

It is clear from the preceding discussion that phagocytosis is essential for the elimination of a variety of foreign particles from the tissues of invertebrates and, as in vertebrates, invertebrate phagocytes do not ingest particles indiscriminately. In fact, to function effectively within a multicellular organism a phagocytic cell must select only unwanted or foreign material for ingestion. These cells therefore require a capacity for self/non-self discrimination.

The nature of this discrimination is not known but it is possible to erect a model which accounts for both recognition by vertebrate cells through the products of the major histocompatibility complex (MHC) and the requirement of invertebrate phagocytes for self/non-self discrimination.

The simplest form of self/non-self discrimination would be a specific recognition of self. Evidence has been presented indicating that a number of invertebrates appear to actively recognise "self" (Theodor, 1976; Oka, 1970). Although these studies were not referring directly to phagocytic cells, it is nevertheless likely that phagocytes do recognise self. In fact, self-recognition has been proposed to be a general property of all cells and the possibility of co-operation between specialised cells in the same individual requires that self-recognition be an active process (Dausset, 1981). Others have postulated the recognition of self by phagocytic cells as an active process occurring via a set of histocompatibility markers (Hildemann, 1977; Langman, 1978), the self marker being designated H and its recognition structure on the phagocyte as anti-H (Langman, 1978). A phagocyte would be free to ingest any particle, to which it became attached, not carrying the self H marker, while the association of anti-H receptors on the phagocyte with complementary H molecules on all self cells would initiate a "don't kill" signal. If H is modified by antigen or aging such that recognition by anti-H is not possible then ingestion of self can occur, as, for example, during insect metamorphosis when degenerating tissue is phagocytosed (Wigglesworth, 1954). A protein-carbohydrate system similar to thatr observed in sponge cell recognition has been proposed as the molecular structure of the invertebrate self recognition unit (Rothenberg, 1978). As the invertebrate self recognition system(s) may represent the beginning of

the vertebrate MHC, it is particularly relevant that carbohydrate-protein interactions are viewed with an increasing importance within the murine H-2 system (Lengerova et al., 1977; Sia & Parish, 1981).

The selective ingestion of invertebrate phagocytes operates not only with respect to self/non-self but also between different non-self particles (see section 1.3.3). The selection of non-self particles in the absence of a humoral component could occur either via a cell surface receptor or through non-specific means, i.e. a physical attraction caused, for example, by hydrophobicity. Despite the fact that conclusive evidence for a cell surface receptor is restricted to the Helix pomatia study (Renwrantz et al,. 1981), it is probable that both mechanisms operate. As firm attachment of the foreign particle to the phagocyte is probably required for efficient ingestion, one can envisage that a receptor able to bind those particles/molecules that do not stick to the phagocyte non-specifically would be favoured by selection. The notion that primitive macrophages have the capacity to recognise non-self is not new, as Langman (1978), in a discussion on T cell killing, proposed that a non-self recognition structure (anti-X) evolved on the surface of phagocytic cells as a result of selection imposed by intracellular parasites. A primitive non-self receptor could well have a broad specificity directed against carbohydrate determinants found commonly on the surfaces of a variety of pathogens and, possibly, also on self cells. If a phagocyte did bind a self cell, then any "don't kill" signal generated by the union of H with anti-H would need to override the postulated phagocytosis signal generated by adhesion of anti-X to the surface.

The requirement by a number of invertebrates of serum opsonins for phagocytosis is a further example that, in some species,

recognition of foreignness is an active and specific process. It is postulated that in this case phagocytosis is induced by the binding of an opsonin-coated foreign particle to the phagocytic cell via an opsonin receptor.

There are a number of ways that self-reactivity (auto-immunity) could be avoided in animals with opsonins without postulating the necessity for a "don't kill" signal on recognition of self:-

(1) Self cells may be saturated with low affinity-binding opsonin, while opsonin binds non-self particles, through the same binding site, with high affinity. One could envisage that the attachment of the opsonised particles to the phagocytes is stabilised by multi-point binding. To ensure that all opsonins bind self with low affinity the production of opsonins binding self with high affinity would need to be suppressed. This is the mechanism that is beleived to operate in vertebrates to eliminate the sensitisation of self cells with antibody (Karush, 1976; Pike et al., 1982).

(2) Self cells other than the phagocytes may not bind opsonin, while phagocytes possess a receptor common to all opsonins. Multi-point binding of the opsonised particles to the phagocytes could again be envisaged as producing a stable attachment.

(3) All self cells may be unable to bind uncomplexed opsonin. The adhesion of the opsonin to foreign particles could generate or expose another opsonin binding site for a receptor on the phagocytes.

Whether one or more of these mechanism operate within the invertebrates is open to question. To date no invertebrate opsonins have been shown to undergo a conformational change upon attachment to a

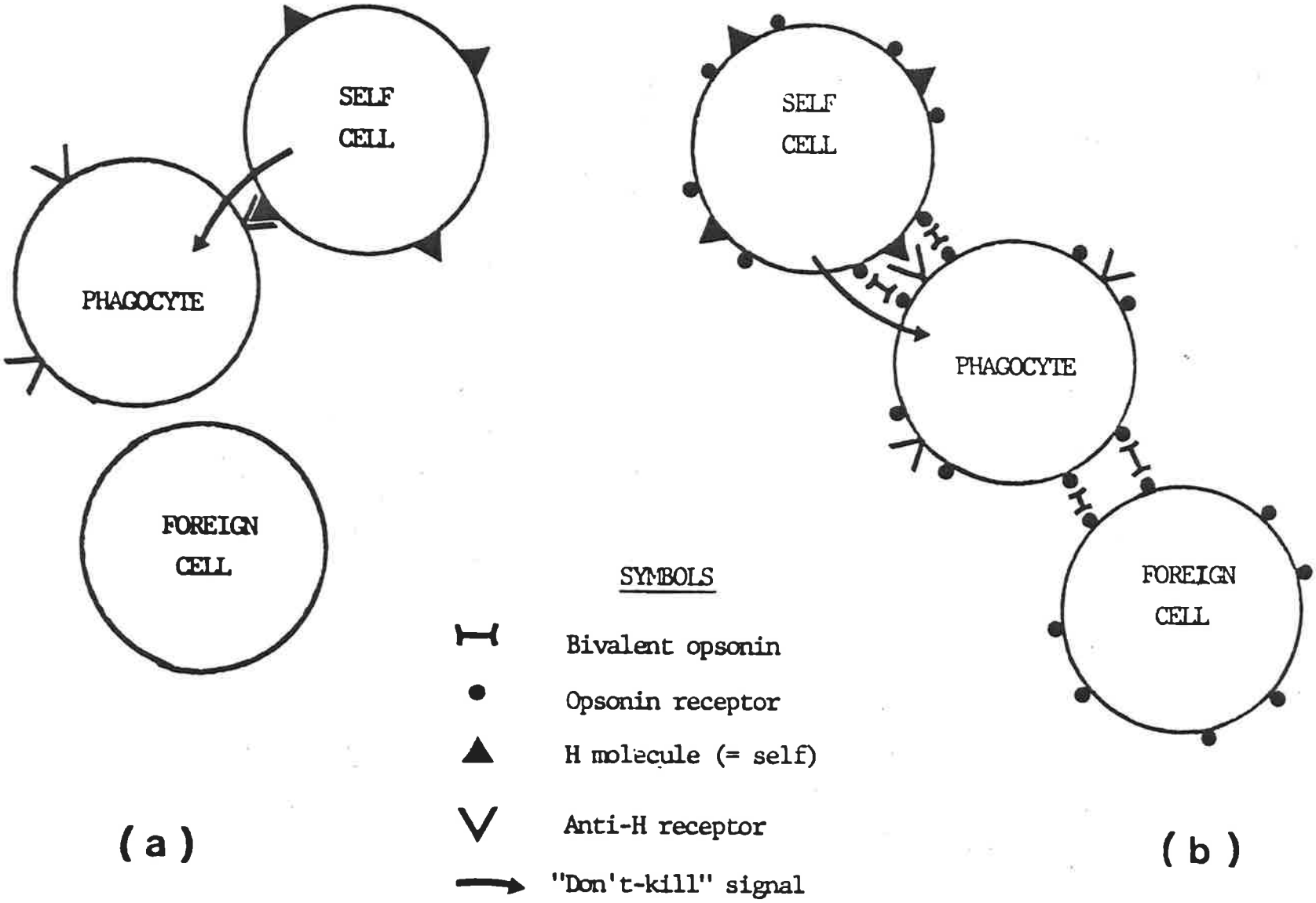
foreign particle. More immportantly, invertebrates appear to lack a variety of highly specific opsonins and the possession of many opsonins each with a fine specificity base is a requirement for all three proposals. In order for a limited number of opsonins to bind a wide range of potential pathogens opsonins would need to be of broad specificity. It is unlikely therefore, that all such opsonins would bind self determinants with low affinity. It is worth noting that washed cells from some invertebrates appear to be coated with opsonin (Tyson & Jenkin, 1974). One may expect that opsonins coating phagocytic cells would act like anti-X receptors and hence facilitate phagocytosis of foreign particles. However the potential would also exist for phagocytes to bind functioning self cells, if the self cells had exposed opsonin receptors. It therefore seems more likely that the control of phagocytosis in invertebrates may require two signals:

(1) phagocytosis being induced by the linkage of particles through either an opsonin or a cell bound anti-X receptor (signal 1), unless

(2) the phagocyte simultaneously receives a "don't kill" signal (signal 2) through the occupancy of its self recognition receptors (Fig 1.2).

A refinement of the specificity of both the anti-X receptors and opsonins would reduce the problem of self-reactivity and probably increase the affinity/avidity with which these molecules bound unwanted material, provided the non-self material had the corresponding X marker. However an increased number of the more specific opsonins and anti-X receptors would be required if the same variety of particles is to be recognised. Nevertheless, providing the benefits of specificity outweighed the metabolic cost of producing more opsonins and/or anti-X receptors, it is conceivable that some of the complex invertebrates may

FIGURE 1.2 A model proposed to explain phagocytosis by invertebrate cells where self-reactivity is controlled by the recognition of self through a self marker (H) and a recognition structure on the phagocyte (anti-H). Self-recognition is proposed to control phagocytosis in the absence of an opsonin (a), or when phagocytosis is initiated by the binding of opsonised cells (b). In both cases it is envisaged that any cell/particle to which the phagocyte becomes attached would be ingested unless self is specifically recognised.


SELF
CELL
PHAGOCYTE
FOREIGN
CELL
(a)
SYMBOLS
Bivalent opsonin
Opsonin receptor
H molecule (= self)
Anti-H receptor
"Don't-kill" signal
SELF
CELL
PHAGOCYTE
FOREIGN
CELL
(b)

possess a family of such molecules. Prowse & Tait (1969) have presented evidence that two opsonins may exist in the haemolymph of Helix aspersa. As recognition is investigated in more invertebrates using a wide range of foreign particles, particularly those from the animals' native environment, other examples of animals utilising a range of opsonins are likely to arise.

Unfortunately the study of immunity in invertebrates has been so far restricted to a very few animals and most of these have been investigated in little detail. Although evidence is available to support aspects of the model, many questions remain unanswered. The prevalence of opsonins and the properties of these molecules, their binding specificities, source of production and importance in immunity need to be examined thoroughly. The precise means by which invertebrate phagocytes recognise particles has yet to be elucidated for any particular case. For instance, what is the relative importance of adhesion through either a surface receptor or physical attractions in recognition by phagocytic cells and at what stage in the phylogeny did surface receptors arise? These and other questions can only be answered by detailed systematic studies on animals within different phyla. Although the lack of reliable information makes the construction of generalities both difficult and to some extent unwise, the presentation of models is necessary to offer both a basis for experimentation and a direction for this research.

1.5 The experimental approach undertaken in an investigation of cell recognition in B. leachii.

At the commencement of this work virtually nothing was known about the defence response of B. leachii to foreign material. B. leachii colonies were known to possess amoeboid cells which were

found to be involved in the removal of unwanted material (Burighel et al., 1976) but nothing was known about the recognition mechanisms utilised by these cells. A study on the recognition of con-specifics by Botryllus primigenus, a related species, suggested that this reaction was controlled by factors in the haemolymph (Mukai, 1967) but whether cellular and/or molecular elements were involved was not known. In two other studies (Fuke & Sugai, 1972; Wright, 1974) the suggestion was made that haemagglutinins detected in the haemolymph of some ascidians could be involved in recognition by the phagocytic cells of these species, but the experimental evidence was inconclusive. In view of these studies it was decided to address the question of cell recognition in B. leachii by firstly, examining the haemolymph of this animal for haemagglutinins, if possible purifying and characterising any haemagglutinins detected and secondly, investigating the function of the agglutinins. My preliminary work suggested that there were at least two haemagglutinins in B. leachii haemolymph. These two haemagglutinins, termed HA-1 and HA-2, and a third molecule (LBP-3) which, although binding to certain vertebrate erythrocytes was a very poor agglutinin, were purified and characterised by Sam Schluter (see Schluter, 1982). An investigation of the functional aspects of these molecules formed the basis of this thesis.

The function of the agglutinins was addressed at three levels:-

(1) If agglutinins did perform an essential function in ascidians then one might expect molecules of a similar structure also to exist in other ascidian species. This possibility was examined using haemolymph from a wide range of ascidian species.

(2) Both in vivo and in vitro studies were undertaken in an effort to obtain unambiguous data as to whether or not the

haemagglutinins from B. leachii function as opsonins in this species.

(3) Finally, the possibility that the B. leachii haemagglutinins may be structurally related to vertebrate antibody was considered. As the phylogenetic position of the ascidians is intermediate between the vertebrates and the non-chordate invertebrates, it was possible that a molecule with an opsonic function in B. leachii colonies may also function as an opsonin for the phagocytic cells from a vertebrate. If so, it could be argued that the invertebrate opsonin may have some structural homology with immunoglobulin.

Chapter 2

MATERIALS AND METHODS

2.1 Media and reagents

Deionised, filtered water (Milli-RO60; Millipore Corporation, U.S.A.) was used to make the solutions and buffers. The following solutions were prepared and stored at 4° before use.

Saline: 0.9% w/v (0.154 M) dispensed in 500 ml aliquots and autoclaved at 120° for 20 min.

Alsever's solution: NaCl (4.2 g), D-glucose (20.5 g), sodium citrate dihydrate (8 g) and citric acid (0.8 g) were dissolved in water to a final volume of one litre. The solution was adjusted to pH 6.1 with 10% citric acid, dispensed in 100 ml aliquots and autoclaved at 120° for 20 min.

Phosphate-buffered saline with azide (PBS-azide): NaCl (16.2 g), Na_2HPO_4 (3.04 g), $NaH_2PO_4.2H_2O$ (0.78 g) and NaN_3 (1 g) were dissolved in water to a final volume of two litres.

Tris-buffered saline with azide (TSA): Stock 0.5 M Tris buffer was prepared by dissolving 121.1 g of Tris(hydroxymethyl)amino-methane (Tris) and 1 g of NaN_3 in 1800 ml of water, adjusting the pH to pH 7.5 with 32% w/w HCl and adjusting the final volume to two litres with water.

A second stock solution (3 M NaCl) was prepared by dissolving 154.9 g of NaCl and 0.5 g of NaN_3 in water to a final volume of one litre. TSA was prepared by mixing together 50 ml of each solution plus 10 ml of 10% NaN_3 with 900 ml of water.

General reagents were obtained as indicated: Sephadex G-200, Sepharose 4B, Sephacryl S-200 and Sephacryl S-300 (Pharmacia Fine Chemicals); Standard proteins (Sigma Chemical Company and Calbiochem-Behring Corporation); Sodium iodide-125 (IMS:30) and sodium

chromate-51 (The Radiochemical Centre, Amersham); 199 Medium, foetal calf serum, bovine serum albumin (Cohn fraction V) and rabbit antiserum specific for sheep erythrocytes (Commonwealth Serum Laboratories, Melbourne); Dulbecco's modified Eagles medium (Flow Laboratories, Virginia); Concanavalin A and phytohaemagglutinin (Pharmacia); Wheat germ lectin (Calbiochem, Australia).

The following reagents Mouse IgG, Purified HA-1 and HA-2 agglutinins and LBP-3 from B. leachii and haemolymph from Cherax destructor were supplied by Drs P.L. Ey and S.F. Schluter of Dept. of Microbiology and Immunology, The University of Adelaide.

Except where indicated, sugars were purchased from BDH Chemicals Ltd., Laboratory grade: D-arabinose, L-arabinose. Analytical grade: D-galactose, D-glucose, lactose, mannose, melibiose (A grade, Calbiochem), maltose and sucrose. D-Fucose (Sigma Chem. Co.) was kindly donated by Dr J. Redmond, Chemistry Dept., Maquarie University, New South Wales.

2.2 Collection and storage of animals.

Colonies of B. leachii were collected from pier pilings at Edithburgh, South Australia (unless otherwise indicated) using SCUBA. The other ascidians used in this study were collected in a similar manner from a variety of locations around the South Australian coast. All animals were maintained at 15° in holding tanks of aerated sea water.

2.3 Preparation of ascidian specimens for dissection and identification.

As soon as possible after collection the animals were narcotised. This was performed by transferring the animals to a small volume of sea water to which was added, over a number of hours,

increasing quantities of $MgSO_4$. When the animals were completely immobilised they were transferred to a buffered solution of salt water formalin: 100 ml Formalin, 900 ml sea water, $NaH_2PO_4.H_2O$ (4 g) and Na_2HPO_4 (6.5 g) for at least 48 hr before dissection. Dissection was carried out according to the directions of Monniot & Monniot (1972) and identification was on the basis of keys and descriptions given in the following publications: Kott (1952; 1957; 1962; 1963; 1968; 1972 a & b; 1975; 1976); Millar (1963); Monniot & Monniot (1972). Voucher specimens with corresponding in situ photographs are held at the Dept. of Zoology, The University of Adelaide.

2.4 Collection of ascidian haemolymph.

2.4.1 B. leachii and Botryllus sp..

After removing surface water with a paper towel, incisions were made in the tunic matrix beneath the zooids and the exuding fluid was collected in a large petri dish. Gentle squeezing by hand usually produced as much as 20 ml of haemolymph from a colony 8 cm in diameter. The haemolymph was centrifuged (900 g, 5 min) to remove cells and other debris and was stored at -20°. When required it was thawed and clarified by centrifuging at 30,000 g for 30 min at 4° in a Sorvall RC-5 centrifuge using a SS34 rotor (Dupont Instruments U.S.A.).

2.4.2 Other ascidian species.

Haemolymph was collected from other ascidians in a manner similar to that described above (2.4.1); however differences existed in the quantity of haemolymph obtained and the position of the incisions. Although incisions were again made in the tunic, often it was impossible to avoid either slicing zooids (e.g. in compound ascidians

like the Didemnidae) or piercing the body wall (e.g. Podoclavella cylindrica and Stolonica australia), hence it was difficult to estimate the relative proportions of haemolymph, coelomic fluid and interstitial fluid that was collected. Nevertheless this liquid was termed haemolymph for convenience.

All samples collected were centrifuged (900 g, 5 min) and stored at -20°.

2.5 Preparation of erythrocytes.

Sheep blood was drawn aseptically into Alsever's solution and stored at 4° for no longer than 3 weeks. Blood collected by cardiac puncture from guinea pigs, trout and chickens was mixed with 0.15 M NaCl containing 0.013 M trisodium citrate (citrate-saline) and stored at 4° for a maximum of three days. Tortoise (Chelodina longicolis) blood was obtained by puncturing the jugular veins and it was treated in the above manner. When required, the erythrocytes were centrifuged at 900 g for 5 min, washed twice in saline and stored at 4° as a 10% v/v suspension for no longer than 3 days. Blood collected from LACA mice was mixed with citrate saline (0.168 M NaCl). Mouse erythrocytes were washed in 0.168 M NaCl and used immediately.

2.6 Haemagglutination assays.

Two-fold serial dilutions of haemolymph were mixed with an equal volume (50 µl) of an 0.5% erythrocyte suspension in the wells of a microtitre tray (Linbro Scientific Co., U.S.A., Model 1S-FB-96). Titres were expressed as the greatest dilution of the sample which caused complete agglutination. In situations where greater accuracy was required the titre given represents the maximum dilution which caused any agglutination, agglutination being evaluated as a fraction

of 1, i.e. 0, 1/4, 1/2, 3/4 or 1 where 1 = complete agglutination in a particular well, e.g. a titre of $1/2^{2.75}$ indicates that 2.75 wells were agglutinated. A variety of diluents was used according to the particular agglutinins being assayed. Physiological saline, isotonic trisodium citrate (3.8%), 25 mM tris-buffered saline, pH 7.6 (TSA) supplemented with bovine serum albumin (BSA, 0.01 mg/ml) and either 5 mM $CaCl_2$ (HA-1 diluent) or 5 mM EDTA (HA-2 diluents) were used as diluents.

2.7 Inhibition of haemagglutination by sugars.

For testing the specificity of agglutinins, haemagglutination assays were performed in the presence of a variety of sugars, each at a final concentration of 67 mM. The sugars were dissolved in distilled water to give solutions iso-osmotic with physiological saline and then diluted in either TSA plus 5 mM $CaCl_2$ and 5 mM $MgCl_2$ or 3.8% trisodium citrate. Serial two-fold dilutions (25 µl) of the sample were made across the rows of a microtitre tray. Twenty five microlitres of the first sugar solution were then added to each well in the first row, while subsequent rows received 25 µl of a different sugar. A row containing 25 µl of diluent was the control. Finally 50 µl of a 0.5% suspension of erythrocytes were added to all wells. The effect of a sugar on the agglutination reaction could be determined by a comparison of the titres obtained with and without sugar. Where precise sugar inhibition data were required, this procedure was repeated with a range of sugar concentrations and the concentration of sugar which reduced the agglutinating titre by one well (50%) was determined.

2.8 Concentration of samples by ultrafiltration.

Samples were concentrated using Amicon (Amicon Coorporation, U.S.A.) stirred cells and Diaflo ultrafiltration membranes. A 200 ml model 202 stirred cell was used with a PM 10 membrane and a 50 ml cell (model 52) was used with a YM 10 membrane. Samples of less than 10 ml were concentrated on a Minicon-C515 (Amicon) unit. Before use, this unit was briefly washed with Tris-buffered saline supplemented with 0.01 mg/ml BSA in order to reduce non-specific adsorbance of the agglutinins to the sides of the unit.

2.9 Iodination of proteins.

Purified HA-1 and HA-2 agglutinins and LBP-3 (from B. leachii) were radio-labelled with iodine-125 (IMS:30, Amersham) by Dr P.L. Ey, using a solid-phase oxidising agent (Iodogen) as described by Salacinski et al. (1981).

2.10 Column chromatography.

2.10.1 Sephacryl S-200 chromatography of haemolymph.

Sephacryl S-200 was mixed with TSA and the slurry poured into a 2.5 cm diameter glass tube. The column was allowed to pack under gravity flow with extra slurry being occasionally added until the bed was 84 cm long. The column was equilibrated at 4° with TSA containing 0.1 M glucose, 0.05 M galactose and 0.52 M NaCl (eluate buffer). It was necessary to use a buffer with a tonicity equivalent to sea water as a number of the agglutinins were shown to be unstable at lower salt concentrations. The column was calibrated with Blue Dextran 2000, I^{125}-labelled mouse IgG1, BSA and horse heart myoglobin. A mixture of these markers was applied to the column in 8 ml of buffer. The column was washed at a flow rate of 12 ml/hr set by a LKB

(LKB-Productor AB) peristaltic pump and 5.7 ml fractions were collected using an LKB Ultrorac fraction collector. The elution positions of the various markers were determined by measuring optical absorbance at 280 nm and radioactivity (Packard Autogamma counter) in the various fractions. The markers were identified by a previous knowledge of their molecular weights.

2.10.2 Sephacryl S-300 chromatography of affinity purified agglutinins.

A 40 x 2 cm column of Sephacryl S-300, prepared in a fashion identical to that described above (2.10.1), was equilibrated at 4° with TSA containing 0.5 mM EDTA, 0.5 mM EGTA and for some fractionations 0.1M lactose, depending on the nature of the agglutinin(s) being chromatographed. Two millitre samples were loaded onto the column and the eluate was collected as 1.9 ml fractions at a flow rate of 6.8 ml/hr. The eluent buffer was that used to equilibrate the column. The column was calibrated with Blue Dextran 2000, myoglobin, BSA, mouse IgG1 and IgM antibodies, and tyrosine. Various combinations of these markers were included as intrinsic markers in subsequent 2 ml samples containing affinity purified agglutinins.

2.11 Purification of agglutinins from B. leachii and Botryllus sp. haemolymph.

The agglutinins from B. leachii haemolymph were purified by Drs. S.F. Schluter and P.L. Ey, using methods slightly modified from those described by Schluter (1982). The initial step for all proteins (HA-1, HA-2, & LBP-3) was affinity chromatography using an adsorbent consisting of acid treated, epoxy-activated Sephrose 4B to which lactose had been conjugated (Lac-acid-Sepharose). The adsorbent was

prepared by Dr S.F. Schluter (Schluter, 1982) by the method of Uy & Wold (1977).

The HA-2 agglutinin was purified in three steps, by affinity chromatography on the Lac-acid-Sepharose column, followed by ion-exchange chromatography on DEAE-cellulose (DE-52, Whatman; 25 mM Tris-HCl, 1 mM EDTA, 20 mM lactose, 8 mM NaN_3, pH 8.3) where the pure agglutinin was collected in the effluent, and finally by gel filtration chromatography on Sephacryl S-200 (TSA & 1 mM EDTA & 0.1 M lactose), where the agglutinin eluted as a single, homogenous peak. This was concentrated and dialysed. The purified HA-2 yielded a single polypeptide band (approx. mol. wt. = 33,000) upon analysis by polyacrylamide gel electrophoresis in sodium dodecyl sulphide (SDS-PAGE), both in reducing and non-reducing conditions. A 1 mg/ml solution had an agglutinating titre against sheep erythrocytes (0.5%) of 1:3,500.

The HA-1 agglutinin and the related lactose-binding protein-3 (LBP-3) were similarly purified by affinity chromatography, ion-exchange (both proteins were eluted together using a salt gradient) and chromatography on Sephacryl S-200 (TSA plus 1 mM EDTA). They were separated in the latter step and each protein was rerun through the Sephacryl column to reduce cross-contamination to negligible levels ($< 1\%$). SDS-PAGE analysis showed that the HA-1 consisted of a single disulphide-bonded subunit (approx. mol. wt. = 28,500) and the LBP-3 of two disulphide-bonded subunits (approx. mol. wts. = 22,500 & 28,500). Both proteins reacted strongly with rabbit antisera raised against either protein, but neither reacted with an antiserum against HA-2. A 1 mg/ml solution of the HA-1 and LBP-3 exhibited an agglutination titre against 0.5% guinea pig erythrocytes of approximately 1:700,000 and 1:400 respectively.

The methods used to purify the agglutinins from Botryllus sp. haemolymph were based on those described above. EDTA had been found to reversibly inactivate the B. leachii HA-1 agglutinin without affecting the activity of the HA-2 agglutinin (Schluter, 1982). Hence when B. leachii haemolymph containing excess EDTA was passed through the Lac-acid-Sepharose column, the HA-1 agglutinin passed through in the effluent while the HA-2 agglutinin bound to the column. It was assumed that this would also apply for the Botryllus sp. agglutinins as their divalent cation requirements were apparently identical to those of the B. leachii agglutinins.

Botryllus sp. haemolymph (75 ml) was dialysed against TSA containing 5mM EDTA and passed at 4° through a column conatining 15 ml of Lac-acid-Sepharose. The column was washed at 21 ml/hr with TSA plus EDTA to collect unbound substances in the effluent. Fractions of 8.8 ml were collected. The "HA-2-like" agglutinin was subsequently eluted with lactose (0.1 M in TSA). To purify the "HA-1-like" agglutinin, the effluent haemolymph from above was made 10 mM with respect to $CaCl_2$ and then re-passaged through the affinity column which had been equilibrated with TSA plus 5mM $CaCl_2$. The column was washed with TSA/$CaCl_2$ and the agglutinin was then eluted with TSA/$CaCl_2$ and 10 mM lactose.

2.12 Preparation of isokinetic sucrose gradients

The apparatus used for making these gradients was an adaptation of the gradient maker described by Noll (1967) and is illustrated in Schluter (1982). It consisted of a stoppered mixing chamber containing a magnetic stirring bar. This chamber was connected to a vessel containing the reservoir solution by silicon tubing that passed through the air-tight stopper. The reservoir solution was pumped into the

mixing chamber by a peristaltic pump. The sucrose solution from the mixing vessel was delivered to the bottom of a cellulose nitrate gradient tube so that the less dense fluid introduced first floated above the fluid of increasing density pumped in subsequently. The sucrose solutions were prepared by dissolving weighed amounts of sucrose in half the required final volume of 20 mM phosphate-buffered 0.3 M NaCl (pH 7.2) containing 0.1% NaN_3 and then diluting with distilled water to the final volume. This ensured that the salt concentration was the same throughout the gradient.

To make a gradient, 12 ml of sucrose solution, from the reservoir, at a concentration (C_r) of 31.31% w/v was pumped into the mixing vessel containing 10.49 ml (V_m) of sucrose solution at a concentration (C_t) of 4.06% w/v. The pumping of the reservoir solution into the mixing vessel forced an equal volume of sucrose solution out of the mixing chamber into the gradient tube and as more of the reservoir solution was pumped into the mixing chamber the density of the solution added to the gradient increased. Gradients produced as described have a sucrose density range of 4.06% to 22.63% w/v. Details concerning the calculation of C_r and V_m when using a Beckman SW41 (6 bucket) rotor are given in Johns & Stanworth (1976).

2.13 Immunochemical anlaysis.

2.13.1 Rocket Immunoelectrophoresis.

Immunoelectrophoresis (IEP) buffer was prepared as a five-times concentrated solution as follows:-

Solution 1:	Glycine	140.5 g
	Tris	113.0 g
	n-Butanol	50. ml
	Water to	2500. ml

Solution 2:	Barbitone	5.18 g
	Dissolve in approximately 1000 ml of hot water	
	Add 32.5 g sodium barbitone.	

Solutions 1 and 2 were combined and the volume adjusted to 5000 ml with water. The stock solution was diluted five-fold and the pH was adjusted to exactly pH 8.6 to produce the working buffer. One percent agarose was prepared by dispersing 1 g of agarose (Seakem HGT, Marine Colloids) in 100 ml of IEP buffer plus 1 mM EDTA at 100°.

To prepare a plate, 0.1 ml of anti-LBP-3 rabbit immunoglobulin was mixed with 13 ml of 1% agarose in a 56° water bath and poured onto a 7 cm x 10 cm glass plate. After the agarose had gelled, holes (3 mm diameter) were punched at 6 mm intervals in a line 1 cm from the long edge of the plate using a Bio-Kad gel puncher. The plates were connected to the buffer reservoirs with surgical lint. Power was applied at 2 volts/cm and 10 µl samples were loaded into each well. Electrophoresis was continued overnight. The agarose was pressed to a thin film under layers of Whatman filter paper and paper towelling, rinsed in saline for 15 min and pressed again before being dried and stained.

2.13.2 Ouchterlony tests.

The plates were prepared by pouring 6.7 ml of 1% agarose dispersed in IEP-buffer containing either 1 mM EDTA (HA-1 analysis) or 0.2 M lactose (HA-2 analysis) onto a 5 x 7.6 cm glass plate. The agarose was allowed to gel and then six, 3 mm or 6 mm diameter wells were cut at 8 mm spacing around a central well (3 mm in diameter). The wells were filled with either 10 µl (3 mm wells) or 20 µl (6 mm wells) of the various antigen and antibody solutions. After incubation at 37° for 48 hr the plates were washed and stained.

2.13.3 Staining procedure (for Ouchterlony and immunoelectrophoresis plates).

The glass plates were placed for 12 min in an aqueous solution of 45% ethanol/10% acetic acid containing 0.005% w/v Coomassie Brilliant Blue R-250 dye. Destaining was achieved by rinsing the plates for 5 - 10 min in several changes of an aqueous solution of 45% ethanol/10% acetic acid.

2.14. Vertebrate cell culture.

2.14.1 Mouse macrophage monolayers.

Peritoneal exudate cells were collected from outbred LACA mice by the method of Rowley and Whitby (1959) and placed in siliconized tubes. The cells were washed once with 199 medium supplemented with foetal calf serum (M199-FCS) at a final concentration of 10% and resuspended to give approximately 3 x 10^6 cells/ml in the same medium. To Leighton tubes containing a flying coverslip was added 1 ml of the cell suspension. The tubes were incubated overnight at 37° to allow the cells to spread. The cells were then washed with M199-FCS and used for in vitro assays.

2.14.2 Sensitisation of erythrocytes.

Erythrocytes were sensitised by mixing an equal volume of dialysed haemolymph or solutions of the agglutinins to be tested, at the required dilution, with a suspension of washed erythrocytes (1% (v/v) in saline). The suspensions were incubated at 37° for 30 min. The cells were then washed in saline and resuspended in M199-FCS for use in adhesion or phagocytosis assays. Unsensitised erythrocytes used as controls in these experiments were similarly incubated as a 0.5% (v/v) suspension in saline at 37° for 30 min, washed and resuspended in M199-FCS.

2.14.3 Adhesion of erythrocytes to macrophage monolayers.

To washed monolayers were added 1.5×10^8 erythrocytes, either sensitised or unsensitised, in 1 ml of M199-FCS. The monolayers were then incubated overnight at 4°. After incubation the coverslips were removed, rinsed in M199-FCS and finally in saline. Cells attached to the coverslips were fixed in methanol and stained with Wright's stain. For each slide, macrophages visible in three to five fields (depending on cell density) chosen at random (mag 600x) were scored for the number of erythrocytes adhering to each cell.

2.14.4 In vitro phagocytosis assay.

A 1 ml suspension in M199-FCS of Cr^{51}-labelled sheep erythrocytes (1.5×10^8 cells/ml), either sensitised or unsensitised, was added to spread and washed mouse macrophage monolayers. The monolayers were incubated for 1 hr at 4° to allow adhesion of erythrocytes to the macrophages. They were then transferred to 37° for a specified time, ranging from 0 - 120 min. After incubation at 37°, the coverslips were removed, washed in saline and immersed in

Tris-NH_4Cl solution for 10 min at room temperature. This procedure (Boyle, 1968) lysed those erythrocytes not ingested by the macrophages. The Tris-NH_4Cl was prepared by adding 9 vol of 0.83% aqueous ammonium chloride to 1 vol of 0.18 M Tris-HCl buffer, pH 7.2. The coverslips were finally washed three times in saline and the radioactivity associated with each coverslip was measured in a Packard Auto Gamma Spectrometer.

2.15 Radiolabelling erythrocytes.

A 10% (v/v) suspension of washed erythrocytes was centrifuged and the cells were resuspended in saline such that on addition of Cr^{51} (Na_2CrO_4, Radiochemical Centre, Amersham, England), the final concentration of erythrocytes was 50% (v/v). One hundred microcuries of Cr^{51} were added per 7.5×10^9 cells. The cells and Cr^{51} label were vortexed to ensure thorough mixing then incubated at 37° for 30 min. The labelled cells were washed twice with 10 ml of saline and resuspended to a final concentration of 2% (v/v) in saline.

2.16 Clearance of radiolabelled erythrocytes from the circulation of mice.

Outbred LACA mice were injected intravenously (in the tail vein) with 0.2 ml of a 2% (v/v) suspension in saline of Cr^{51}-labelled sheep erythrocytes. At 1, 2, 5, 10 and 15 min after injection, 0.1 ml of blood was collected from each mouse from the retro-orbital sinus using a calibrated capillary tube. Each blood sample was assayed for radioactivity using a Packard Auto Gamma Spectrometer. The rate of elimination of sheep erythrocytes from the circulation followed an exponential curve where the phagocytic index K was given by -

$$\frac{\log_{10} C_1 - \log_{10} C_2}{T_2 - T_1} \qquad \text{or } \log_{10} C = KT + \underline{a}$$

where C_1 and C_2 are the radioactivity levels expressed as counts / min at times T_1 and T_2 (Benaceraf et al., 1959) and a is the intercept on the y-axis.

Mice were blockaded by injecting 0.2 ml of a 10% (v/v) suspension of unlabelled sheep erythrocytes intravenously (in the tail vein). After 20 min the mice were challenged with 0.2 ml of 2% Cr^{51}-labelled sheep erythrocytes and the fate of the challenge dose was followed as described above.

When mouse erythrocytes were used in this assay, the cells were maintained in a buffered salt solution isotonic with mouse serum throughout the labelling and injection procedure. Mouse salt solution consisted of 0.168 M NaCl buffered with 0.02 M HEPES to pH 7.4.

2.17 Solutions for handling B. leachii haemocytes.

2.17.1 Botrylloides salt solution (BSS)

The concentration of major cations in B. leachii haemolymph was calculated by dialysing a known volume of haemolymph against a known volume of deionised distilled water for 24 hr at 4° and then measuring the concentrations of the cations in the external water phase using an absorption spectrophotometer. The osmolarity (1,190 mOs) of haemolymph was measured with an osmometer. The concentration of the major cations in the final solution equalled that calculated for haemolymph while the anions were chosen such the osmolarity of haemolymph was maintained. The final solution consisted of:-

Group A	556.5 mM NaCl	Group B	46 mM $MgSO_4$
	16.4 mM KCl		30 mM $MgCl_2$
	11.3 mM $CaCl_2$		

Group A and group B salts were dissolved separately to avoid precipitation but on mixing their final concentrations were as given above. The solution was buffered at pH 7.0 with 20 mM HEPES (sodium salt), sterilised by millipore filtration using a 0.45 µ filter and stored at -20°.

2.17.2 Botrylloides cell culture medium (BCCM).

The culture medium was a modification of that used by Warr et al. (1977) for culturing Pyura stolonifera haemocytes. Dulbecco's modified Eagles medium (DME) was used as a nutrient source with extra salts being added to adjust the osmolarity to that of B. leachii haemolymph. Stock solutions were sterilised by millipore filtration and stored at -20°.

Stock solution 1: 13.45 g DME powder dissolved in 200 ml distilled water and stored in 20 ml aliquots (5x concentrated).

Stock solution 2:	Ingredients	Amount
Solution A	NaCl	13.67 g
	KCl	0.412 g
	$CaCl_2.2H_2O$	0.721 g
	dissolved in 125 ml of distilled water	
Solution B	$MgSO_4.7H_2O$	5.57 g
	$MgCl_2.6H_2O$	3.05 g
	dissolved in 125 ml of distilled water	

Solutions A and B were mixed to give a final volume of 250 ml. Stock solution 2 was stored in 50 ml aliquots.

Botrylloides culture medium was made by mixing:-

20 ml stock solution 1

50 ml stock solution 2

26 ml double distilled water (autoclaved)

2 ml 200 mM L-Glutamine

2 ml 1 M HEPES buffer (pH 7.0)

40,000 units of Benzylpenicillin Sodium BP (Crystapen)

0.2 ml 200 mg/ml Streptomycin Sulphate BP

0.35 ml of 1% 2-mercaptoethanol (final concentration of 5×10^{-5} M).

The glutamine and HEPES solutions were made up in 20 ml aliquots, millipore-filtered and stored at 4° until required.

2.18. B. leachii haemocyte culture.

2.18.1 Preparation of coverslips for cell culture.

Glass coverslips (13 mm in diameter) were cleaned by soaking for 30 min in a 1% solution of 7X Cleaning Solution (Linbro) heated to 78° in a water bath. They were then rinsed five times in distilled water, dried and autoclaved. One coverslip was placed in each 1.7 x 1.6 cm well (capacity 3.5 ml) of a 24-well plate for tissue culture (Linbro).

2.18.2 Haemocyte monolayers.

Haemolymph was collected into siliconised tubes on ice. The cells were washed twice in BSS by centrifuging at 150 g for 5 min at 4° and gently resuspending. The cells were finally resuspended in BCCM and 1×10^6 cells in 0.6 ml of BCCM were added to wells containing a prepared coverslip. These cells were incubated 1 hr at 4° to allow adhesion, then the coverslips were removed, washed in BSS and placed for experimentation in fresh wells containing 1 ml of BCCM. Every type

of cell found in haemolymph seemed to adhere to the glass coverslip and although the total number of cells adhering varied depending on the colony from which haemolymph was collected, on average 75 - 80% of the cells added would adhere.

2.18.3 Adherence of sheep erythrocytes to ascidian haemocytes.

To washed haemocyte monolayers in 1 ml of BCCM were added 3×10^7 washed sheep erythrocytes. The monolayers were incubated for 1 hr at 4° after which time the coverslips with their adherent cells were washed twice in BSS. The cells were then fixed in 2.5% gluteraldehyde in BSS for at least 30 min at room temperature, following which they were washed and stained with Wright's stain.

2.19 Production of antisera.

Antisera, raised in rabbits against purified B. leachii HA-2, HA-1 or LBP-3 and various other purified proteins, were generously donated by Dr P.L. Ey. They were prepared by emulsifying antigen (adsorbed to alumina Cγ gel [Calbiochem-Behring]) with a mixture of Span 85 detergent (Koch-Light Lab) and parafin oil (BDH) according to the method of Franck & Simek (1971). Each rabbit was injected subcutaneously at 6 - 8 sites with 0.1 - 0.5 mg of antigen in a total volume of about 2 ml. A second, identical dose was given 4 weeks after the first and booster injections at 2 - 3 month intervals thereafter. The animals were bled at regular intervals (2 - 3 weeks), commencing about 2 weeks after the second immunisation. The sera from different bleeds were pooled, sodium azide was added to a final concentration of 1% and the sera was stored at 4°.

2.20 Purification of rabbit IgG.

The IgG immunoglobulins used for immunofluorescent studies and in erythrocyte adhesion assays were isolated from rabbit antisera by the method of Steinbuch and Audran (1969).

2.21 Immunofluorescence.

2.21.1 Fluorescein isothiocyanate (FITC) labelling of agglutinins and IgG.

This was performed using a modification of the method of Rinderknecht (1962). Each protein was diluted to 2 mg/ml in 0.1 M carbonate buffer (pH 9.0) and mixed with Fluorescein isothiocyanate (10%) on celite (FITC-celite) (Calbiochem, La Jolla, California), 2 mg of FITC-celite being added per millilitre of protein solution. The mixture was stirred magnetically in the dark for 2 hr at room temperature. It was then centrifuged (1,200 g for 7 min) and the supernatant immediately chromatographed on a column of Sephadex G-25 (38 cm x 2.5 cm) equilibrated with PBS containing 0.05% sodium azide. The labelled protein eluted in the void volume, while free FITC-celite bound to the gel. The B. leachii HA-2 agglutinin was labelled in the presence of 50 mM lactose to protect the binding site against inactivation. Labelling efficiencies were determined by the formula (Hudson & Hay, 1980):-

$$\text{Fluorescein : Protein ratio} = \frac{2.87 \times OD_{495\ nm}}{OD_{280\ nm} - 0.35 \times OD_{495\ nm}}$$

Fractions having a Fluorescein : protein ratio above 4 were pooled for use in immunofluorescence assays.

Two hundred microlitres of 0.2 M ethanolamine (pH 9.0) and 0.5 ml of 0.25 M Tris-buffer (pH 6.8) were added to the labelled protein before

chromatography on Sephadex G-25 in PBS-azide containing 0.1 M lactose.

2.21.2 Fixation of cells for immunofluorescence.

The method used was a modification of that employed by Schwarz & Koepler (1979). Paraformaldehyde (2.5% w/v) was dissolved in BSS by incubating at 60° overnight. The resulting clear solution were kept at 4°. Ascidian haemocyte monolayers were incubated at 4° for 30 min with 0.5 ml of the 2.5% paraformaldehyde solution for fixation. They were then washed with BSS and incubated in 0.5 ml of 0.2 M glycine in BSS, during the 60 min incubation at room temperature the glycine was changed three times. The glycine reacted with free aldehyde groups remaining after fixation and so markedly reduced the level of background fluorescence. The coverslips were finally washed with BSS and immersed in 0.5 ml of a 1:10 dilution in BCCM of the appropriate fluorescein-labelled molecule for labelling.

2.21.3 Fixation of fluorescent labelled cells.

After being incubated with the fluorescent proteins, the monolayers of B. leachii haemocytes were washed three times in fresh 10 ml aliquots of BSS and then immersed in 0.5 ml of 2.5% paraformaldehyde/BSS for 30 min at 4°. They were then washed and mounted in BSS for observation.

Chapter 3.

A COMPARISON OF THE HAEMAGGLUTININS FROM A VARIETY OF ASCIDIANS WITH THE HAEMAGGLUTININS FROM *Botrylloides leachii*.

3.1 Introduction.

Although the presence of haemagglutinins in the haemolymph of a variety of invertebrate species has been known for some time (Table 1.1 and Ey & Jenkin, 1982), very little is understood about the function of these molecules. Two functions for invertebrate haemagglutinins have been suggested, an opsonic function (Boyden, 1966) and a nutritional function (Uhlenbruck & Steinhausen, 1977). The notion that haemagglutinins have an opsonic role has been embraced by a number of workers (see Chapter 1, Section 1.3.4) however the supporting evidence has been largely circumstantial. The work of Renwrantz and his collegues (Renwrantz *et al.*, 1981) is exceptional in that they presented good evidence to support an opsonic function for an agglutinin from *Helix pomatia* (a detailed discussion is presented in Chapter 1, Section 1.3.4). In view of this finding and in the absence of any evidence to support an alternative role for these molecules, the idea that haemagglutinins found within the haemolymph of protochordates may also function as opsonins was pursued.

When this study was initiated, haemagglutinins had been detected in a number of ascidians (Tyler, 1946; Uhlenbruck *et al.*, 1970; Fuke & Sugai, 1972; Bretting & Renwrantz, 1973; Renwrantz & Uhlenbruck, 1974; Wright, 1974; Anderson & Good, 1975; Parrinello & Patricolo, 1975; Wright & Cooper, 1975; Form *et al.*, 1979). However, information about the structure of these molecules and in particular their binding site specificities was very limited. It seemed reasonable to expect that a greater understanding of the structure of the haemagglutinins might shed light not only on the functional capacities of these molecules but also provide information as to their evolution, such as has been shown by studies on other proteins (e.g. β_2-microglobulin: Shalev *et al.*, 1981). It is possible that the structure of a recognition molecule

would have been conserved throughout the evolution of the Ascidiacea in much the same way as the basic structure of antibody has been conserved within the vertebrate classes (review: Marchalonis, 1977). For this reason it was of interest to determine the distribution of haemagglutinins within the Ascidiacea and to obtain some idea of the structural variations of these molecules. To determine whether or not they are opsonins would, nevertheless, require further experimentation.

My early work suggested that B. leachii haemolymph contained two agglutinins, one specific for guinea pig erythrocytes and the other binding to a variety of vertebrate erythrocytes. This result led to the characterisation and purification of these two agglutinins, termed HA-1 and HA-2 respectively, and a third protein, Lactose Binding Protein Three (LBP-3), in a concurrent study by Sam Schluter (Schluter et al., 1981; Schluter, 1982). The binding activity of the HA-1 molecule (mol. wt. 152,000) was shown to be calcium-dependent, specific for guinea pig erythrocytes and inhibited by lactose, melibiose, D-galactose and D-fucose with similar efficiency. The HA-2 molecule agglutinated erythrocytes from a variety of vertebrate species without requiring divalent cations for activity and had considerably greater affinity for lactose than for any other sugar examined. Its molecular weight was 65,000. The third protein, LBP-3, had an apparently identical specificity to the HA-1 agglutinin although it only weakly agglutinated guinea pig erythrocytes. The HA-1 agglutinin and LBP-3 co-purified during affinity chromatography but could be separated by chromatography on Sephacryl S-300 in the presence of a chelating agent (Schluter, 1982).

In view of the possibility that one or more of these agglutinins act as recognition molecules for phagocytes in B. leachii and because

there was a lack of rigorous data concerning agglutinins in other ascidian species, it was decided to investigate whether agglutinins of a similar structure or specificity were common among the Ascidiacea. Accordingly, a detailed comparative study of the haemagglutinating activity of haemolymph from a variety of ascidian species was undertaken.

3.2 The haemagglutinins found in the haemolymph of a number of ascidian species.

Ascidian specimens were collected using SCUBA from a variety of locations around the South Australian coast and were either bled for haemolymph or fixed in salt water buffered formalin for identification (Methods given in Chapter 2, Section 2.4 & 2.3). Species were chosen from as many of the major families within the three orders of the Ascidiacea as was possible, so that the haemagglutinating activity of haemolymph from closely related and phylogenetically distinct species could be compared (Fig 3.1). The haemolymph samples were titrated against sheep, mouse, guinea pig, tortoise (Chelodina longicolis) and trout erythrocytes (all 0.5% v/v in saline) using diluents both supplemented with or devoid of Ca^{++} and Mg^{++} ions (TSA plus 5 mM $CaCl_2$ and 5 mM $MgCl_2$, or 0.13 M trisodium citrate respectively). These results are presented in Table 3.1.

Haemagglutinins were found in most but not all of the haemolymph samples examined and considerable variations in titre were recorded for the positive samples. Aplidium australiensis haemolymph, for example, agglutinated all cell types and did not require Ca^{++} or Mg^{++} ions for activity, while haemolymph from Didemnum patulum (an ascidian belonging to the same order) agglutinated only sheep and mouse erythrocytes. In addition, divalent cations were required by the

FIGURE 3.1 Proposed phylogenetic relationships between the families and large subfamilies of the Urochordata (after Miller, 1966). The species investigated in Chapter 3 belong to the groups designated (*).

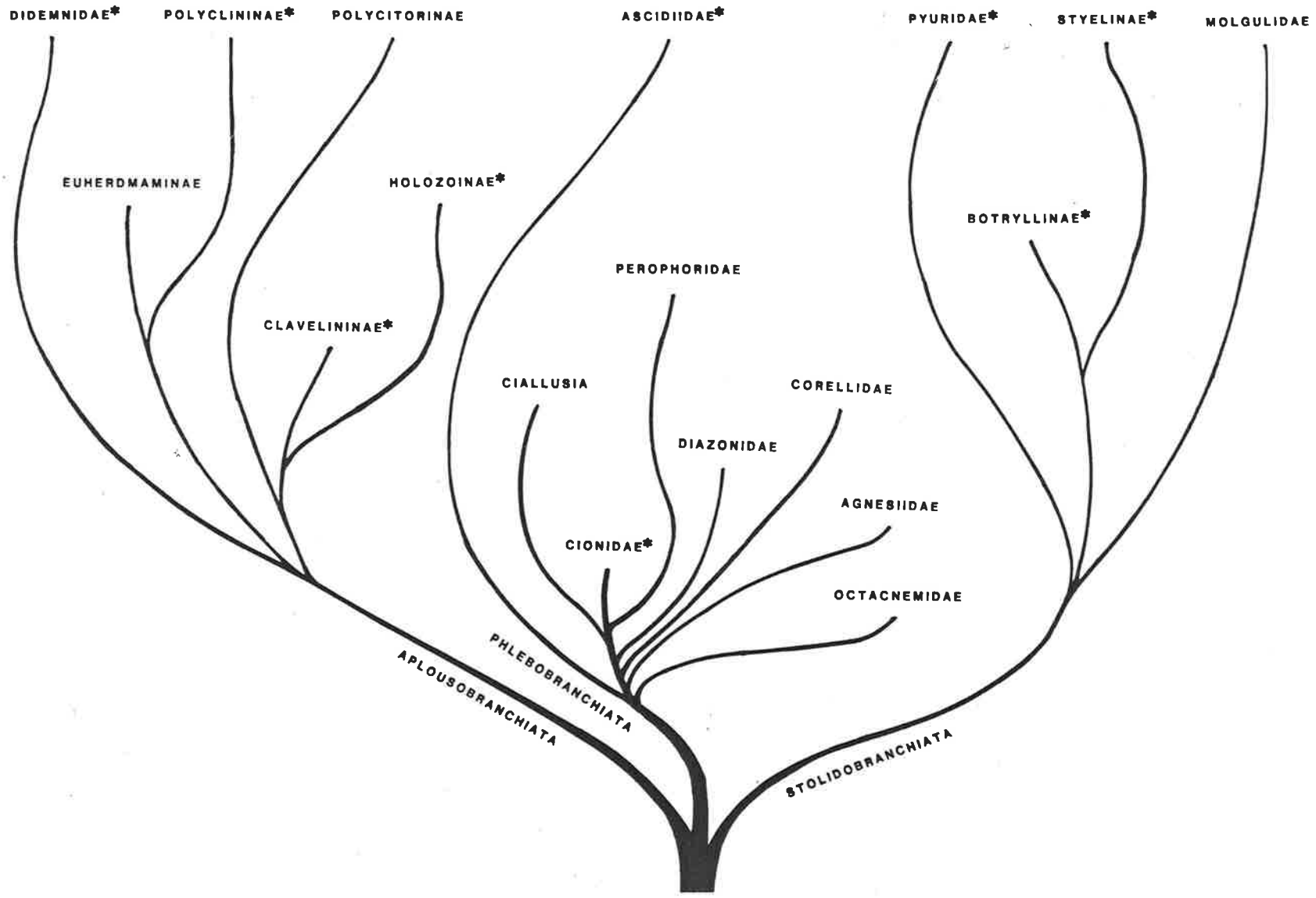

DIDEMNIDAE*
POLYCLININAE*
POLYCITORINAE
ASCIDIIDAE*
PYURIDAE*
STYELINAE*
MOLGULIDAE
EUHERDMAMINAE
HOLOZOINAE*
BOTRYLLINAE*
PEROPHORIDAE
CLAVELININAE*
CIALLUSIA
CORELLIDAE
DIAZONIDAE
AGNESIIDAE
CIONIDAE*
OCTACNEMIDAE
PHLEBOBRANCHIATA
APLOUSOBRANCHIATA
STOLIDOBRANCHIATA

TABLE 3.1 Agglutination titres of the haemplymph of a variety of ascidians*.

Classification	Species	Type of erythrocyte				
		Sheep	Mouse	Guinea pig	Trout	Tortoise
APLOUSOBRANCHIA						
Didemnidae	Didemnum patulum	16(2)	32(32)	0	0	0
	Diplisoma sp.	8(8)	0?(64)	2(0)	4(2)	0
Polyclinidae	Aplidium australiensis	32(32)	128(64)	128(128)	16(16)	16(16)
Clavelinidae						
(Holozoinae)	Sycozoa tenuicaulis	0	0	0	0	0
	Atapozoa fantasiana	4(2)	4(2)	4(2)	8(2)	4(0)
(Clavelininae)	Podoclavella cylindrica	0	0	0	0	0
PHLEBOBRANCHIATA						
Ascidiidae	Phallusia despressiuscula	2(2)	4(4)	4(4)	0	0
	Ascidia thompsoni	2(2)	0	0	2(2)	0
Cionidae	Ciona intestinalis	2(2)	0	0	0	0
STOLIDOBRANCHIATA						
Pyuridae	Microcosmus nichollsi	8(8)	4(0)	0	2(0)	0
	Pyura praeputialis	4(8)	2(2)	0	0	0
	Pyura irregularis	0	32(0)	2(0)	0	0
	Halocynthia hispida	16(4)	8(8)	512(2)	0	0
	Herdmania momus	2(2)	2(2)	0	0	0
Styelidae						
(Botryllinae)	Botrylloides leachii	64(64)	64(64)	12,800(128)	64(64)	16(16)
	Botrylloides magnicoecus	2(2)	0	4(4)	0	0
	Botryllus sp.	32(32)	32(32)	25,600(32)	16(16)	0
(Styelinae)	Cnemidocarpa etheridgii	4(4)	64(4)	128(8)	32(32)	0
	Polycarpa pedunculata	0	0	0	0	0
	Polycarpa obtecta	2(2)	2(2)	0	0	0
	Polycarpa papillata	4(4)	64(64)	32(32)	2(2)	0
(Polyzoinae)	Stolonica australis	2(2)	2(2)	2(2)	0	0

* Agglutination was assessed by mixing two-fold serial dilutions of each haemolymph sample with an equal volume (50 µl) of a 0.5% erythrocyte suspension. The diluent was either TSA containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ or 0.13 M trisodium citrate. Reciprocal titres are given. The titres in parentheses were obtained using citrate as the diluent.

D. patulum agglutinin(s) for activity with sheep erythrocytes. Variations in titre were particularly apparent within the Stolidobranchiata. Very high titres were recorded when haemolymph from both Botrylloides leachii or Botryllus sp. were titrated against guinea pig erythrocytes in the presence of Ca^{++} and Mg^{++} ions, although haemolymph from a specimen of Botrylloides magnicoecus exhibited very little activity against any cell type. Halocynthia hispida and Cnemidocarpa etheridgii haemolymph also required Ca^{++} and/or Mg^{++} for activity with guinea pig erythrocytes but both exhibited much lower titres. In contrast, divalent cations were necessary for C. etheridgii haemolymph to agglutinate both mouse guinea pig erythrocytes but not trout erythrocytes.

As the first step in assessing whether similarities exist between the HA-1 or HA-2 molecules from B. leachii and the haemagglutinins from these other species, the specificities of the more active samples were examined by testing the inhibitory effects of a number of sugars. Lactose, D-galactose, sucrose, D-arabinose and melibiose were chosen to enable the detection of molecules with specificities similar to the B. leachii agglutinins (see Schluter et al., 1981). Each sugar was tested at a final concentration of 67 mM.

Marked differences in the sensitivity of the various haemagglutinins to the sugars were observed (Table 3.2). Within the Styelidae, for example, the Cnemidocarpa etheridgii and the Polycarpa papillata haemagglutinins showed no inhibition with any of the sugars while the activity of haemolymph from Botryllus sp. was markedly inhibited by a number of sugars. Moreover, the pattern of inhibition by lactose, D-galactose, melibiose, D-arabinose and sucrose obtained with the Botryllus sp. haemolymph, titrated against guinea pig erythrocytes in the presence of divalent cations, was virtually

TABLE 3.2 The binding specificity of haemagglutinins from a variety of ascidians*.

Species	Type of erythrocyte					
	Sheep	Mouse	Trout	Tortoise	Guinea pig plus Ca^{++},Mg^{++}	Guinea pig citrate
Didemum patulum	NI (16)	NI (32)	-	-	-	-
Diplosoma sp.	NI (8)	-	-	-	-	-
Aplidium australiensis	NI (32)	NI (64)	L,A,M = 4 (16)	L = 4 (16)	L = 32 (128)	L = 32 (128)
Microcosmus nichollsi	L,M < 4 (8)	-	-	-	-	-
Pyura irregularis	-	all < 4 (32)	-	-	-	-
Halocynthia hispida	NI (16)	-	-	-	L,S = 64; G = 128 (512)	-
Botrylloides leachii	L = 2 (64)	L = 2 (64)	L < 4 (64)	L,G,M < 4 (16)	L,G,M < 200; A = 1600; S = 6400 (12800)	L < 4 (128)
Botryllus sp.	L,G < 4; M = 4 (32)	L,G < 4; M = 4 (32)	L,G,M < 4; (16)	-	L,G,M < 200; A = 1600; S = 12800 (25600)	-
Cnemidocarpa edtheridgii	-	NI (64)	NI (32)	-	NI (128)	NI (8)
Polycarpa papillata	-	NI (64)	-	-	NI (32)	-

* To assess binding specificity titrations were performed in diluents containing D-arabinose (A), D-glalactose, lactose (L). melibiose (M), or sucrose (S). The final concentration of all sugars was 67 mM. The diluents were TSA supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$ or 0.13 M trisodium citrate. The sugar inhibition patterns obtained for the agglutination of guinea pig erythrocytes are shown with both diluents. The inhibition patterns obtained with the other erythrocyte types did not differ with the diluent used; the results shown were obtained using TSA, Ca^{++},Mg^{++} diluent. Inhibiting sugars and reciprocal titres are shown.

NI = Not inhibited

The control titres, i.e. without sugar are given in parentheses.

identical to that observed with B. leachii haemolymph. The Botryllus sp. haemolymph, like that of B. leachii, also appeared to contain two agglutinins. The agglutinins in the haemolymph of Halocynthia hispida, an ascidian belonging to the Pyuridae and so more distantly related to B. leachii, seemed to possess different specificities. The Ca^{++} or Mg^{++} ion-dependent agglutination of guinea pig erythrocytes with haemolymph from this species was inhibited equally well by lactose and sucrose and to a lesser degree by D-galactose, while the agglutination of sheep erythrocytes was not inhibited by any of the sugars tested. Haemolymph from Aplidium australiensis contained agglutinins sensitive to yet another combination of sugars. From these results, it was clear that a variety of agglutinins occurs in the haemolymph of many different ascidians.

3.3 Sephacryl S-200 fractionations.

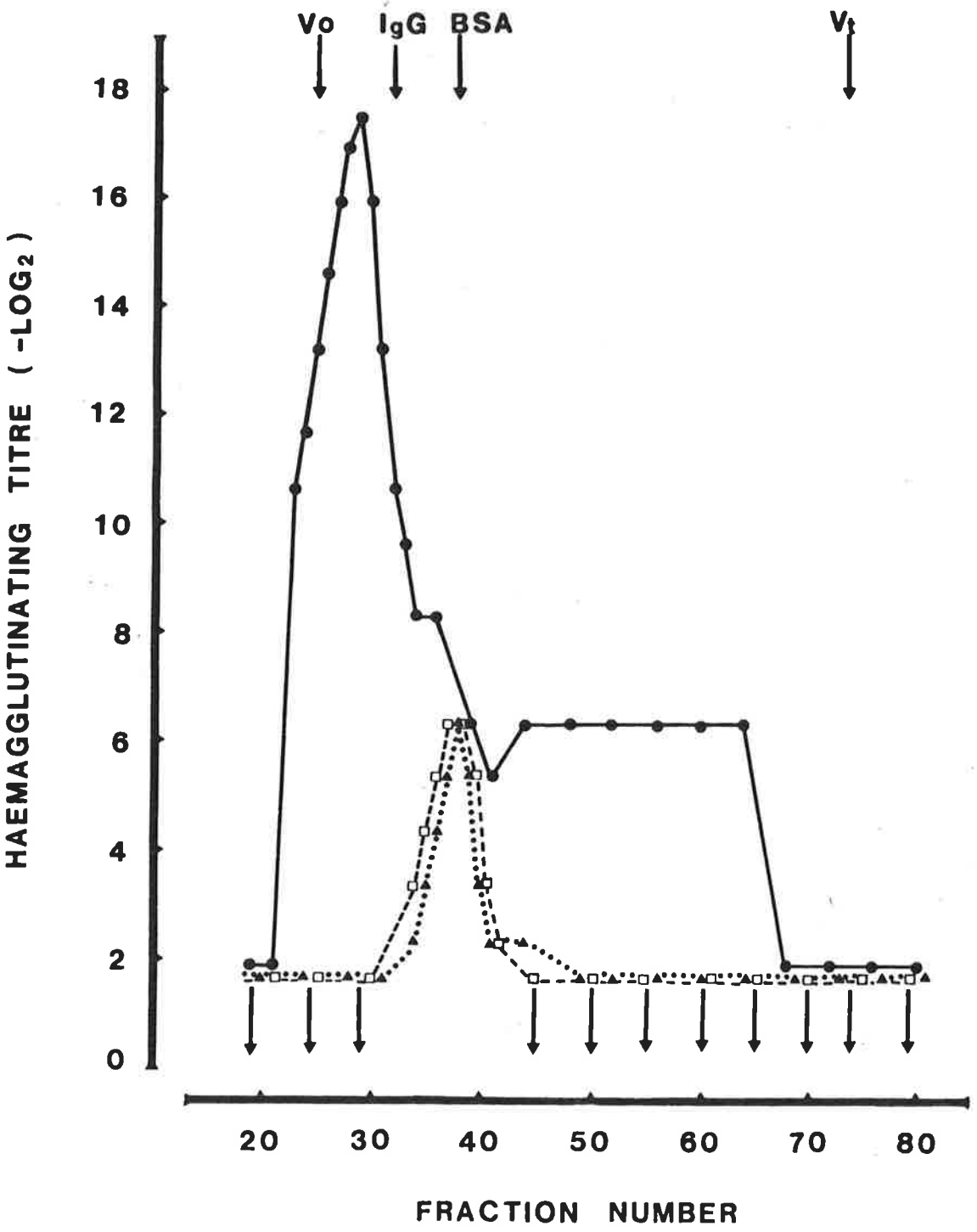
In order to resolve agglutinins of different size in the haemolymph from a particular ascidian and to estimate the molecular weights of these agglutinins, haemolymph samples containing 0.05 M galactose and 0.1 M glucose were subjected to chromatography on a column of Sephacryl S-200 (see Chapter 2, Section 2.10.1).

3.3.1 The fractionation of Botryllus sp. haemolymph.

Botryllus sp. haemolymph was concentrated 8-fold by ultrafiltration against a PM 10 membrane. The sheep erythrocyte agglutinin titre of the concentrated sample was 1:256. An 8 ml sample of haemolymph containing 0.05 M galactose and 0.1 M glucose was applied to the Sephacryl S-200 column, which was eluted at 4° with TSA containing 0.05 M galactose, 0.1 M glucose and an additional 0.52 M NaCl. The fractions from this and all subsequent fractionations using

FIGURE 3.2 Chromatography of Botryllus sp. haemolymph on Sephacryl S-200.

Sample: 8 ml concentrated haemolymph (sheep erythrocyte agglutinating titre 1:256) containing dissolved galactose (0.05 M) and glucose (0.1 M). Eluent: TSA containing 0.05 M galactose, 0.1 M glucose and an additional 0.52 M NaCl. Flow rate: 23 ml/hr. Temperature: 4°. Fraction size: 5.7 ml. Fractions were diluted 1:5 in distilled water and titrated for haemagglutinating activity in 0.13 M trisodium citrate (sheep erythrocytes, □ ; guinea pig erythrocytes, ▲) or TSA containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ (guinea pig erythrocytes, ●). The elution positions of blue dextran (Vo), mouse IgG1 (IgG) and bovine serum albumin (BSA) and the bed volume of the column (Vt) are shown. The arrows indicate a titre of less than 1:4.


Vo
IgG
BSA
Vt
18
16
14
12
10
8
6
4
2
0
HAEMAGGLUTINATING TITRE (-LOG2)
20
30
40
50
60
70
80
FRACTION NUMBER

this buffer were diluted 1:5 in distilled water to restore their tonicity to that of physiological saline. They were then tested for agglutinating activity with guinea pig and sheep erythrocytes (0.5% v/v in saline). Both 0.13 M trisodium citrate and TSA/Ca^{++}-Mg^{++} were used as diluents.

The results (Fig 3.2) indicated that there were at least two agglutinins within the Botryllus sp. haemolymph. One agglutinin had an apparent size greater than that of IgG (mol. wt. = 150,000) and agglutinated guinea pig erythrocytes specifically, but only in the presence of Ca^{++} and Mg^{++} ions. The agglutinin(s) in the second peak agglutinated both guinea pig and sheep erythrocytes and exhibited no dependence on divalent cations. Their size appeared to be similar to that of BSA (mol. wt. = 67,000). These apparent molecular weights for the Botryllus sp. agglutinins correspond to those of the B. leachii HA-1 and HA-2 agglutinins (Schluter et al., 1981; Schluter, 1982).

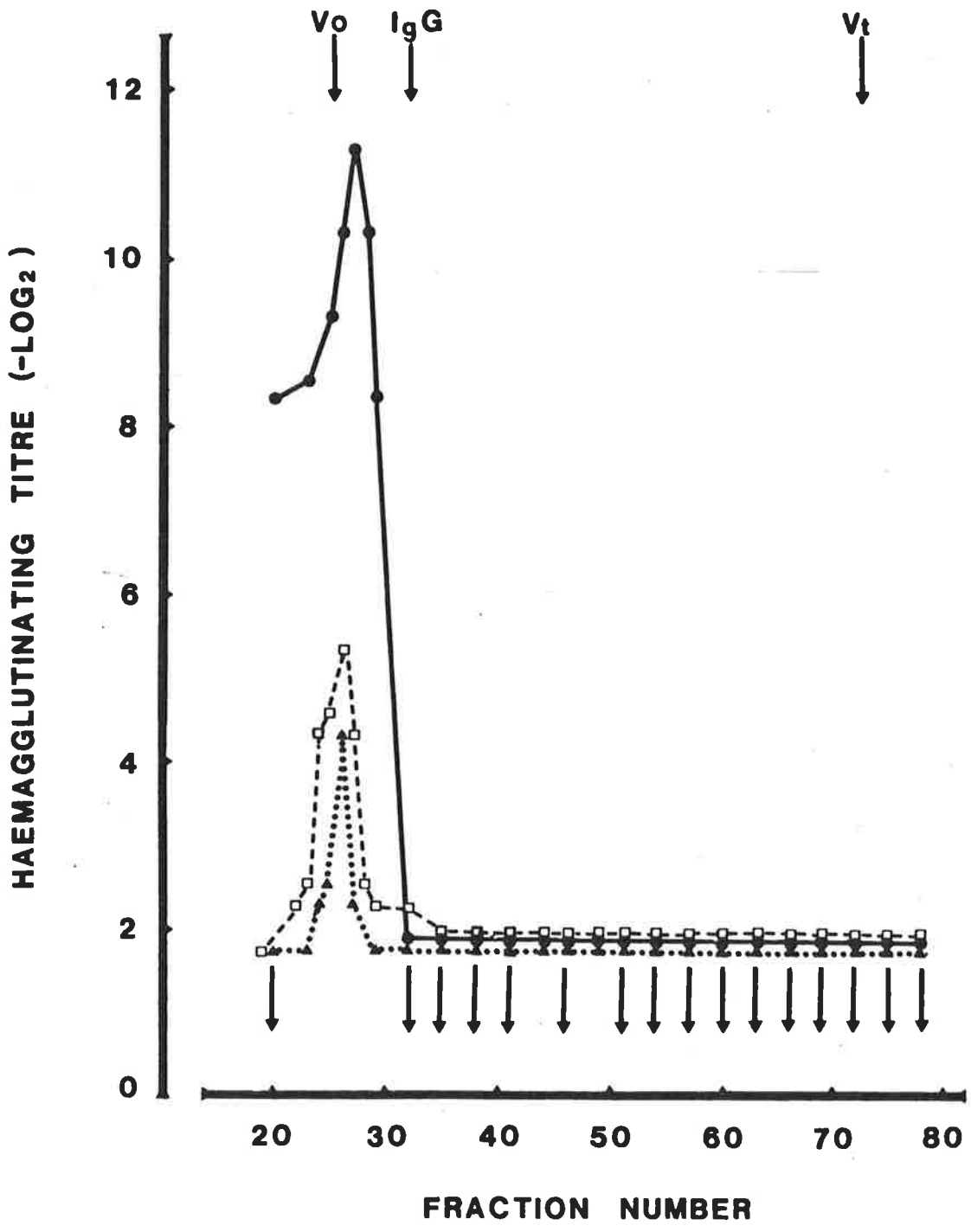
3.3.2 The fractionation of Polycarpa papillata haemolymph.

Eight millilitres of Polycarpa papillata haemolymph, previously concentrated 4-fold to give a guinea pig erythrocyte agglutinating titre of 1:256, was chromatographed on the Sephacryl S-200 column. The fractions were titrated in both citrate or TSA/Ca^{++}-Mg^{++} to determine their agglutinating activity with mouse and guinea pig erythrocytes.

The results (Fig 3.3) suggested that two haemagglutinins were present in the haemolymph. Both activities eluted almost coincidentally (peak fractions: 25-27), consistent with agglutinins having a size near the exclusion limit of the gel (approx. 400,000 mol. wt.). However they could be distinguished by their divalent cation requirements: the titre of fraction 26 against guinea pig

FIGURE 3.3 Chromatography of Polycarpa papillata haemolymph on Sephacryl S-200.

Sample: 8 ml concentrated haemolymph (guinea pig erythrocyte agglutinin titre in citrate diluent, 1:256) containing 0.05 M galactose and 0.1 M glucose. Eluent: TSA supplemented with 0.05 M galactose, 0.1 M glucose and 0.52 M NaCl. Flow rate: 17 ml/hr. Temperature: 4°. Fraction size: 5.6 ml. Fractions were diluted 1:5 in distilled water and titrated for agglutinating activity in 0.13 M trisodium citrate (mouse erythrocytes, □; guinea pig erythrocytes, ▲) or TSA containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ (guinea pig erythrocytes, ●). The elution positions of blue dextran (Vo) and mouse IgG1 (IgG) and the bed volume of the column (Vt) are shown. The arrows indicate a titre of less than 1:4.


Vo
IgG
Vt
12
10
8
6
4
2
0
HAEMAGGLUTINATING TITRE (-LOG2)
20
30
40
50
60
70
80
FRACTION NUMBER

erythrocytes was 2^{-11} in Ca^{++}-Mg^{++} diluent but only 2^{-4} in citrate diluent, whereas the titre against mouse erythrocytes was the same in both diluents. The detection of a divalent cation dependent agglutinin that was very active with guinea pig erythrocytes was unexpected, for there had been no suggestion of such an activity in the unfractionated haemolymph (Table 3.1).

Although fraction 26 contained the peak of both activities, the inhibitory effects of sugars on each activity could be measured independently by performing the assay in diluent containing citrate or Ca^{++} & Mg^{++} ions. As before (Table 3.2), lactose, D-galactose, sucrose, D-arabinose and melibiose were each tested at a final concentration of 67 mM. The two activities (agglutination of guinea pig and mouse cells in the absence of Ca^{++}-Mg^{++} and of guinea pig cells in the presence of Ca^{++}-Mg^{++}) appeared to have different specificities. The sugar specificity of the peak fraction when tested against guinea pig/mouse erythrocytes in citrate (i.e. in the absence of Ca^{++} and Mg^{++} ions) resembled that of the agglutinin originally detected in unfractionated haemolymph (Table 3.2), i.e. none of the sugars tested caused any inhibition of agglutination. However, when Ca^{++} and Mg^{++} ions were included in the diluent the pattern of agglutinating activity against guinea pig erythrocytes was markedly similar to that observed with the HA-1 agglutinin from B. leachii haemolymph (Table 3.3).

3.3.3 The fractionation of Cnemidocarpa etheridgii haemolymph.

A fresh sample of C. etheridgii haemolymph was collected from Edithburgh, South Australia. The guinea pig erythrocyte agglutinating titre of this sample was 1:512 when titrated in Ca^{++}-Mg^{++} diluent, but the titre against mouse erythrocytes, in contrast to that obtained

TABLE 3.3 The effect of different sugars on the activity of the Ca^{++}-dependent haemagglutinins to guinea pig erythrocytes of different ascidians.

Sugar tested (67 mM)	Ascidians from which agglutinin* was obtained				
	Botryllus sp.	*P. papillata*	*C. etheridgii*	*H. hispida*	*B. leachii*§
None (control)	256,000 (100)	1920 (100)	80 (100)	2,480 (100)	12,800 (100)
Lactose	2,000 (<1)	20 (<1)	20 (<25)	160 (6.5)	200 (<1.5)
D-galactose	2,000 (<1)	20 (<1)	20 (<25)	1,240 (50)	200 (<1.5)
Sucrose	128,000 (50)	800 (42)	80 (100)	160 (6.5)	6,400 (50)
D-arabinose	16,000 (6)	240 (12.5)	20 (<25)	1,240 (50)	1,600 (12.5)
Melibiose	2,000 (<1)	20 (1)	20 (<25)	2,480 (100)	200 (<1.5)

* The samples tested were fractions obtained by Sephacryl S-200 fractionation of haemolymph. Agglutination was assessed by mixing 2-fold serial dilutions of the agglutinins with an equal volume (25 µl) of 0.27M sugar and then adding 50 µl of a 0.5% erythrocyte suspension to each well. The diluent was TSA containing 5 mM $CaCl_2$ plus 5 mM $MgCl_2$. Titres are expressed as the greatest dilution producing complete agglutination. Reciprocal titres are shown.

§ Unfractionated haemolymph.

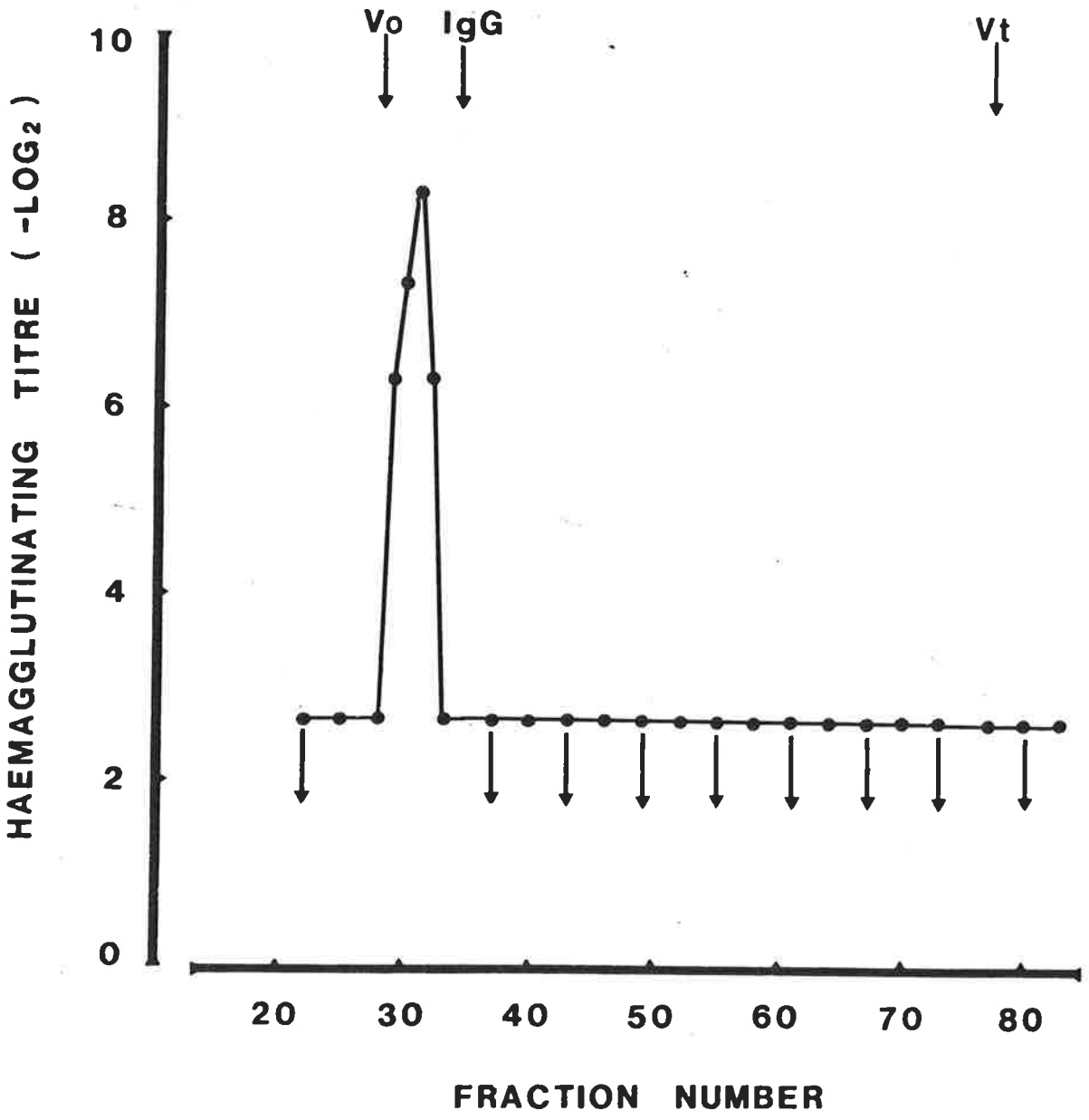
¶ The figures in parentheses are the haemagglutinating activity as a % of the control.

previously (1:64; Table 3.1) was only 1:4. This difference may have been due to the presence of distinct agglutinins against each cell type or merely to differences in the erythrocytes used on each occasion. An 8 ml sample of this haemolymph was chromatographed on the Sephacryl S-200 column and the fractions were tested for haemagglutinating activity against guinea pig erythrocytes in Ca^{++}-Mg^{++} diluent. A single peak of agglutinating activity was detected (Fig 3.4). The position of the peak suggested that the agglutinin had a molecular weight of 200,000-400,000. The activity of the peak fraction with various sugars was examined. Lactose, D-galactose, D-arabibose and melibiose, at a final concentration of 67 mM, all reduced the titre by at least 3 wells, whereas sucrose at the same concentration had no effect (Table 3.3).

This sample of C. etheridgii haemolymph differed therefore from the one examined previously (see Tables 3.1 & 3.2) not only in exhibiting a very low titre for mouse erythrocytes but also in possessing a guinea pig erythrocyte agglutinin having a different sugar specificity profile. It should be noted that the sample of haemolymph examined in Tables 3.1 & 3.2 and the sample analysed by chromatography (Fig 3.4) were collected from two separate locations, Rapid Bay (138° 11' E, 35° 31' S) and Edithburgh (137° 45' E, 35° 5' S) respectively. It appears that the concentration of the agglutinins in the haemolymph varied from locality to locality. A similar variation in the agglutinating titre has also been recorded for B. leachii haemolymph (see Chapter 4, Section 4.2) and Botryllus sp. haemolymph (data not shown).

FIGURE 3.4 Chromatography of Cnemidocarpa editheridgii haemolymph on Sephacryl S-200.

Sample: 8 ml of haemolymph (guinea pig erythrocyte agglutinin titre, 1:512) with galactose and glucose added to concentrations of 0.05 M and 0.1 M respectively. Eluent buffer: TSA supplemented with 0.05 M galactose, 0.1 M glucose and 0.52 M NaCl. Flow rate: 19 ml/hr. Temperature: 4°. Fraction size: 5.4 ml. Fractions were diluted 1:5 in distilled water and tested for agglutinating activity with guinea pig erythrocytes using TSA containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ as the diluent. The elution positions of blue dextran (Vo) and mouse IgG1 (IgG) and the bed volume of the column (Vt) are shown. The arrows indicate a titre of less than 1:8.


Vo
IgG
Vt
10
8
6
4
2
0
HAEMAGGLUTINATING TITRE (-LOG2)
20
30
40
50
60
70
80
FRACTION NUMBER

3.3.4 The fractionation of Halocynthia hispida and Aplidium australiensis haemolymph.

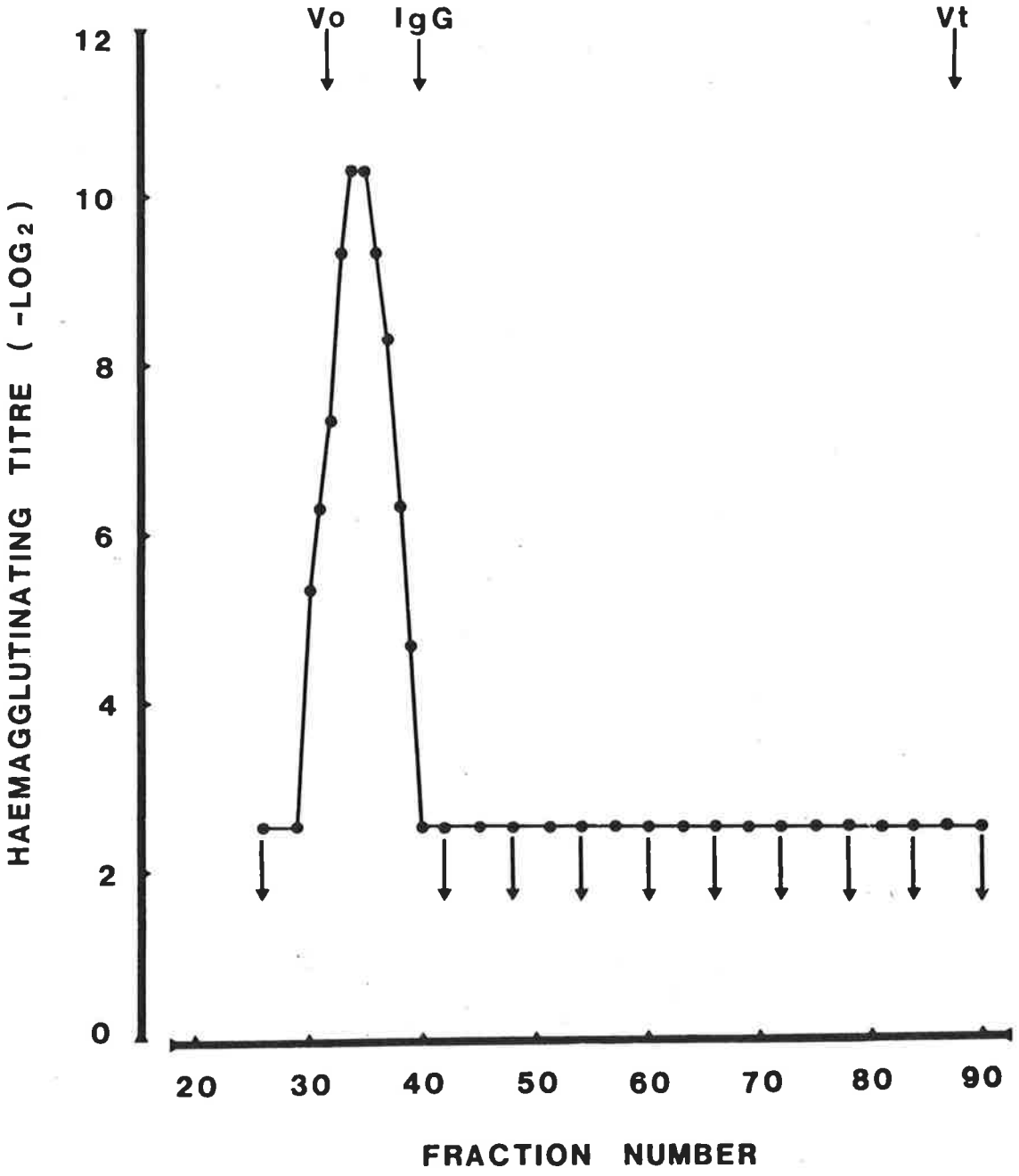
The fractionations described to date were performed on samples of haemolymph collected from ascidians within the family Styelidae, to which B. leachii belongs. To obtain further comparative data, samples of haemolymph from the ascidians Halocynthia hispida and Aplidium australiensis, which belong to the families Pyuridae and Polyclinidae respectively, were chromatographed on Sephacryl S-200.

Although the A. australiensis haemolymph had a reasonable titre with guinea pig erythrocytes (1:128), no agglutinating activity could be detected in any of the column fractions. It is possible that the agglutinin was very unstable when separated from other components of the haemolymph. Alternatively, it may have adhered to the column.

The fractionation of H. hispida haemolymph proved more successful. A sample of 4-fold concentrated haemolymph with a guinea pig agglutinating titre dependent on divalent cations of 1:2000 was loaded onto the Sephacryl column. Haemagglutination assays on the fractions, using guinea pig erythrocytes and Ca^{++}-Mg^{++} diluent, revealed a single peak of agglutinating activity (Fig 3.5) which eluted before mouse IgG in a position similar to that obtained with the divalent cation-dependent guinea pig erythrocyte agglutinins identified in the haemolymph of the styelid ascidians (Table 3.3). However, the results of the sugar inhibition experiments performed with this agglutinin differed from the others in two ways. Firstly, sucrose was as effective an inhibitor as lactose and secondly, the inhibitory capacity of D-galactose was only marginal (Table 3.3). In all other respects the H. hispida agglutinin resembled the divalent cation-dependent guinea pig erythrocyte agglutinins obtained from the three styelid ascidians.

FIGURE 3.5 Chromatography of Halocynthia hispida haemolymph on Sephacryl S-200.

Sample: 8 ml of concentrated haemolymph (guinea pig erythrocyte agglutinin titre, 1:2000) containing 0.05 M galactose and 0.1 M glucose. Eluent buffer: TSA supplemented with 0.05 M galactose, 0.1 M glucose and 0.52 M NaCl. Flow rate: 17.5 ml/hr. Temperature: 4°. Fraction size: 4.6 ml. Fractions were diluted 1:5 in distilled water and tested for agglutinating activity with guinea pig erythrocytes using TSA containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ as the diluent. The elution positions of blue dextran (Vo) and mouse IgG1 (IgG) and the bed volume of the column (Vt) are shown.


Vo
IgG
Vt
12
10
8
6
4
2
0
HAEMAGGLUTINATING TITRE (-LOG2)
20
30
40
50
60
70
80
90
FRACTION NUMBER

3.4 Detailed comparison of the agglutinins from B. leachii and Botryllus sp.

It was of interest that B. leachii and the three styelid ascidians examined here all possessed guinea pig erythrocyte agglutinins that:

(a) required divalent cations for activity,

(b) had a size consistent with a mol. wt. of 200,000-400,000 and

(c) exhibited similar although not identical inhibition patterns with the sugars tested (Table 3.3).

Moreover, the ascidian believed to be most closely related to B. leachii, Botryllus sp. (Fig 3.6) possessed an additional agglutinin resembling the B. leachii HA-2 molecule. Because of the apparent similarities between the Botryllus sp. and the B. leachii agglutinins, the properties of the Botryllus sp. agglutinins were studied in greater detail.

3.4.1 Affinity purification of the Botryllus sp. agglutinins.

The purification procedures (described in detail in Chapter 2, Section 2.11) were based on those previously developed for the B. leachii agglutinins (Schluter, 1982). The HA-1 and HA-2 agglutinins from B. leachii were separated by utilising the common affinity of these molecules for lactose and their different requirements for Ca^{++} ions. Because the Botryllus sp. agglutinins exhibited similar properties it was anticipated that these molecules could be purified by the same technique.

A column of acid-treated, lactose-substituted Sepharose 4B was equilibrated with TSA containing 5 mM EDTA. Seventy five millilitres of Botryllus sp. haemolymph (agglutinating titres: sheep erythrocytes = 1:40; guinea pig erythrocytes = 1:25,600 in the presence of Ca^{++})

FIGURE 3.6 Botryllus sp. growing subtidally on a pier piling at Stenhouse Bay.

were first dialysed against 2.5 litres of TSA/EDTA and then passed down the affinity column. The column was washed with the TSA/EDTA buffer. It was expected that under these conditions the sheep erythrocyte agglutinin would adhere to the column, since it did not require divalent cations for activity and that the effluent would contain all of the Ca^{++}-dependent guinea pig erythrocyte agglutinin. The sheep erythrocyte agglutinin could then be recovered from the column by elution with lactose.

Haemagglutination assays performed on the fractions indicated that although the greater proportion of the sheep erythrocyte agglutinin had bound to the column and was subsequently eluted by lactose, a small proportion also appeared in the effluent haemolymph (Fig 3.7). The effluent fractions (# 8-12) containing residual agglutinin were therefore pooled and re-applied to the column, which was washed and the agglutinin eluted as before. On this occasion there was no agglutinin in the effluent. All the eluate fractions containing purified agglutinin were pooled (73 ml) and retained for analysis.

After the sheep erythrocyte agglutinin had been eluted, the column was equilibrated with TSA supplemented with 5 mM $CaCl_2$. The effluent haemolymph fractions from the EDTA washes were pooled (120 ml) and made 10 mM with respect to $CaCl_2$ before being applied to the column. Although the guinea pig erythrocyte agglutinin could be detected in the TSA/$CaCl_2$ wash (effluent), approximately 98% of agglutinin had bound to the column and could be eluted with 10 mM lactose (Fig 3.8).

The column was washed with TSA/EDTA to ensure that all of the bound agglutinin had been removed. It was then re-equilibrated with TSA/$CaCl_2$ and loaded with those effluent fractions containing the residual guinea pig erythrocyte agglutinin (fractions # 4-23, 120 ml).

FIGURE 3.7 Purification of Botryllus sp. haemagglutinins by affinity chromatography on Lac-acid-Sepharose-4B.

Stage 1: Isolation of the sheep erythrocyte agglutinin.

A 75 ml sample of haemolymph (agglutination titres: 1:40 and 1:25,600 with sheep and guinea pig erythrocytes respectively) was dialysed overnight against 2.5 L TSA plus 5 mM EDTA (TSA/EDTA)and then applied at 4° to the Lac-acid-Sepharose column (see Chapter 2, Section 2.11) which had been equilibrated with TSA/EDTA. The column was washed with TSA/EDTA (21 ml/hr) and bound material was eluted with TSA + 0.1 M lactose. A second adsorption was then carried out. The column was re-equilibrated with TSA/EDTA and fractions 8-12 inclusive (total volume = 44 ml) were reapplied, the column being washed as before. Fraction size: 8.7 ml. Fractions were analysed without dialysis by measuring absorbance at 280 nm (o·····o) and assaying for sheep erythrocyte agglutinating activity, (●——●). The diluent was TSA/EDTA supplemented with 0.01 mg/ml bovine serum albumin.

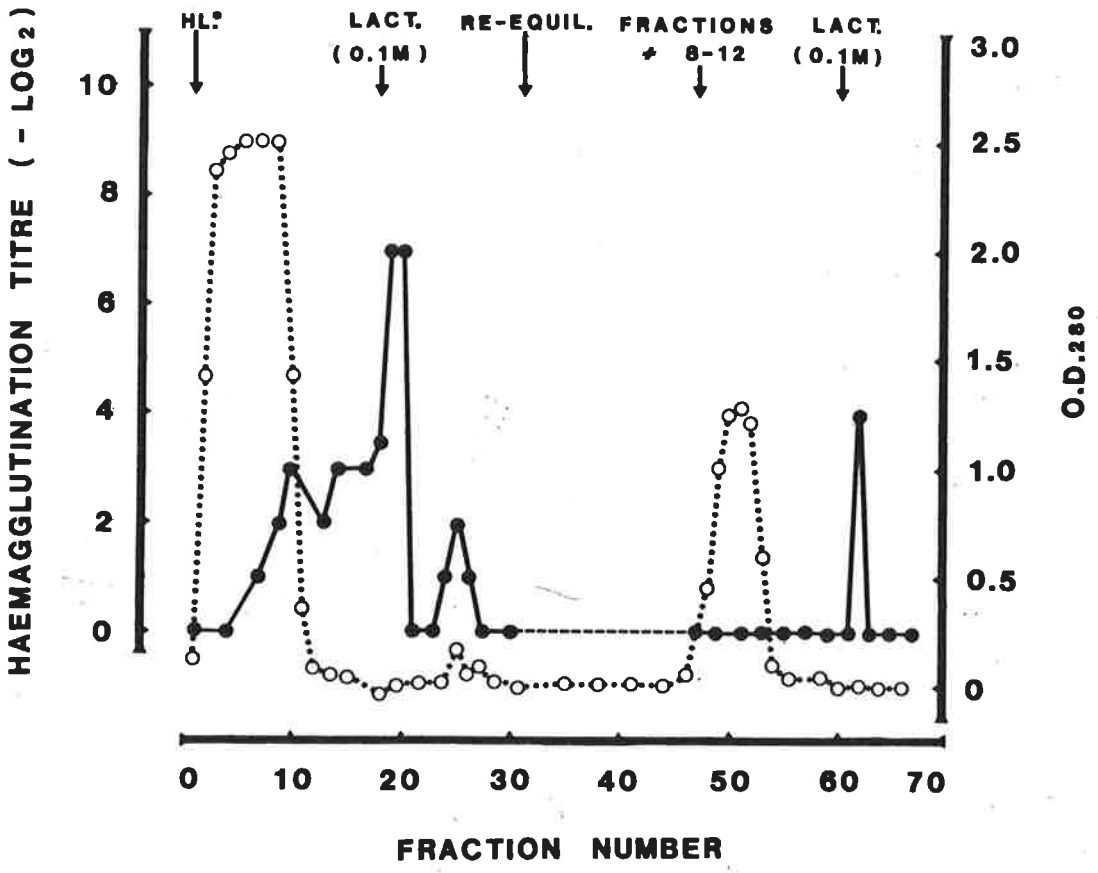
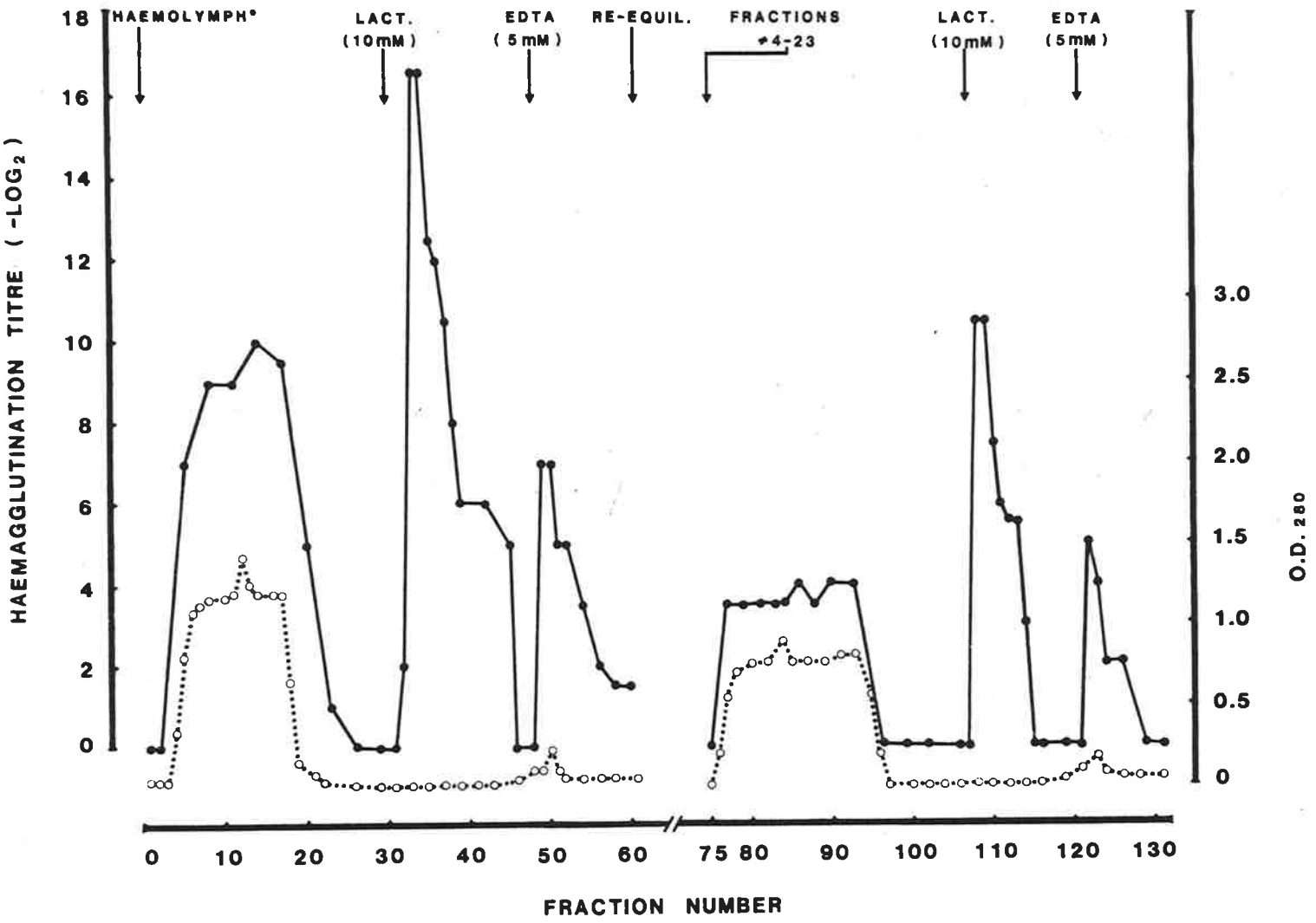

HL.
LACT. (0.1M)
RE-EQUIL.
FRACTIONS + 8-12
LACT. (0.1M)
HAEMAGGLUTINATION TITRE (- LOG2)
10
8
6
4
2
0
3.0
2.5
2.0
1.5
1.0
0.5
0
O.D.280
0
10
20
30
40
50
60
70
FRACTION NUMBER

FIGURE 3.8 Purification of Botryllus sp. haemoagglutinins by affinity chromatography on Lac-acid-Sepharose 4B.

Stage 2: Isolation of the guinea pig erythrocyte agglutinin.

The effluent haemolymph from stage 1 (Fig 3.7; fractions 1-7 and 48-54 inclusive, pooled volume = 120 ml) was reconstituted with $CaCl_2$ (to 10 mM) and then reapplied to the affinity column which had been washed exhaustively with TSA + 0.1 M lactose and equilibrated with TSA + 5 mM $CaCl_2$. After the column had been washed with TSA/Ca^{++}, bound material was eluted first with TSA/Ca^{++} plus 10 mM lactose and finally with TSA plus 5 mM EDTA (TSA/EDTA). A second adsorption step was then carried out. The column was re-equilibrated with TSA/Ca^{++} (re-equil.) and fractions 2-23 (pooled volume = 120 ml) were re-applied, the column being washed as before. Flow rate: 21 ml/hr. Fraction size: 8.7 ml. Fractions were analysed without dialysis by measuring absorbance at 280 nm (o·····o) and assaying for guinea pig erythrocyte agglutinating activity (●——●). The diluent was TSA/Ca^{++} supplemented with 0.01 mg/ml bovine serum albumin.


HAEMOLYMPH*
LACT. (10mM)
EDTA (5mM)
RE-EQUIL.
FRACTIONS #4-23
LACT. (10mM)
EDTA (5mM)
HAEMAGGLUTINATION TITRE (-LOG2)
O.D. 280
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
0.5
1.0
1.5
2.0
2.5
3.0
0
10
20
30
40
50
60
75
80
90
100
110
120
130

The column was washed and the agglutinin eluted as described. A small quantity of agglutinin was still detectable in the effluent (Fig 3.8; fractions 77-95, titres $< 2^{-4}$), but this was not considered sufficient to warrant a third passage down the column. The fractions containing the eluted guinea pig erythrocyte agglutinin were pooled (179 ml) and stored at 4°.

3.4.2 Sephacryl S-300 chromatography of the affinity purified agglutinins.

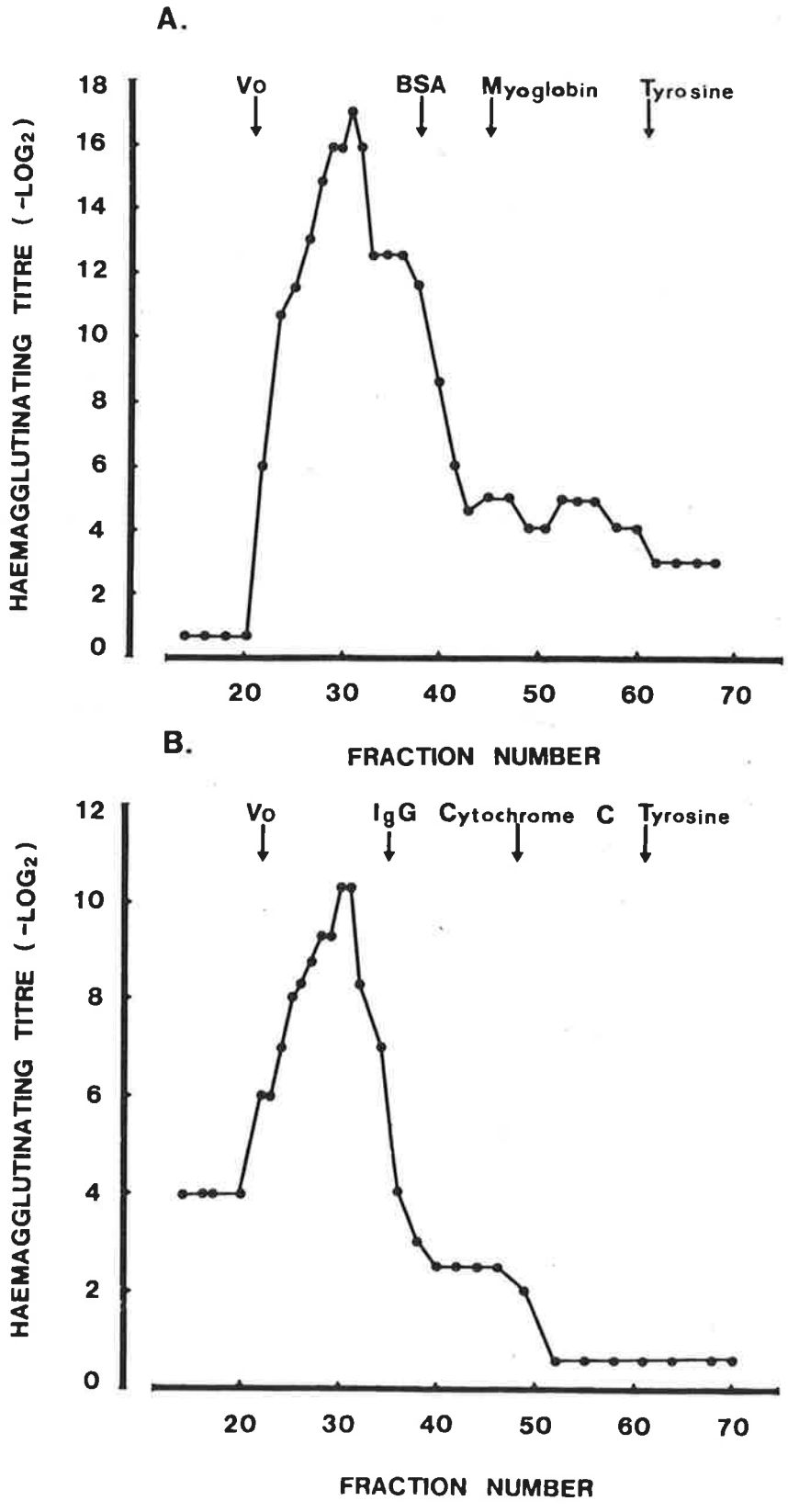
The Botryllus sp. agglutinins were further purified by chromatography on Sephacryl S-300. Using this gel, it was possible to compare the chromatographic behaviour of the Ca^{++}-dependent Botryllus sp. guinea pig erythrocyte agglutinin with that of affinity purified HA-1 agglutinin from B. leachii. The elution position of the sheep erythrocyte agglutinin from Botryllus sp. and the HA-2 agglutinin could also be compared.

The pool of Botryllus sp. guinea pig erythrocyte agglutinin from the affinity column was concentrated by ultrafiltration and a sample containing the agglutinin plus molecular weight markers was chromatographed on the Sephacryl S-300 column. The eluent buffer contained EDTA and EGTA to prevent interaction of the agglutinin with the Sephacryl (see Chapter 2, Section 2.10.2). The fractions were titrated against 0.5% guinea pig erythrocytes using a Ca^{++}-supplemented diluent. A single peak of agglutinating activity was resolved, running ahead of BSA and peaking at fractions 30 and 31 (Fig 3.9A). Affinity-purified HA-1 agglutinin from B. leachii, chromatographed under identical conditions, also eluted from the column with a peak at fractions 30 and 31 (Fig 3.9B). Hence the HA-1

FIGURE 3.9 Sephacryl S-300 chromatography of affinity-purified guinea pig erythrocyte agglutinins from B. leachii and Botryllus sp..
The column was equilibrated and washed with TSA plus 0.5 mM EDTA and 0.5 mM EGTA (TSA/EDTA/EGTA). Flow rate: 6.8 ml/hr. Fraction size: 1.9 ml. All fractions were assayed without dialysis for guinea pig erythrocyte agglutinating activity. The diluent was TSA containing 5 mM $CaCl_2$ and 0.01 mg/ml bovine serum albumin.

A. Botryllus sp. guinea pig erythrocyte agglutinin. Sample: 1.5 ml of purified agglutinin ($O.D._{280}$ = 0.1, haemagglutinating titre = 1:320,000) + 0.5 ml buffer containing markers. The agglutinin was obtained by concentrating the lactose-eluates of Fig 3.8 (fractions 33-45 and 108-119, inclusive; pooled volume = 179 ml) by ultrafiltration. The elution positions of blue dextran (Vo), bovine serum albumin (BSA), myoglobin and tyrosine are shown.

B. B. leachii guinea pig erythrocyte agglutinin (HA-1). Sample: 2 ml containing 0.02 ml purified HA-1 agglutinin ($O.D._{280}$ = 0.49; agglutinating titre = 1:160,000) and molecular weight markers. The elution positions of blue dextran (Vo), mouse IgG (IgG), cytochrome c and tyrosine are shown.


A.
Vo
BSA
Myoglobin
Tyrosine
HAEMAGGLUTINATING TITRE (-LOG2)
18
16
14
12
10
8
6
4
2
0
20
30
40
50
60
70
FRACTION NUMBER
B.
Vo
IgG
Cytochrome C
Tyrosine
HAEMAGGLUTINATING TITRE (-LOG2)
12
10
8
6
4
2
0
20
30
40
50
60
70
FRACTION NUMBER

agglutinin and the guinea pig erythrocyte agglutinin from Botryllus sp. have the same apparent molecular size.

To fractionate the sheep erythrocyte agglutinins, which are not inactivated by chelating agents, the column was equilibrated with TSA plus 0.1 M lactose, this being used as the eluent. The lactose was included to saturate the binding sites of the agglutinins and thereby prevent any interaction of the agglutinins with the Sephacryl. A sample containing the affinity-purified Botryllus sp. sheep erythrocyte agglutinin was loaded onto the Sephacryl S-300 column. The fractions were tested, without dialysis, for agglutinating activity with sheep erythrocytes (0.5% v/v in saline) using TSA/EDTA/BSA as the diluent. The agglutinin eluted off the column in four fractions, well ahead of cytochrome c and peaking at fraction 38 (Fig 3.10A). This coincided with the elution position of purified B. leachii HA-2 agglutinin (Fig 3.10B).

3.4.3 Comparison of the sedimentation coefficients of the B. leachii and Botryllus sp. agglutinins.

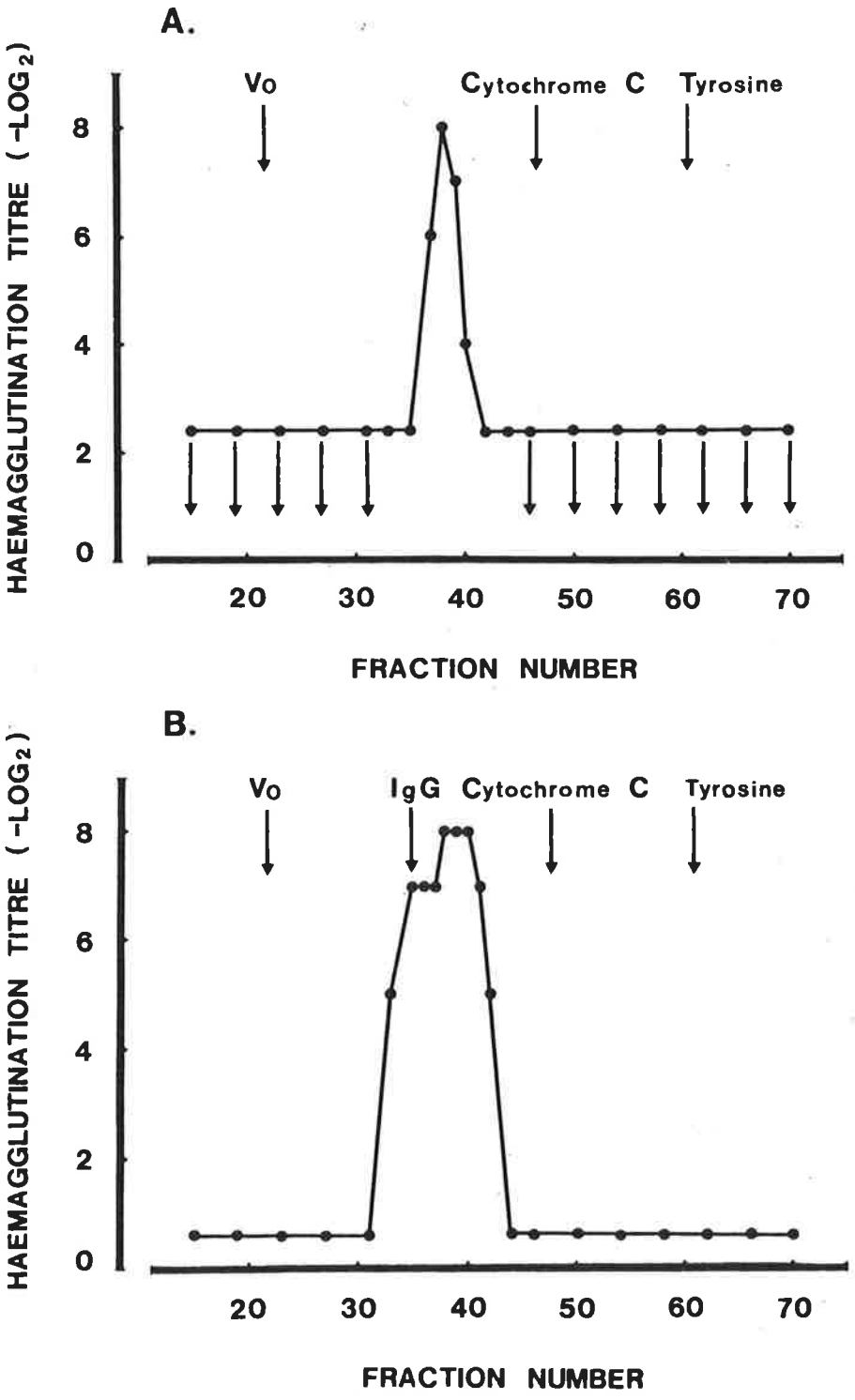
It appeared from the Sephacryl S-300 chromatography results that the HA-1 agglutinin and the Botryllus sp. guinea pig erythrocyte agglutinin were similar in size, as were the HA-2 agglutinin and the Botryllus sp. sheep erythrocyte agglutinin. These results however, were subject to the assumption that none of the agglutinins had interacted with the gel and that their passage through the column had accordingly been unretarded. An independent and more accurate estimate of the molecular size of these agglutinins was therefore sought by velocity centrifugation. The LBP-3 from B. leachii was also included as a marker, since this molecule is related to the HA-1 agglutinin and

FIGURE 3.10 Sephacryl S-300 chromatography of purified sheep erythrocyte agglutinins from B. leachii and Botryllus sp..

The column was equilibrated and washed with TSA containing 0.5 mM EDTA, 0.5 mM EGTA and 0.1 M lactose. Flow rate: 6.7 ml/hr. Fraction size: 1.9 ml. All fractions were assayed without dialysis for sheep erythrocyte agglutinating activity. The diluent was TSA containing 5 mM EDTA and 0.01 mg/ml bovine serum albumin.

A. Botryllus sp. sheep erythrocyte agglutinin. Sample: 1.5 ml of purified agglutinin ($O.D._{280}$ = 0.23; haemagglutination titre = 1:640) plus 0.5 buffer containing markers. The agglutinin was obtained by concentrating the lactose-eluates of Fig 3.7 (fractions 13-20 and 62 inclusive, pooled volume = 73 ml) by ultrafiltration. The elution positions of blue dextran (Vo), cytochrome c and tyrosine are shown.

B. B. leachii sheep erythrocyte agglutinin (HA-2). Sample: 2 ml containing of 0.5 ml purified HA-2 agglutinin ($O.D._{280}$ = 0.47, agglutinating titre = 1:1600) and molecular weight markers. The elution positions of blue dextran (Vo), mouse IgG1 (IgG), cytochrome c and tyrosine are shown.


A.
Vo
Cytochrome C
Tyrosine
HAEMAGGLUTINATION TITRE (-LOG2)
8
6
4
2
0
20
30
40
50
60
70
FRACTION NUMBER
B.
Vo
IgG
Cytochrome C
Tyrosine
HAEMAGGLUTINATION TITRE (-LOG2)
8
6
4
2
0
20
30
40
50
60
70
FRACTION NUMBER

it was possible that the Botryllus sp. agglutinin was similar to this molecule.

Sedimentation coefficients (S) were measured using isokinetic sucrose gradients (Noll, 1967; Johns & Stanworth, 1976). The sedimentation velocity of a particle is constant throughout the length of these gradients and the S value is therefore proportional to the distance the molecule travels. Hence the S value of an unknown molecule can be calculated by direct comparison with an internal marker. Details regarding the construction of the gradients are given in Chapter 2, Section 2.12.

The results from a preliminary set of gradients where the sedimentation velocity of each of the four agglutinins and LBP-3 was compared to that of I^{125}-labelled IgG suggested that the Botryllus sp. guinea pig erythrocyte agglutinin and the B. leachii HA-1 agglutinin had different S values, whereas the HA-2 molecule and the Botryllus sp. sheep erythrocyte agglutinin had the same S value. The sedimentation velocity of LBP-3 was the same as that of the HA-1 agglutinin. A second set of gradients were run to confirm these results.

Bovine serum albumin, radiolabelled with iodine-125, was used as the internal marker for the second set of gradients, with one of the following additional radiolabelled proteins also included in each gradient: HA-1 agglutinin, IgG or LBP-3. Haemagglutination assays were used to detect the Botryllus sp. agglutinins and the unlabelled B. leachii molecules. The unlabelled LBP-3 was also detected by rocket immunoelectrophoresis in gels containing anti-LBP-3 antibodies. The results of the five gradients (Fig 3.11A-E) show that in most cases the proteins moved through the gradients as well-defined, symmetrically shaped zones. Although some spreading seems apparent, particularly

when compared to the radiolabelled markers, (e.g. Figs 3.11A, C, D & E), this is mostly due to the agglutination titres having been plotted on a logarithmic rather than a linear scale. If this is taken into account, it can be seen that the bulk of each agglutinin preparation sedimented as a homogenous band. The position of LBP-3 in the gradient was determined by haemagglutination assays, but since this molecule is a very inefficient agglutinin (Schluter, 1982; Ey, unpublished data) the peak fractions were also analysed by rocket immunoelectrophoresis. The results (Fig 3.12) indicated that fractions 18 and 19 contained the most LBP-3 and hence represented the centre of the sedimented zone. This result corresponded exactly with that determined by haemagglutination assays (Fig 3.11E).

Sedimentation distances were calculated as the distance (number of fractions) between the centre of the sample at the start of the experiment and the sedimented zone at the completion of the experiment. These values and the corresponding sedimentation coefficients are given in Table 3.4. The conclusions derived from the preliminary set of gradients were confirmed by these data. It is of interest to note that although the Botryllus sp. guinea pig erythrocyte agglutinin and the HA-1 molecule eluted from the Sephacryl S-300 column in apparently identical positions (Fig 3.9), the difference in their sedimentation coefficients ($S_{20,w}$ = 5.43, vs 6.05 $\pm$ 0.18, respectively) indicates that the HA-1 agglutinin probably has a slightly greater molecular weight than the Botryllus sp. agglutinin. The latter may be a more aglobular molecule than the HA-1 agglutinin, which is known to be highly asymmetric (Schluter, 1982).

FIGURE 3.11 Velocity sedimentation ultracentrifugation in isokinetic sucrose gradients.

Gradients (12 ml) were centrifuged at 5° for 17 hr at 40,000 rpm and then fractionated by upward displacement into 0.2 ml aliquots. All samples contained I^{125}-labelled bovine serum albumin (BSA, 82,000 cpm) and usually an additional labelled protein, as intrinsic markers. Sedimentation was from left to right. Only the upper half of the gradient is shown (total length of the gradient = 60 fractions).

A. Sedimentation of B. leachii HA-2. Sample was 0.2 ml of purified HA-2 containing radiolabelled BSA and radiolabelled HA-1 (43,000 cpm). Fractions were assayed for sheep erythrocyte agglutinating activity (diluent: TSA + 5 mM EDTA + 0.01 mg/ml BSA) (▲·····▲) and for radioactivity (●——●).

B. Sedimentation of Botryllus sp. sheep erythrocyte agglutinin. Sample was 0.2 ml of purified agglutinin containing radiolabelled BSA and 40,000 cpm of radiolabelled mouse IgG. Fractions were assayed for sheep erythrocyte agglutinating activity (diluent: TSA + 5 mM EDTA + 0.01 mg/ml BSA) (▲·····▲) and for radioactivity (●——●).

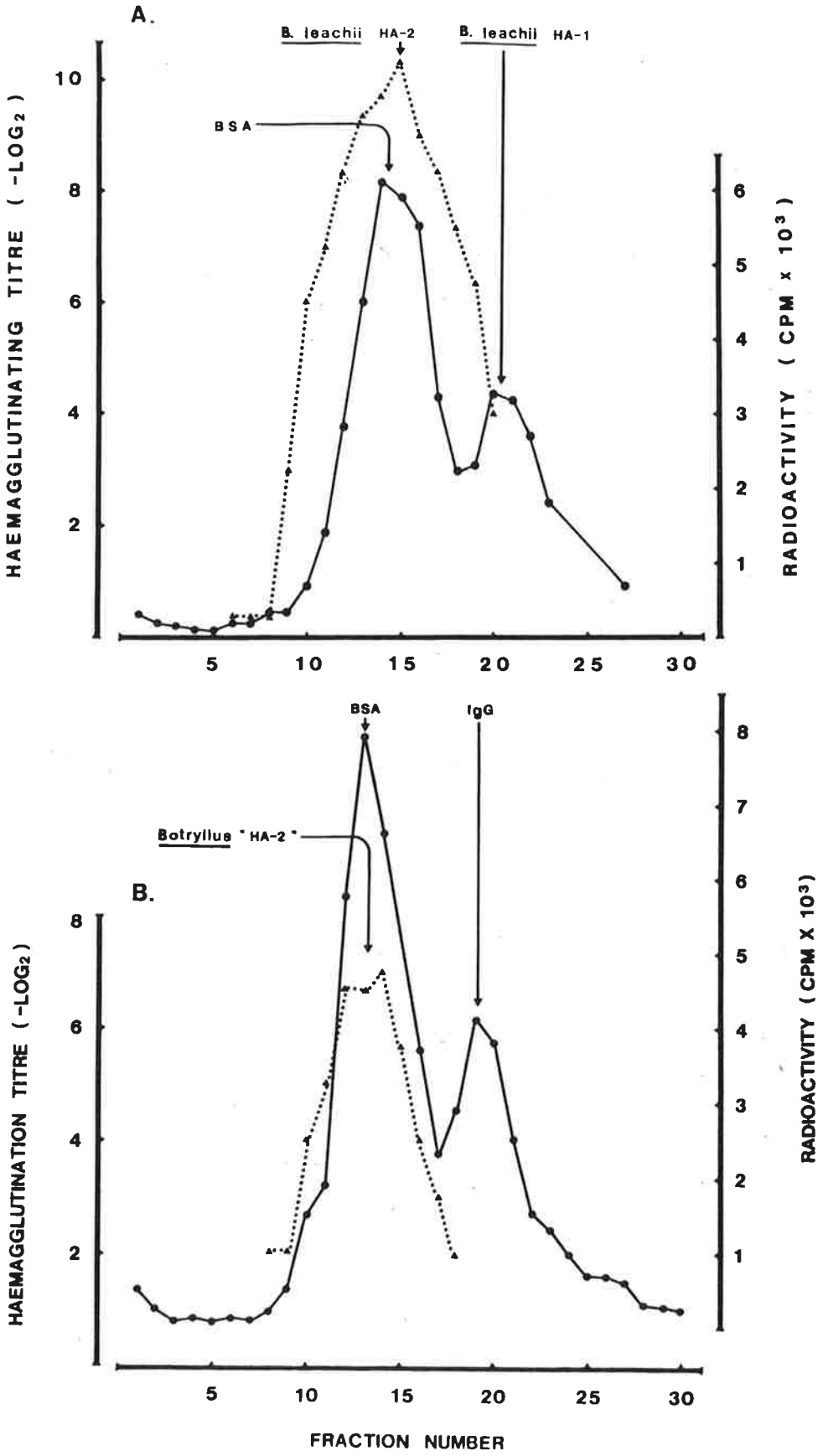

A.
B. leachii HA-2
B. leachii HA-1
BSA
HAEMAGGLUTINATING TITRE (-LOG2)
RADIOACTIVITY (CPM x 10³)
B.
BSA
IgG
Botryllus "HA-2"
HAEMAGGLUTINATION TITRE (-LOG2)
RADIOACTIVITY (CPM X 10³)
FRACTION NUMBER

FIGURE 3.11 Velocity sedimentation ultracentrifugation in isokinetic sucrose gradients.

C. Sedimentation of B. leachii HA-1. Sample was 0.2 ml of purified HA-1 containing radiolabelled BSA and 33,000 cpm of radiolabelled LBP-3. Fractions were assayed for guinea pig erythrocyte agglutinating activity (diluent: TSA + 5 mM $CaCl_2$ + 0.1 mg/ml BSA) (▲·····▲) and for radioactivity (●——●).

D. Sedimentation of Botryllus sp. guinea pig erythrocyte agglutinin. Sample was 0.2 ml of purified agglutinin containing radiolabelled BSA and 43,000 cpm of radiolabelled HA-1 . Fractions were assayed for guinea pig erythrocyte agglutinating activity (diluent: TSA + 5 mM $CaCl_2$ + 0.1 mg/ml BSA) (▲·····▲) and for radioactivity (●——●).

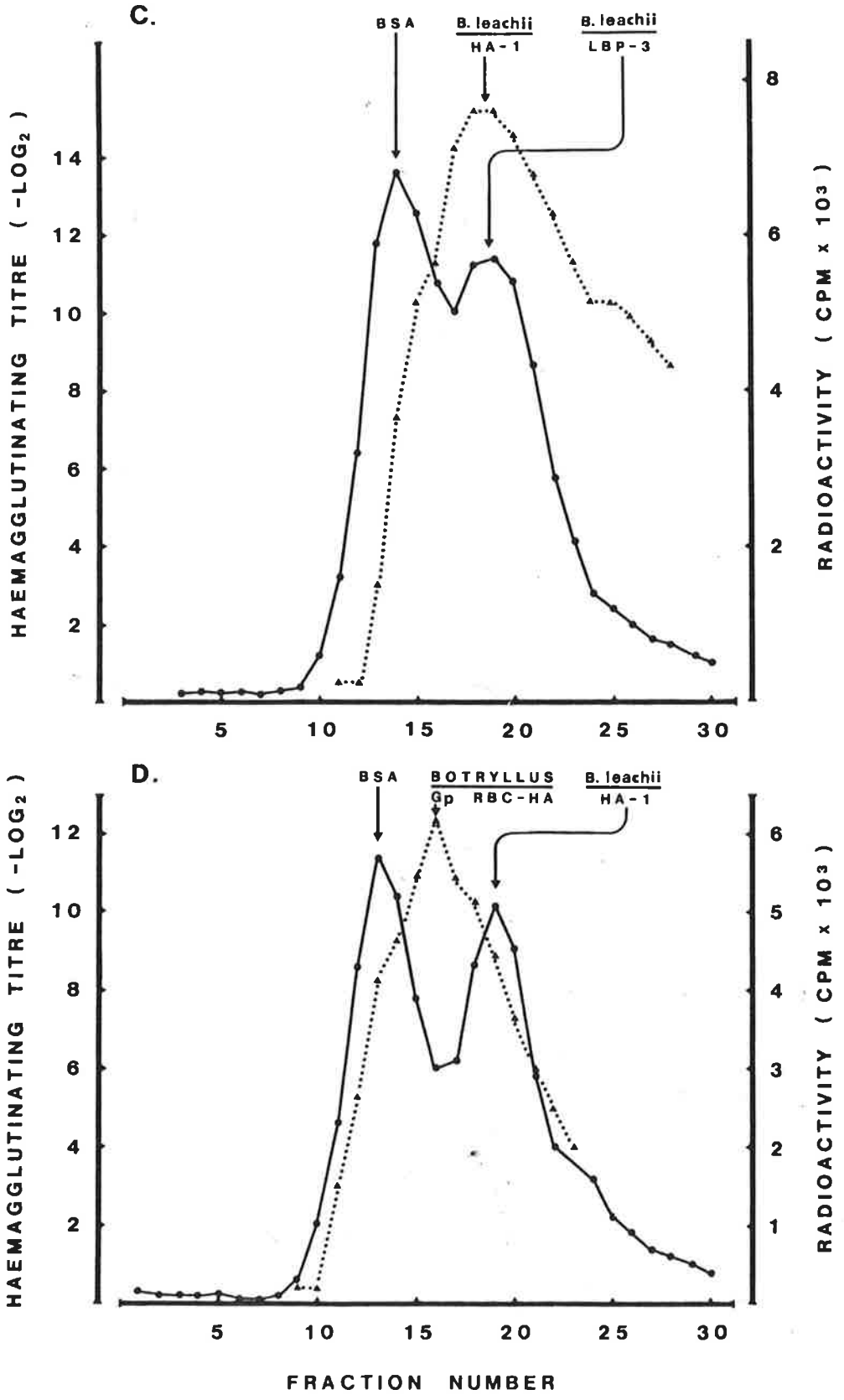

C.
BSA
B. leachii
HA-1
B. leachii
LBP-3
HAEMAGGLUTINATING TITRE (-LOG2)
RADIOACTIVITY (CPM x 10³)
D.
BSA
BOTRYLLUS
Gp RBC-HA
B. leachii
HA-1
HAEMAGGLUTINATING TITRE (-LOG2)
RADIOACTIVITY (CPM x 10³)
FRACTION NUMBER

FIGURE 3.11 Velocity sedimentation ultracentrifugation in isokinetic sucrose gradients.

E. Sedimentation of B. leachii LBP-3. Sample was 0.2 ml of purified LBP-3 containing radiolabelled BSA and 43,000 cpm of radiolabelled HA-1. Fractions were assayed for guinea pig erythrocyte agglutinating activity (diluent: TSA + 5 mM $CaCl_2$ + 0.1 mg/ml BSA) (▲·····▲) and for radioactivity (●——●).

E.

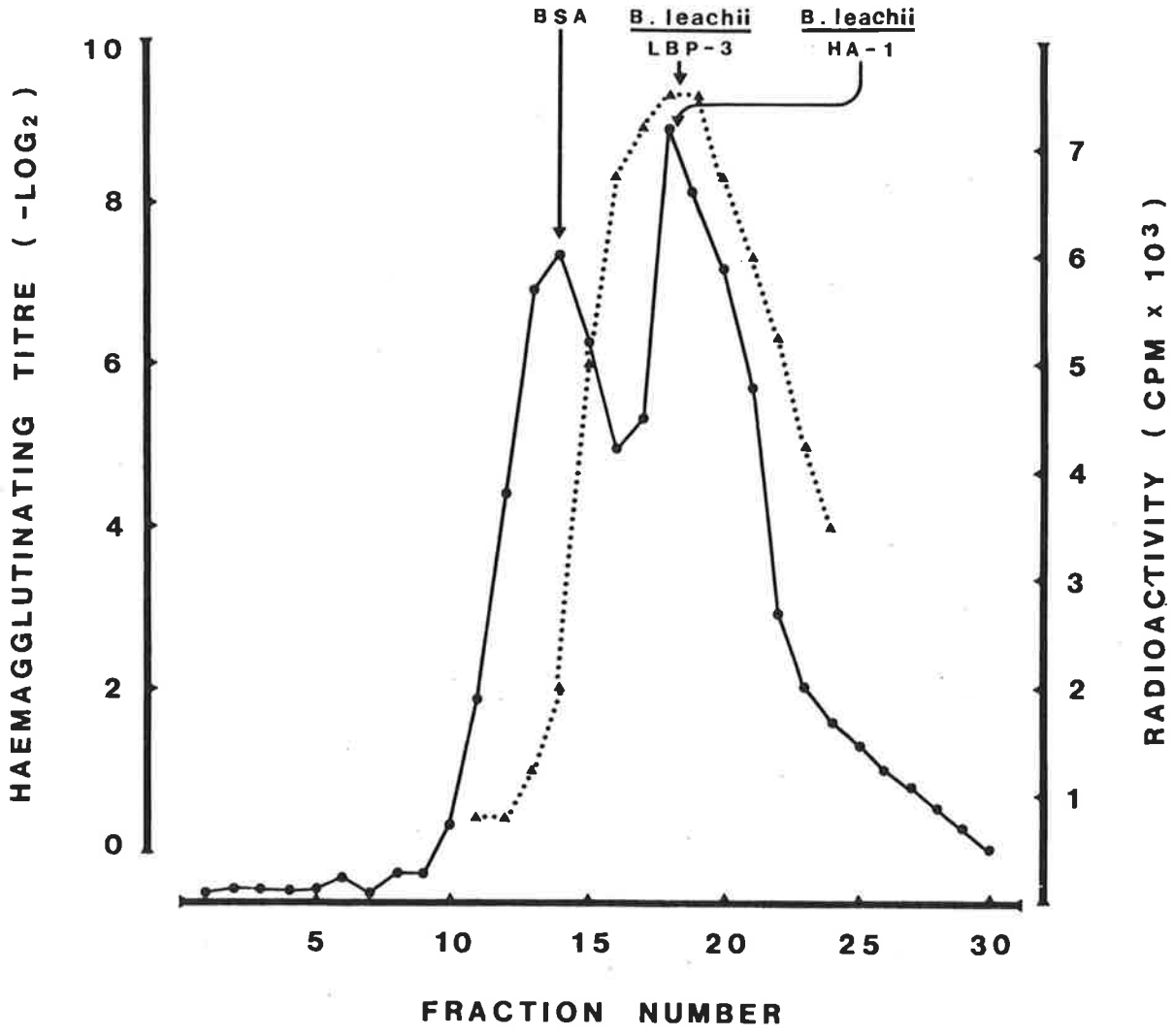

BSA
B. leachii
LBP-3
B. leachii
HA-1
HAEMAGGLUTINATING TITRE (-LOG2)
RADIOACTIVITY (CPM x 103)
FRACTION NUMBER
0
2
4
6
8
10
1
2
3
4
5
6
7
5
10
15
20
25
30

FIGURE 3.12 Rocket immunoelectrophoresis of the LPB-3 peak fractions obtained after velocity sedimentation ultracentrifugation. Samples (10 µl) of fractions 11-25 (Fig 3.11E) were loaded into wells on a prepared plate of agarose containing anti-LBP-3 immunoglobulin and electrophoresed according to the procedure described in Chapter 2, Section 2.13.2.

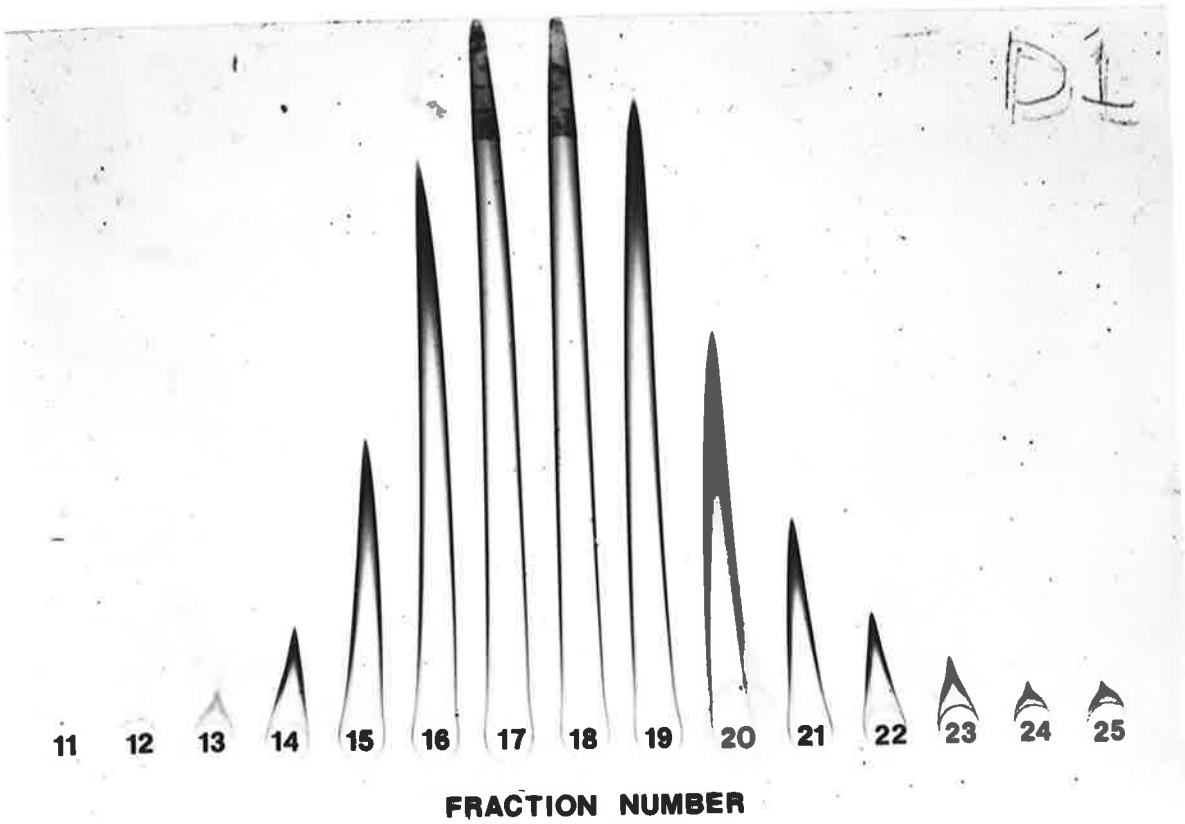

D1
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
FRACTION NUMBER

TABLE 3.4 The distance travelled by various proteins during ultracentrifugation in isokinetic sucrose gradients and the calculated sedimentation coefficients.

Gradient number	BSA* (marker)	Proteins analysed				
		Botryllus sp.		*B. leachii*		
		GpRBC-HA¶	SRBC-HA§	HA-1$	HA-2	LBP-3
		distance sedimented ($S_{20,w}$)				
1	13	16(5.43)#	-	19(6.45)	-	-
2	13	-	13(4.41)	-	-	-
3	14	-	-	18(5.67)	-	18.5(5.83)
4	14	-	-	18.5(5.84)	-	19(5.98)
5	14.5	-	-	20.5(6.23)	14(4.26)	-

* Bovine serum albumin.

¶ Guinea pig erythrocyte agglutinin.

§ Sheep erythrocyte agglutinin

Sedimentation coefficient (Svedberg units, $S_{20,w}$) = (Distance travelled by test protein / Distance travelled by BSA) x Sedimentation coefficient of BSA. The value used for BSA was 4.41S (Phelps & Putnam, 1960; Squire *et al.*, 1968).

$ Mean $S_{20,w}$ = 6.05 $\pm$ 0.18. Value estimated by Schluter (1982) was 5.68 $\pm$ 0.22.

3.4.4 The binding specificities of the Botryllus sp. and B. leachii agglutinins.

An important aspect of a comparative study of molecules is the investigation of functional similarities. Although not unequivocal, one way of examining functional similarities is to determine the specificity of the binding site(s). A method commonly employed with invertebrate agglutinins is to measure the inhibitory effects of various sugars on agglutination. The free sugars probably compete with the erythrocyte binding sites for the agglutinin, hence a sugar most resembling the erythrocyte binding site is expected to be the best inhibitor.

Earlier experiments, testing the effect of various sugars on the agglutination titres of haemolymph from Botryllus sp. and B. leachii, had indicated that some sugars were more effective inhibitors than others. In order to compare in a quantitative manner the effectiveness of the different sugars, the concentration of sugar required to halve the titre of a particular agglutinin preparation was determined and this was used as the measure of inhibitory capacity. A panel of nine sugars was chosen. The capacity of these sugars to inhibit the agglutination of sheep and guinea pig erythrocytes by B. leachii haemolymph ranged from very good (e.g. lactose) to not detectable (e.g. mannose, with guinea pig erythrocytes) (Table 3, Schluter et al., 1981). It was hoped by using sugars with a range of inhibitory abilities that any fine differences in the specificities of the B. leachii and Botryllus sp. agglutinins might be detected. As a precaution against random variation, the inhibition experiments for the HA-1 agglutinin and the Botryllus sp. guinea pig erythrocyte agglutinin were performed in parallel, on the same day with the same suspension of erythrocytes. Experiments with the HA-2 molecule and the Botryllus sp.

sheep erythrocyte agglutinin were performed similarly. The agglutinins were titrated initially in sugar at a final concentration of 67 mM and the agglutination titre compared to that of the control (no sugar added). If inhibition occurred, the concentration of sugar was reduced by diluting 1:2 in saline and the agglutinins were retitrated. This procedure was repeated until the concentration of sugar that reduced the haemagglutination titre by approximately 50% could be estimated. In each case purified agglutinins were used.

The results (Table 3.5) revealed no difference in the binding specificity of the B. leachii HA-1 agglutinin and Botryllus sp. guinea pig erythrocyte agglutinin. However, the HA-2 agglutinin and the sheep erythrocyte agglutinin from Botryllus sp. appeared to have distinct specificities, since lactose, melibiose and D-galactose were all more effective inhibitors of the Botryllus sp. agglutinin than of the HA-2 molecule. These two agglutinins had similar specific agglutination activities, a 1 mg/ml solution of each exhibiting a titre of 1:4,000-5,000 against 0.5% sheep erythrocytes.

3.4.5 Antigenic cross-reactivity of the Botryllus sp. and B. leachii agglutinins.

The possibility that the guinea pig and sheep erythrocyte agglutinins from Botryllus sp. are structurally related to the B. leachii HA-1 and HA-2 molecules was examined serologically by testing for antigenic cross-reactivity. Purified agglutinins from both of the ascidian species were analysed by the double immunodiffusion method of Ouchterlony (Weir, 1978) using rabbit antisera against the purified B. leachii agglutinins. The results are shown in Figs 3.13 and 3.14.

TABLE 3.5 The capacity of sugars to inhibit the agglutination of erythrocytes by agglutinins from *B. leachii* and *Botryllus* sp.[¶].

Sugar	Concentration of sugar (mM) required to inhibit by 50% the agglutination of:			
	guinea pig erythrocytes*		sheep erythrocytes§	
	HA-1 agglutinin	*Botryllus* agglutinin	HA-2 agglutinin	*Botryllus* agglutinin
Lactose	0.5	0.5 - 0.25	4 - 8	0.5
Melibiose	0.5 - 0.25	0.5	>67	32
D-galactose	0.5	0.5 - 1	>67	16
L-arabinose	2	2	ND	ND
Glucose	8	8	>67	>67
Maltose	8	8	>67	>67
D-arabinose	32	32	>67	>67
Sucrose	67	67	>67	>67
D-fucose	ND	ND	>67	>67
Mannose	>67	>67	ND	ND

¶ The agglutinins used had been affinity purified and chromatographed on Sephacryl S-300. The HA-2 agglutinin and the Botryllus sp. sheep erythrocyte agglutinin were dialysed overnight against TSA before use. Agglutination was assessed by mixing 2-fold serial dilutions of the agglutinin with an equal volume (40 µl) of sugar plus 80 µl of a 0.5% erythrocyte suspension. The maximum concentration of sugar tested was 67 mM. If inhibition occurred, the sugar solution was diluted in saline and the assay was repeated with lower concentrations of sugar until that concentration which decreased the agglutination titre by approximately 50% could be estimated.

* Diluent: TSA containing 5 mM $CaCl_2$ and 0.01 mg/ml BSA.

§ Diluent: TSA containing 5 mM EDTA and 0.01 mg/ml BSA.

ND = Not done.

The absence of any precipitin line between the Botryllus sp. guinea pig erythrocyte agglutinin and the anti-(HA-1) serum, compared with the very intense line found with the HA-1 agglutinin, suggested that there was little or no antigenic cross-reactivity between these two agglutinins (Fig 3.13). However the lack of any precipitin line with the Botryllus sp. agglutinin could well have been due to the low protein content of the sample (< 0.1 mg/ml). Unfortunately there was insufficient material left at this stage to attempt further studies on a more concentrated sample.

In contrast to the preceding results, the anti-(HA-2) antibodies were reactive with both the B. leachii and the Botryllus sp. sheep erythrocyte agglutinin (Fig 3.14). The partial fusion of the HA-2 and Botryllus sp. sheep erythrocyte agglutinin precipitin lines (Fig 3.14C & D) indicated that these molecules indeed share some antigenic determinants. Furthermore, the formation of a spur on the HA-2 precipitin line showed that the HA-2 agglutinin possessed in addition a number of unique determinants, i.e. determinants not expressed on the Botryllus sp. agglutinin. No spur was detected on the Botryllus sp. agglutinin precipitin line since the antibodies had been raised against the B. leachii (HA-2) agglutinin and there would have been none which recognised determinants unique to the Botryllus sp. agglutinin. These determinants, if they exist, would be detected in a reciprocal test using antibodies raised against the Botryllus sp. sheep erythrocyte agglutinin.

FIGURE 3.13 Analysis of Botryllus sp. guinea pig erythrocyte agglutinin (GpRBC-HA) by double radial immunodifusion. Samples were loaded into 3 mm diameter wells in a 1% agarose gel containing TSA + 1 mM EDTA. The plates were incubated at 37° for 48 hr and then pressed, washed, dried and stained with Coomassie blue. The centre well of each plate contained 10 µl of anti-(HA-1) rabbit IgG (12.26 mg/ml).

A. B. leachii HA-1

$O.D._{280}$ = 1.0

Well #	Sample applied
1	10 µl, diluted 1/2
2	10 µl, diluted 1/4
3	10 µl, diluted 1/8
4	10 µl, diluted 1/16
5	10 µl, diluted 1/32
6	10 µl, diluted 1/64

B. GpRBC-HA

$O.D._{280}$ = 0.1

Well #	Sample applied
1	20 µl, neat
2	20 µl, neat
3	10 µl, neat
4	10 µl, neat
5	10 µl, diluted 1/2
6	10 µl, diluted 1/2

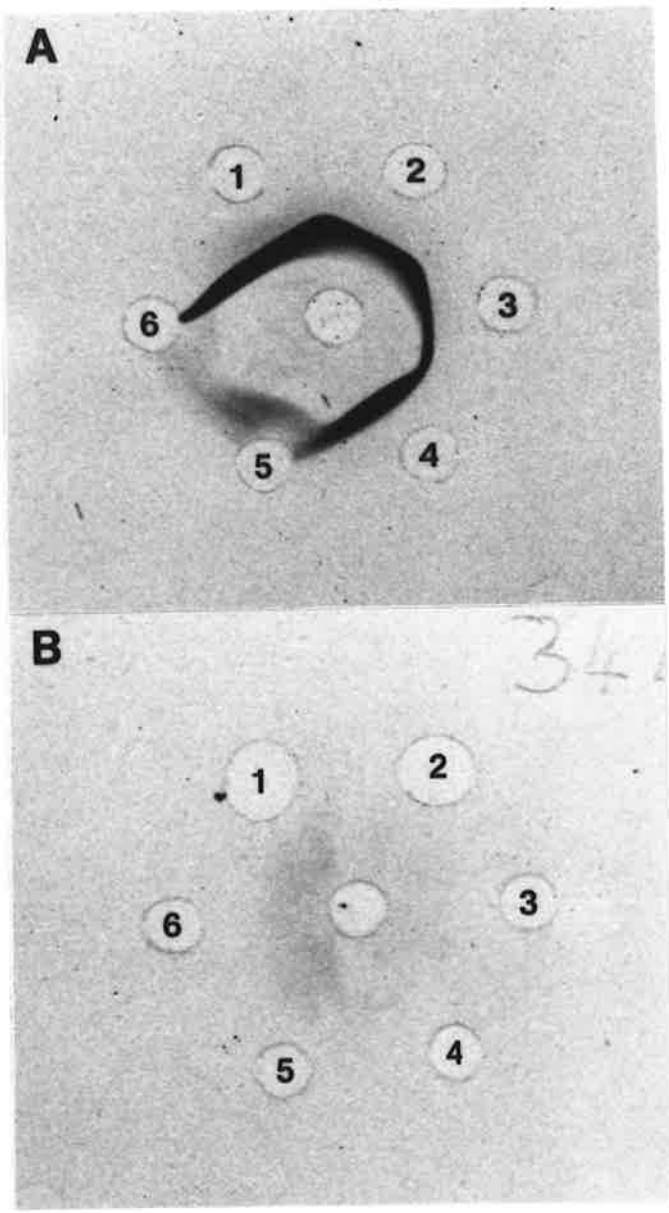

A
1
2
3
4
5
6
B
1
2
3
4
5
6

FIGURE 3.14 Analysis of Botryllus sp. sheep erythrocyte agglutinin by double radial immunodiffusion. Samples (10 µl) were loaded into 3 mm diameter wells in a 1% agarose gel containing TSA + 1 mM EDTA + 0.2 M lactose. The plates were incubated at 37° for 48 hr and then pressed, washed, dried and stained with Coomassie Blue R-250. The samples were diluted in TSA containing 5 µg/ml BSA (carrier protein).

A,B: Centre well: Affinity-purified rabbit anti-(HA-2) antibody (0.7 mg/ml).

Sample wells: Purified B. leachii HA-2 agglutinin (0.8 mg/ml).

Values represent the reciprocal of the dilution.

C,D: Centre well: Affinity-purified rabbit anti-(HA-2) antibody (0.7 mg/ml).

Sample wells: C = Affinity-purified Botryllus sp. sheep erythrocyte agglutinin (0.05 mg/ml)

D = 8-fold concentrated Botryllus sp. haemolymph.

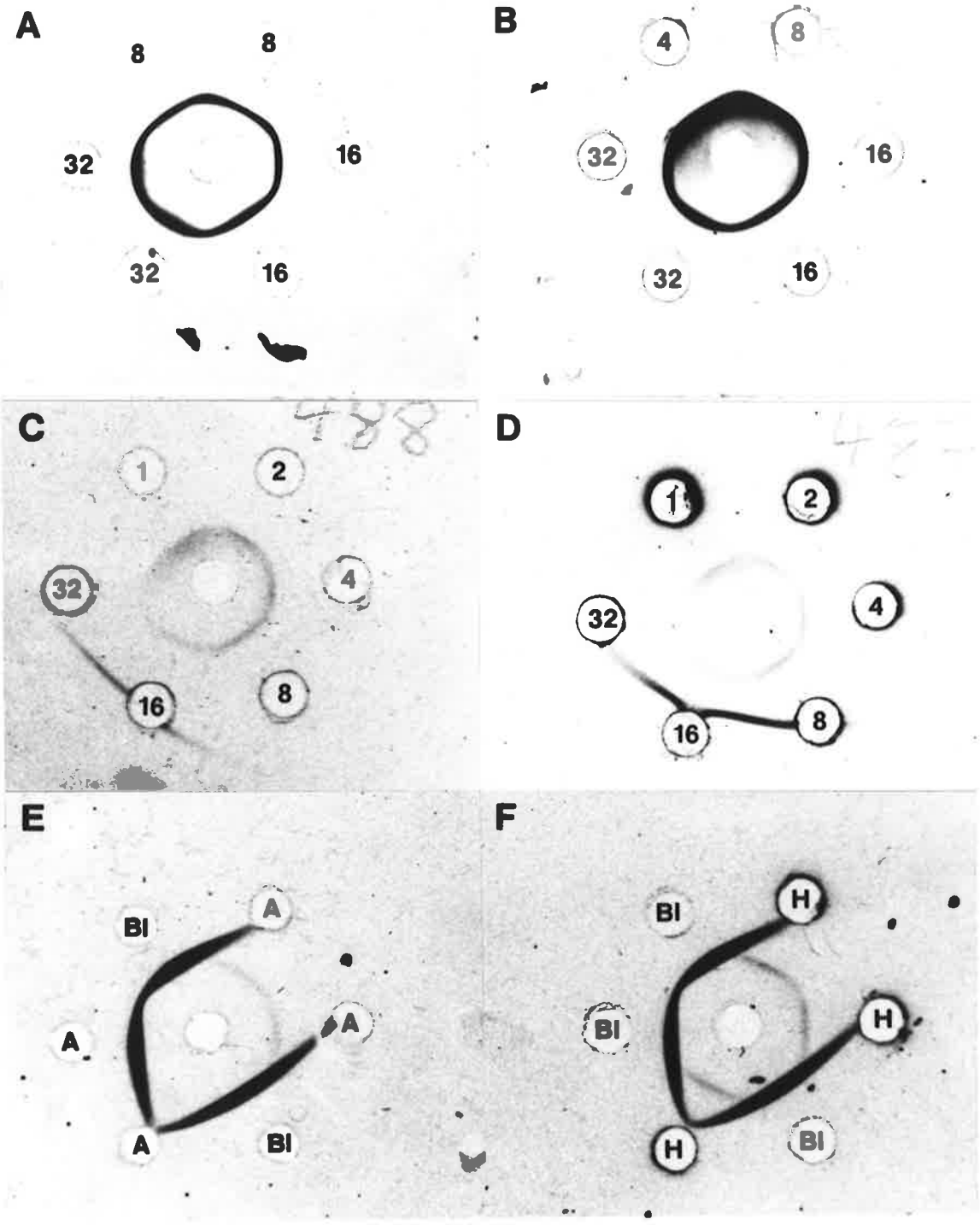
Values represent reciprocal dilutions. The precipitin lines passing through the lower left wells are artifacts caused by the close proximity of wells belonging to other immunodiffusion assays.

E,F: Centre well: Affinity-purified rabbit anti-(HA-2) antibody (0.7 mg/ml).

Sample wells: B. leachii HA-2, (1:16 dilution; 0.05 mg/ml) (B1).

Botryllus sp. agglutinin (purified; 1:4 dilution; 0.0125 mg/ml) (A).

Botryllus sp. haemolymph (8 x concentrated; 1:4 dilution) (H).


A
8
8
32
16
32
16
B
4
8
32
16
32
16
C
1
2
32
4
16
8
D
1
2
32
4
16
8
E
Bl
A
A
A
A
Bl
F
Bl
H
Bl
H
H
Bl

3.5 Discussion.

There are a number of factors to be considered when embarking on a comparative study of this type. For example:

(a) Conclusions as to the phylogenetic relationships between the animals under study are dependent on the taxonomy of the group. Although the phylogenetic relationships that have been proposed for the major (cosmopolitan) ascidian families (Miller, 1966) is presumably well based, the taxonomy of the extensive ascidian fauna in South Australian waters is in its infancy. This means that with more extensive collecting in these waters, new genera and even new families may need to be erected, in which case current ideas about phylogeny would require re-assessment.

(b) The animals (ascidians) examined today have undoubtedly changed from their ancestral forms as they adapted to a variety of different habitats and life history patterns. Thus if an agglutinin is not detected in one member of a particular family it does not necessarily follow that all members lack that molecule. An examination of Table 3.1 shows that this is indeed the case.

(c) The number of agglutinins identified is limited by the detection methods employed. In this study, the agglutinins detected were restricted to those that both occur in reasonable concentration in the body fluids and react with at least one of the five types of erythrocyte used in the haemagglutination assays. Additional agglutinins, that react with other cells or organisms, e.g. bacteria or protozoa, may well occur. In fact, preliminary data have suggested that B. leachii haemolymph contains agglutinins for various bacteria which appear different from the HA-1 and HA-2 molecules (results not shown). Some agglutinins could be concentrated within particular organs, like the Helix pomatia

erythrocyte agglutinin which is located predominantly within the snail's albumin gland (Uhlenbruck & Prokop, 1966; Prokop et al., 1968; Ishiyama & Uhlenbruck, 1972).

With considerations such as these in mind, it is clear that data such as are presented in Table 3.1 must be viewed with some caution. Nevertheless, information of this nature is valuable in providing base line data for more extensive studies, particularly those aimed at characterising and purifying the agglutinins.

A number of other workers have detected erythrocyte agglutinins in the haemolymph of ascidians (review: Ey & Jenkin, 1982). However, virtually no information is available as to the structure or binding site specificity of these molecules. In the absence of such information it is impossible to determine whether any of the agglutinins detected in this study are structurally related to those described in the literature, particularly as the agglutinating titre of haemolymph, at least for some species, appears to vary depending on the locality from which the animal is collected. The only ascidian agglutinin to have been isolated previously is one from the haemolymph of Halocynthia pyriformis. Analysis by velocity sedimentation in sucrose density gradients and by SDS-polyacrylimide gel electrophoresis (SDS-PAGE) indicated that this molecule has a native molecular weight of 800,000 and is composed of subunits with a molecular weight of 20,000 (Form et al., 1979). Its binding specificity was described as complex. Anderson & Good (1975) had earlier described an agglutinin from H. pyriformis that agglutinated horse and human A erythrocytes and which could be inhibited by N-acetyl-neuraminic acid at a concentration of 0.25 mM. Other sugars tested (D-glucose, D-galactose, D-mannose, L-fucose, L-arabinose, D-xylose N-acetyl-D-galactosamine and N-acetyl-D-glucosamine) were not inhibitory at this concentration. It

is not known at present whether the agglutinin examined by Anderson & Good is the same as that purified by Form et al. (1979). Like the guinea pig erythrocyte agglutinin from the haemolymph of Halocynthia hispida (detected in this study), the H. pyriformis agglutinin was reported to require Ca^{++} ions for activity. However, the data on these molecules is too limited to determine if they may be structurally related.

A paper by Vasta et al. (1982), which appeared in the literature during the final stages of preparing this thesis, indicated that these workers had obtained results similar to those of Tables 3.1 & 3.2 (this thesis) with haemolymph from 10 species of North American ascidians. They found that all the species tested possessed haemagglutinins (lectins) but, like the data of Table 3.1, marked variation existed between the titres of closely related species. It was concluded from both the specificities of the haemagglutinins, as determined by inhibition with sugars and glycoconjugates, and cross adsorbtion data that all but one species possessed multiple haemagglutinins. However, these conclusions were not confirmed by the separation or purification of any of the haemagglutinins.

As more ascidian species are investigated and the methods for detecting and characterising the agglutinins are improved, it may be possible to discern changes in the structure and binding site specificity of a particular agglutinin that have occurred over time as a consequence of divergence and adaptation. The data obtained from a limited number of styelid ascidians indicate that this has already been achieved, to a limited extent, for the HA-1 and HA-2 agglutinins.

Molecules with HA-1 like properties were detected in the haemolymph of Polycarpa papillata, Cnemidocarpa edtheridgii and a species of Botryllus sp.. Chromatographic analysis on Sephacryl S-200

indicated that haemolymph collected from each of the three species contained a guinea pig erythrocyte agglutinin having an apparent molecular weight of 200,000-400,000 and requiring Ca^{++} and/or Mg^{++} ions for activity. Furthermore, the pattern of agglutinating activity obtained when these molecules were tested in the presence of five different sugars resembled that obtained with the B. leachii HA-1 agglutinin, indicating that the different agglutinins had similar if not identical binding specificities. Of particular interest was the finding that Halocynthia hispida, an ascidian from another family (Pyuridae) also possessed a Ca^{++}-dependent, guinea pig erythrocyte agglutinin that chromatographed on Sephacryl S-200 in approximately the same position as the HA-1 molecule. The H. hispida agglutinin did appear to have a slightly different binding site specificity from that exhibited by the other "HA-1-like" agglutinins. The results obtained are in accordance with the notion that the guinea pig erythrocyte agglutinins detected in the haemolymph of these ascidians may be structurally related to the HA-1 agglutinin from B. leachii. However, one would need to obtain accurate molecular weight estimates of the native proteins and detailed information on subunit structure, binding site specificities and antigenic cross-reactivities, before any confident assessment of the similarity of these molecules with the HA-1 agglutinin could be made.

Chromatography of the Botryllus sp. haemolymph on Sephacryl S-200 revealed not only an "HA-1-like" molecule but also a sheep erythrocyte agglutinin which superficially resembled the HA-2 molecule. Fortunately, both of the Botryllus sp. agglutinins could be purified using a techinque developed for the B. leachii agglutinins. This allowed a detailed comparison of the purified guinea pig and sheep

erythrocyte agglutinins from Botryllus sp. with the HA-1 and HA-2 agglutinins, respectively, to be undertaken.

The HA-1 agglutinin and the Botryllus sp. guinea pig erythrocyte agglutinin were similar in at least two respects. The sugar inhibition data indicated that these two molecules had essentially identical binding site specificities. They also eluted at the same position when chromatographed on Sephacryl S-300. Analysis by velocity sedimentation in sucrose density gradients, however, indicated that the HA-1 molecule may be slightly larger than the Botryllus sp. guinea pig erythrocyte agglutinin. There was no detectable antigenic cross-reactivity between these two agglutinins, as determined by immunodiffusion against an anti-(B. leachii HA-1) antiserum, although this negative result may have been a consequence of using the Botryllus sp. agglutinin at an insufficient concentration.

A preliminary analysis by SDS-PAGE (performed by Dr P.L. Ey) of tha partially purified (i.e. by affinity chromatography only) Botryllus sp. guinea pig erythrocyte agglutinin revealed, under reducing conditions, two distinct polypeptide bands having apparent molecular weights of approximately 31,000 and 18,000, respectively. No other bands were evident. If these polypeptides represented the subunits of the agglutinin, rather than contaminants, this agglutinin has a structure quite distinct from that of the B. leachii HA-1 (single subunit, mol. wt. = 28,500) or LBP-3 (2 subunits, mol. wts. 22,500 & 28,500). All three proteins have a strict Ca^{++}-dependent binding specificity for guinea pig erythrocytes. Although the sugar inhibition data (Table 3.5) revealed no difference in apparent specificity between the Botryllus sp. agglutinin and the B. leachii HA-1, a comparison of their specific agglutinating activities (based on $O.D._{280}$) indicated that the Botryllus sp. agglutinin was at least 50 times more active

than the HA-1. These preliminary results suggest that the two agglutinins are not related in gross structure, but they appear to possess very similar if not identical binding sites. The possession of the same binding site within different polypeptide chains is quite conceivable in the light of recent discoveries concerning eukaryotic gene structure (Blake, 1979; Steinmetz et al., 1981). The difference in specific activity might be explained if the Botryllus sp. agglutinin contains more binding-site subunits per molecule than does the HA-1. Unfortunately there was insufficient material to continue these studies. It would seem worthwhile to repeat these experiments using a more highly purified preparation of the Botryllus sp. agglutinin to confirm or negate these results.

The Botryllus sp. sheep erythrocyte agglutinin and the HA-2 molecule also share a number of properties. They are the same molecular size, as determined by both Sephacryl S-300 chromatography and velocity sedimentation, and preliminary SDS-PAGE data (obtained by Dr P.L. Ey) indicate that the Botryllus sp. agglutinin, like the B. leachii HA-2 agglutinin (Schluter, 1982), is a dimer comprising two non-covalently linked, presumably identical subunits which have a molecular weight of about 32,000. Thus on a structural basis, these two agglutinins cannot be distinguished. The finding that they are antigenically cross-reactive but not antigenically identical strongly supports the idea that the genes coding for these two agglutinins have evolved from a common precursor gene. That the two agglutinins, although highly related, are not identical is shown not only by the serological data but also by the slight difference in the binding site specificity of the two proteins, indicated by the sugar inhibition data.

It may be concluded from these results that molecules clearly similar to the HA-1 and HA-2 agglutinins exist in species other than

B. leachii, since a closely related ascidian, Botryllus sp., has in its haemolymph one agglutinin which has similarities with the HA-1 molecule and another agglutinin which is clearly structurally-related to the HA-2 molecule. The haemolymph of two other styelid ascidians, Polycarpa papillata and Cnemidocarpa edtheridgii, also contain agglutinins which appear similar to the B. leachii HA-1 agglutinin, although more detailed structural data are required to assess whether or not these molecules are structurally related. Species from other families (e.g. Halocynthia hispida) could also have molecules structurally related the the B. leachii agglutinins. The apparently widespread occurrence and conserved structure of these molecules suggests that they perform some necessary function in these animals. However, it remains to be determined by further investigations whether or not these molecules have any role in immune recognition or defence in these animals.

Chapter 4.

HAEMAGGLUTININ LEVELS IN HAEMOLYMPH FROM B. leachii FOLLOWING A CHANGE IN ENVIRONMENT OR INJECTION WITH ERYTHROCYTES.

4.1 Introduction

Although it has been known for some time that foreign particles can be phagocytosed by cells circulating in the haemolymph of ascidians (Fulton, 1920; Smith, 1970; Anderson, 1971; Brown & Davies, 1971 and Bang, 1975), the method by which ascidian haemocytes recognise particles as foreign has not been addressed in any detail. Wright (1974) speculated a haemagglutinin found in the haemolymph of Ciona intestinalis could be involved in the phagocytosis of erythrocytes by haemocytes, but little experimental evidence existed to support this claim.

A characteristic of the immune response of vertebrates is the increase in the production of antibody after challenge with antigen. Immunological memory for the priming antigen is usually displayed when a second dose of the antigen results in the production of a significantly greater amount of specific antibody than that induced by the initial immunization. Representatives from all classes of vertebrates, including the primitive fishes, have been shown to exhibit the above mentioned characteristics (Marchalonis, 1977). Since protochordates are believed to occupy the phylogenetic position between the vertebrates and the invertebrates, it was of particular interest to determine whether the production of a protochordate opsonin or agglutinin could also be stimulated following challenge.

As indicated in the preceding chapter, at least two haemagglutinins have been demonstrated in the haemolymph of B. leachii (Schluter, et al., 1981). The HA-1 agglutinin is specific for guinea pig erythrocytes whereas the HA-2 molecule agglutinates a variety of erythrocytes including those from the sheep, chicken and guinea pig and also mouse macrophages. If the haemocytes or other phagocytic cells of B. leachii possess carbohydrate receptor sites for the HA-2 or HA-1

agglutinins, either of these agglutinins could behave as "natural opsonins" for particles bearing the appropriate receptor sites by mediating the adhesion of the particles to the phagocytes. On this basis, it is plausible that one or both of these agglutinins could serve *in vivo* as recognition molecules in this species. This possibility leads one to ask whether the haemagglutinin(s) detected in the haemolymph have been synthesised and secreted in response to previous exposures to stimulatory material. This question was investigated in two ways: firstly by examining the effect of a different external environment on the levels of the HA-1 agglutinin and secondly, by attempting to stimulate the production of the HA-2 agglutinin in a specific manner.

It had been noted previously that the HA-1 agglutinating titre of haemolymph obtained from colonies collected at two distantly separated locations, Edithburgh (137° 45' E, 35° 5' S) and the Outer Harbour of Pt. Adelaide (138° 27' E, 34° 47' S), was different. The differences in HA-1 titre could have been due to differences in the marine environment at the two localities. For example, water quality is likely to be different between the two sites, for Outer Harbour is a busy port with an industrial hinterland while Edithburgh is a small holiday village serving a rural community. The effect of environment was investigated by transferring colonies collected at Edithburgh to Outer Harbour and *vice versa*, the haemolymph from these colonies being assayed for any change in HA-1 titre.

It was thought possible that a specific change in the haemagglutinating titre could be induced by injecting colonies with erythrocytes bearing haemagglutinin receptors on their surface. As guinea pig erythrocytes, the only erythrocytes known to be agglutinated by the HA-1 molecule, had receptors for both the HA-1 and HA-2

agglutinins on their surfaces it was decided to investigate this question using sheep and chicken erythrocytes which are specifically agglutinated by the HA-2 agglutinin. Accordingly, colonies were injected with sheep or chicken erythrocytes and the haemolymph tested for a change in the HA-2 agglutinating titre. As the injected erythrocytes did not have surface receptors for the HA-1 agglutinin it was possible, by titrating the haemolymph for both HA-1 and HA-2 agglutinating activities, to determine whether any change recorded was restricted to the HA-2 agglutinin.

4.2 The effect of environment on the titre of the HA-1 agglutinin.

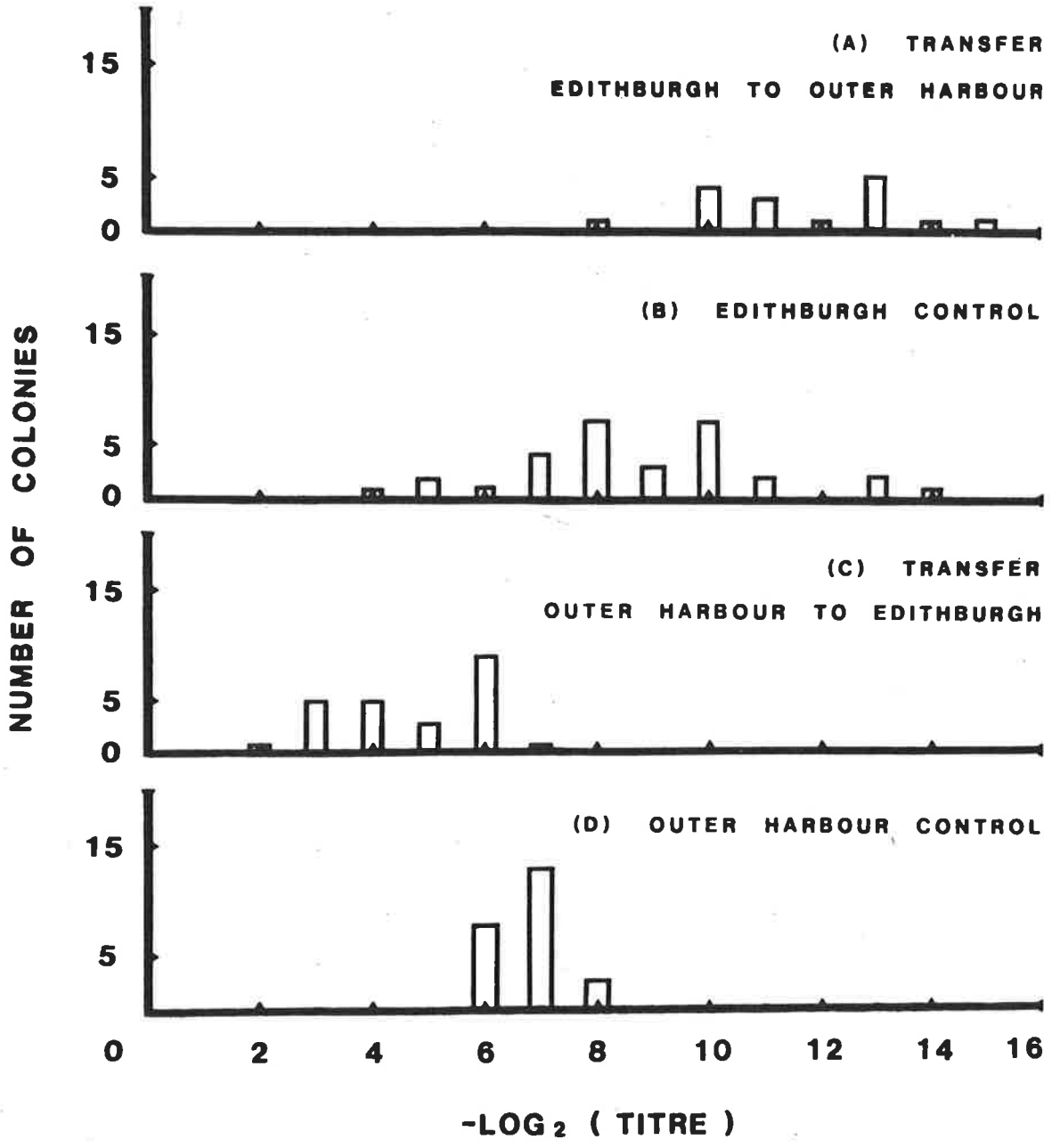
At time zero, eight B. leachii colonies transported from Edithburgh in buckets of aerated sea water were placed in plastic mesh cages attached to pier pilings at Outer Harbour and similarly 11 colonies from Outer Harbour were put in cages fastened to the pilings of Edithburgh pier. Animals which had been removed from the pilings and placed in cages at the same locality served as controls; there were 15 control colonies at Edithburgh and 11 at Outer Harbour. Four weeks after transfer, all the colonies were bled and each haemolymph sample was titrated for HA-1 agglutinating activity with a 1% (v/v) suspension of guinea pig erythrocytes. This experiment was repeated with another batch of control and transferred colonies being placed in cages at the completion of the first experiment. The repeat experiment consisted of 13 colonies that were transferred from Outer Harbour to Edithburgh, 13 that underwent the reciprocal transfer and 15 and 12 control colonies for Edithburgh and Outer Harbour respectively. All colonies were bled at 4 weeks after transfer.

Four of the eight colonies initially transferred from Edithburgh to Outer Harbour died and so haemolymph was collected from the four

remaining healthy colonies and titrated for HA-1 agglutinating activity. The haemolymph titrated for all other groups was obtained from the number of colonies indicated.

The titration results obtained failed to satisfy the assumption of homogeneity of variance ($F_{max} = 15.77$; $P < 0.01$) and a non-parametric analysis was therefore performed. Mann-Whitney U tests indicated that within each treatment category, both experiments were homogeneous. All subsequent analyses were thus performed on the pooled data as displayed in Fig 4.1. A Kruskal-Wallis test, which is analogous to the parametric analysis of variance (Siegal, 1956), indicated that a significant difference in the magnitude of the HA-1 titre did exist between the four groups of colonies ($H = 9.28$; $P < 0.05$). The a posteriori Simultaneous Test Procedure of Dwass as described in Sokal & Rohlf (1969), was then performed in a pairwise manner to identify those groups which were significantly different. This revealed that colonies from each treatment category produced significantly different sets of HA-1 titres from those of the other three groups (in all cases $P < 0.01$). Examination of Fig 4.1 reveals the direction of each difference. The transfer of colonies from Outer Harbour to Edithburgh resulted in a drop in the HA-1 agglutinating activity relative to the level in the control colonies that remained at Outer Harbour, while the reciprocal transfer of Edithburgh to Outer Harbour produced a rise in the HA-1 titre above that of the colonies remaining at Edithburgh. This tended to suggest that the environment at Outer Harbour stimulated the production of HA-1 to a greater extent than did that at Edithburgh. However the two control groups also gave different sets of HA-1 titres, but in this case the median of the Edithburgh data was higher than that of the Outer Harbour data. This result indicates that

FIGURE 4.1 The effect of transfer to a new locality on the guinea pig erythrocyte (HA-1) agglutinating activity of B. leachii haemolymph. Colonies were transferred both from Edithburgh to Outer Harbour and from Outer Harbour to Edithburgh, with control colonies being kept at their original locality. All colonies were bled 4 weeks after transfer. The guinea pig erythrocyte agglutinin titres were determined and these are depicted as a frequency distribution in which the numbers of colonies exhibiting particular titres are shown. The total number of colonies tested was 16, 30, 24 and 24 in Figs 4.1 (A) - (D) respectively.


(A) TRANSFER
EDITHBURGH TO OUTER HARBOUR
(B) EDITHBURGH CONTROL
(C) TRANSFER
OUTER HARBOUR TO EDITHBURGH
(D) OUTER HARBOUR CONTROL
NUMBER OF COLONIES
15
5
0
0
2
4
6
8
10
12
14
16
-LOG2 (TITRE)

environmental stimuli alone are unlikely to account for the quantity of the HA-1 agglutinin in B. leachii haemolymph.

4.3 Haemagglutinin levels in the haemolymph of B. leachii following injection with sheep or chicken erythrocytes

Colonies of B. leachii growing sub-tidally on the pilings of a pier at Edithburgh were used. Colonies were removed from the pilings using SCUBA and cut into portions of approximately equal size (6 cm^2) which were placed randomly in labelled plastic mesh cages that were fastened to the piles. The colonies were then left to acclimatise for two weeks prior to the commencement of the experiment. In this way any artifacts that may have arisen if the animals were maintained in aquaria were eliminated, yet each colony could be readily identified. Unhealthy colonies were not used. The temperature of the sea for the duration of the experiment ranged from 11.2° up to 14.5°.

Immediately prior to injection, the mesh cages containing tunicate colonies were removed from the pilings, returned to the surface and placed in a tray of sea water. The colonies were injected in situ with either 10% erythrocytes (treated groups) or BSS (controls) using a 30 gauge needle. Five injections, given in regions of the colony matrix visibly ramified with blood vessels, were required to bring the volume injected up to 0.15 ml. The colonies were then returned to the pilings in their cages.

4.3.1 The effect of a single injection of erythrocytes

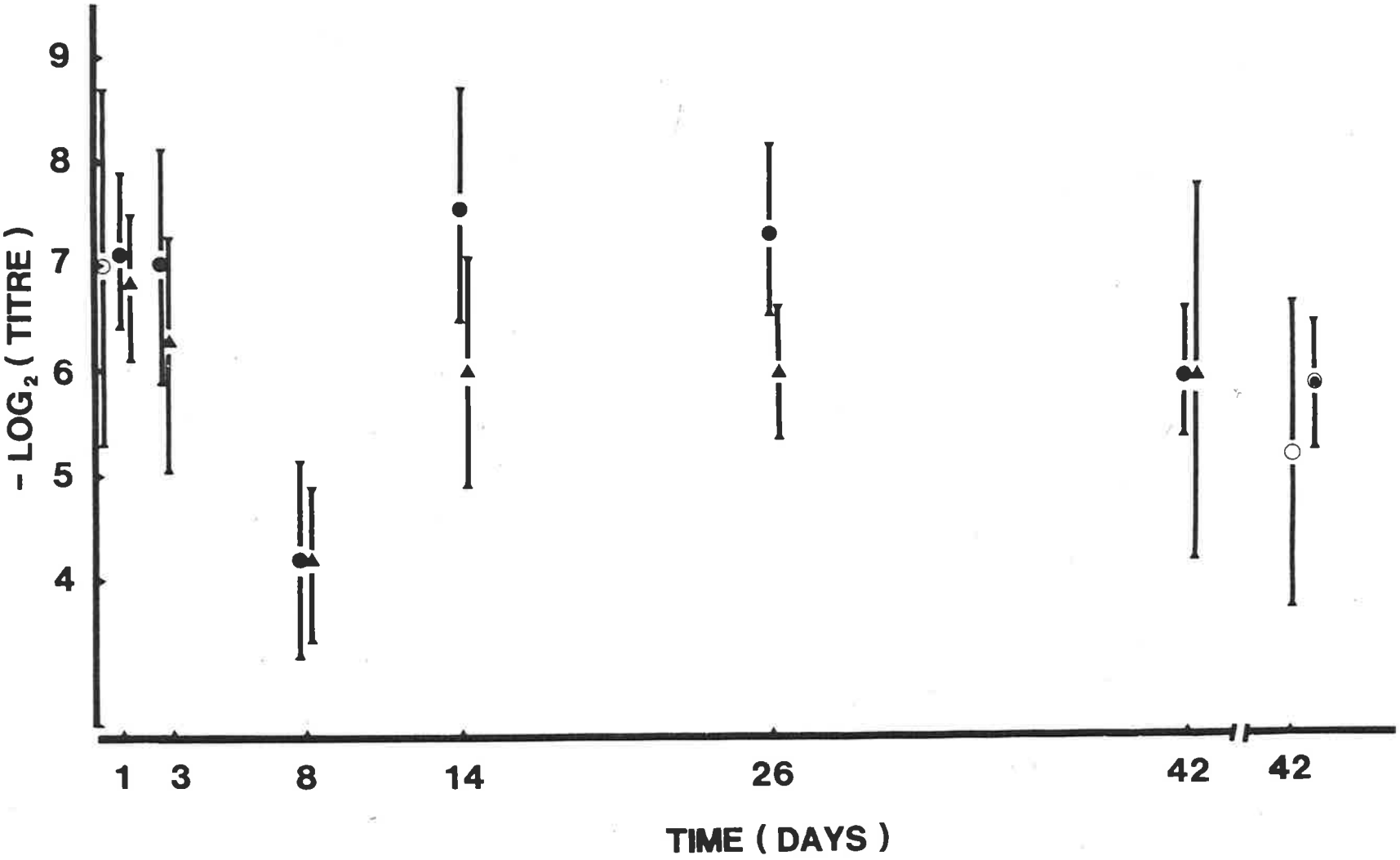
Samples of haemolymph which had been collected from colonies at various times (0 - 48 days) after an injection of sheep erythrocytes were tested for agglutinating activity with sheep erythrocytes. Colonies injected with Botrylloides salt solution (BSS) served as

controls. Each colony had to be killed in order to collect the haemolymph and it was therefore necessary to have sets (consisting of at least 6 colonies per group) of treated and control animals for each time point. Two additional controls were included. The first monitored background variation in the haemagglutinating activity over the duration of the experiment caused by factors which were impossible to control, e.g. environmentally-induced physiological changes in the animals. Thus, haemolymph was collected from undisturbed colonies on both days 0 and 42 and titrated for agglutinating activity against sheep erythrocytes. The second control tested whether the disturbance per se of colonies caused any change in haemagglutinating titres. In this case, colonies were removed from the pier pilings and placed in cages without being injected. After a two week acclimatisation period, these colonies were bled and the haemagglutinating titres were compared with those of the undisturbed colonies sampled on day 42.

Haemagglutinating titres were determined by mixing two-fold serial dilutions of haemolymph (50 µl) in saline with an equal volume of a 0.5% erythrocyte suspension in the wells of a microtitre tray. Agglutination was evaluated as a fraction of 1, i.e. 0, 1/4, 1/2, 3/4 or 1 where 1 = full agglutination (e.g. 2.75 wells), after the cells had settled overnight at room temperature. End points were quite distinct and the titre given for each haemolymph sample represents the maximum dilution ($-\log_2$) which caused complete agglutination.

The removal of colonies from the piles prior to injection had no apparent effect on the sheep erythrocyte agglutinating activity of the haemolymph (Fig 4.2). There was however a slight but significant difference ($P < 0.05$) in the titre of haemolymph collected from undisturbed colonies on days 0 and 42, as shown by the Mann-Whitney U test (Siegel, 1956). This decrease in titre over time would presumably

FIGURE 4.2 The effect of injecting B. leachii colonies with sheep erythrocytes. Injections were given on day 0 and the animals were bled on the days indicated; 6-7 animals were bled at each time point. Sheep erythrocyte agglutinin titres (mean ± standard deviation) are given for groups of animals injected with erythrocytes (●) or BSS (▲). Data from undisturbed (○ ; sample size = 8 & 11) and caged, uninjected colonies (⊙ ; sample size = 8) are also shown. Mann-Whitney U tests (Siegal, 1956) revealed a significant difference between experimental and control values at days 14 and 26 (P = 0.036 and 0.047 respectively).


9
8
7
6
5
4
- LOG2 (TITRE)
1
3
8
14
26
42
42
TIME (DAYS)

be superimposed upon any effect produced by the experimental procedure, thereby highlighting the need to compare treated and control colonies within each time period. The data illustrated in Fig 4.2 show that colonies injected with sheep erythrocytes possessed an elevated haemagglutinating titre 14 days after injection. This increase was still evident after 26 days, but by 42 days the titres of injected and control colonies were again equivalent.

4.3.2 Secondary stimulation with erythrocytes

In vertebrates, a second injection of antigen normally evokes a large and rapid increase in the concentration of specific antibody. To determine whether a similar rise in haemagglutinating activity occurs in *B. leachii*, a second dose of erythrocytes was administered after the first response had subsided. Moreover, colonies were treated such that they received either sheep or chicken erythrocytes for both their primary and secondary injection, while other colonies served as controls. It seemed likely that priming with chicken erythrocytes would produce the same result as that obtained when the animals were primed with sheep erythrocytes, since haemolymph normally contains a single agglutinin (HA-2) for both these cell types (Schluter *et al.*, 1981).

Each colony was cut into three portions (as a series of matched triplets) in an effort to reduce the variation observed earlier (Fig 4.2). One portion acted as a control and received only BSS, whilst the other two received injections of either chicken or sheep erythrocytes. The titre of the haemolymph of a particular colony after injection with erythrocytes could thus be compared to that of a genetically-identical control colony which had been injected with BSS at the same time. The haemolymph samples obtained from these animals

at varying times after injection were titrated against both types of erythrocyte, all agglutination assays being performed "blind" and in duplicate.

A two-way analysis of variance (ANOVA) without replication was used to determine the significance of any difference observed between the erythrocyte-injected colonies and their genetically-identical control colonies. In the case of one missing data point, due to colony death, this value was estimated according to the method given in Sokal and Rohlf (1969). At the 6 week time point, two data points were missing and in this case a Friedman non-parametric two-way ANOVA was used (Siegel, 1956). The ranks assigned to the missing points were such that the possibility of significance was maximised, but despite this there was no significant difference between treated and control animals at this time (Table 4.1).

It is apparent from the results presented in Table 4.1 that there was a slight but definite elevation in the agglutinating titre for both types of erythrocyte 2 weeks after priming. Furthermore, animals that had been primed with sheep erythrocytes showed a corresponding increase in agglutinating activity against chicken erythrocytes and vice versa. The injection of a second dose of erythrocytes 6 weeks after priming produced a response similar to the primary response, i.e. a 2-4 fold increase in titre 2 weeks after challenge.

The data can be displayed in a manner which eliminates variance due to genetic differences between colonies if within each matched triplet the control value is subtracted from both the experimental values. The effect of the experimental treatment is then exhibited as a deviation from zero. The increase in titre 2 weeks after each injection becomes readily apparent (Fig 4.3).

TABLE 4.1 Haemagglutinin levels in haemolymph collected from *B. leachii* colonies injected with sheep or chicken erythrocytes

Type of erythrocyte used in assay	Time of bleeding (Wks p.i.*)	Injected material			
		Sheep erythrocytes	Chicken erythrocytes	BSS (control)	None
		Haemagglutination titre ¶ ($-\log_2$)			
Sheep:	1	6.13 ± 1.02	6.73 ± 0.99	6.23 ± 1.25	ND
1° reaction:	2ø	7.63 ± 0.57	7.23 ± 1.22	5.44 ± 1.10	7.14 ± 1.53
	6	5.05 ± 2.03	4.68 ± 1.69	4.48 ± 1.46	ND
2° reaction:	1	6.92 ± 1.07	6.48 ± 0.72	6.10 ± 0.69	ND
	2§	7.96 ± 0.95	7.98 ± 1.06	6.23 + 0.60	6.38 ± 1.23
Chicken:	1	6.13 ± 1.90	6.40 ± 2.10	5.88 ± 1.82	ND
1° reaction:	2ø	7.67 ± 0.74	7.27 ± 1.32	5.44 ± 1.59	6.38 ± 1.34
	6	5.08 ± 1.47	4.80 ± 1.02	5.07 ± 1.48	ND
2° reaction:	1	5.58 ± 1.16	5.19 ± 0.92	5.10 ± 1.51	ND
	2§	6.31 ± 1.25	6.83 ± 1.14	5.35 ± 0.81	6.13 ± 1.11

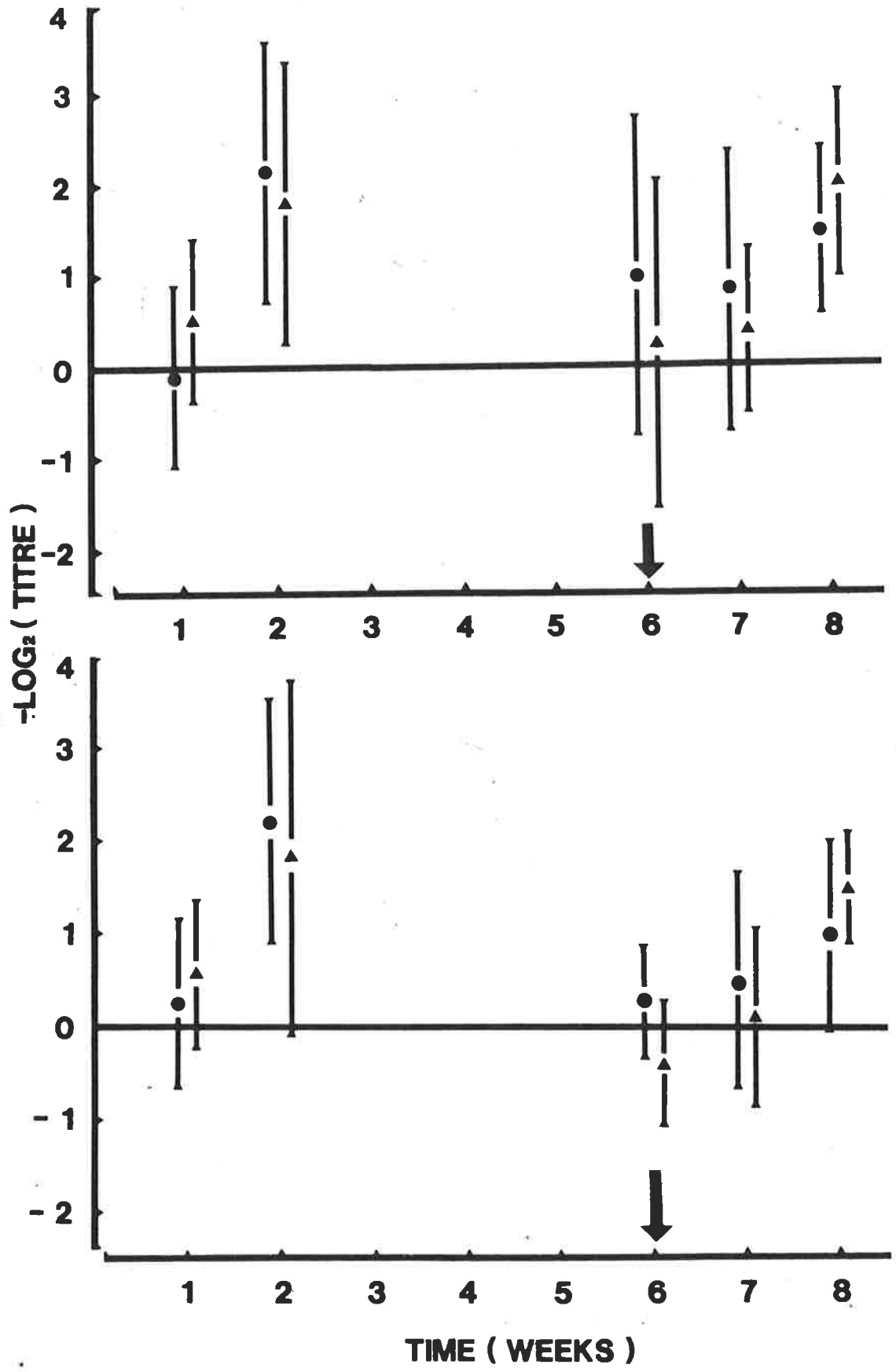
* p.i. = post injection.

¶ Mean ± standard deviation. Sample size = 6, except in 4 cases where one colony died.

ȼ,§ Probability (2-way ANOVA) that data from erythrocytes-injected colonies are equivalent to control values (ȼ, $P < 0.025$; §, $P < 0.005$).

ND = Not done

FIGURE 4.3 Heamagglutinin titres of B. leachii haemolymph following two spaced injections of either sheep or chicken erythrocytes. The animals were injected both on day 0 and on day 42 with either sheep (●) or chicken (▲) erythrocytes. The data have been corrected by subtraction of the appropriate control values (see text). Differences between experimental and control values are indicated by deviation from zero and probabilities are given in Table 4.1. Titrations were carried out against sheep erythrocytes (upper figure) and chicken erythrocytes (lower figure). Vertical bars represent ± one standard deviation.


4
3
2
1
0
-1
-2
1
2
3
4
5
6
7
8
-LOG2 (TITRE)
TIME (WEEKS)

The specificity of the increase in HA-2 titre was examined by using guinea pig erythrocytes to test for any alteration in the HA-1 agglutinin titre. This agglutinin was a particularly useful control, for although guinea pig erythrocytes may be agglutinated by both the HA-1 and HA-2 agglutinins, the titre of these molecules can be measured independently using different assay diluents. Furthermore, the HA-2 agglutinin can be removed from the haemolymph by adsorption with sheep erythrocytes without changing the HA-1 titre (Table 4.2). Thus, to determine whether the injected erythrocytes, which carry determinants for only the HA-2 agglutinin, were causing a specific change in agglutinin levels, i.e. in HA-2 only, those haemolymph samples exhibiting an elevated HA-2 titre (using sheep and chicken erythrocytes; Fig 4.3) were tested for both agglutinins by titration against guinea pig erythrocytes in tris-buffered saline supplemented with 5 mM $CaCl_2$ and 0.01 mg/ml of BSA and also in isotonic sodium citrate (for HA-2).

The data in Table 4.3 indicate that there was no significant rise in the HA-1 agglutinin level in colonies injected with sheep or chicken erythrocytes. However, there was as previously observed an increase in the HA-2 titre. The magnitude of the change in HA-2 titre was the same regardless of the type of indicator erythrocyte used. The results clearly support the conclusion that the altered agglutinating titre observed in haemolymph from animals injected with sheep or chicken erythrocytes was due wholly to an increased concentration of the HA-2 agglutinin.

TABLE 4.2 The effect of the removal of the HA-2 agglutinin on the HA-1 titre.*

Colony Number¶	Titrating Erythrocyte			
	Sheep		Guinea pig	
	adsorbed haemolymph	control	adsorbed haemolymph	control
	$-\log_2$ agglutination titre			
1	1	7	15.5	15
2	1	6.5	11	11
3	1	8	14	14
4	1	5.5	15	15.5
5	1	6	14	14

* Sheep erythrocytes (6×10^8) in 0.4 ml of saline were added to 0.125 ml haemolymph, mixed vigorously and left at room temperature for 1 hour. The samples were centrifuged and the supernatant (adsorbed haemolymph) was titrated in either 3.8% trisodium citrate or TSA supplemented with 5 mM $CaCl_2$ and 0.01 mg/ml BSA for agglutination of both sheep and guinea pig erythrocytes. Controls consisted of 0.125 ml of unadsorbed haemolymph from the same colony, which was added to 0.4 ml of saline and similarly titrated.

¶ Haemolymph was taken from 5 replicates.

TABLE 4.3 Titres of the HA-1 and HA-2 agglutinins, assayed using guinea pig erythrocytes, in haemolymph from *B. leachii* injected with sheep or chicken erythrocytes.

Haemagglutinin measured	Time of bleeding (2 weeks p.i.)	Injected material		
		Sheep erythrocytes	Chicken erythrocytes	BSS (control)
		Haemagglutination titre* ($-\log_2$)		
HA-1	1° reaction	14.68 ± 1.10	14.45 ± 1.25	13.54 ± 2.36
	2° reaction	14.37 ± 1.89	14.67 ± 1.97	14.18 ± 1.14
HA-2	1° reaction¢	10.75 ± 0.55	10.50 ± 0.89	8.90 ± 1.35
	2° reaction¶	9.29 ± 1.03	9.50 ± 0.47	8.27 ± 0.42

* Mean ± standard deviation. The HA-1 agglutinin was titrated in TSA supplemented with 5 mg/ml $CaCl_2$ and 0.01 mg/ml BSA, the HA-2 in 3.8% sodium citrate.

¢,¶ Differences between colonies injected with erythrocytes (chicken or sheep) and BSS (2-way ANOVA): $F_{(2,9)} = 5.02$, $P < 0.05$ (¢); $F_{(2,9)} = 9.12$, $P < 0.025$ (¶).

4.4 Discussion

It is clear from these results that the titres of both the HA-1 and the HA-2 agglutinins in haemolymph can be altered by exposure of B. leachii colonies to particular foreign particles. It was shown that a change of environment evoked a change in the HA-1 agglutinating titre while the injection of colonies with sheep or chicken erythrocytes caused a small rise in titre of the HA-2 agglutinin but not of the HA-1 agglutinin. However from the data presented in this chapter (Section 4.2) it seems unlikely that the quantity of these naturally occurring agglutinins released into the haemolymph is the result of external stimuli alone. Other factors, e.g. genetic composition, are probably influential.

Edithburgh and Outer Harbour appear to represent environments which stimulate to differing degrees the production of the HA-1 agglutinin, the Edithburgh environment providing less of a stimulus than the Outer Harbour environment. However an explanation for the direction of the changes observed in the HA-1 titre (Section 4.2) requires the assumption that B. leachii colonies at Outer Harbour and Edithburgh are genetically isolated and that the capacity for HA-1 production is genetically controlled. One can then propose that Outer Harbour colonies have a low capacity for HA-1 production even in a region where the stimuli for producing this molecule is high, as is reflected in the lower titres of the control colonies at Outer Harbour relative to those at Edithburgh. If the environmental stimuli are reduced, e.g. by transferring these colonies to Edithburgh, the HA-1 titre drops even lower. Edithburgh colonies, on the other hand, produce large amounts of the HA-1 agglutinin under low levels of environmental stimulation; however, upon transfer to the Outer Harbour environment, these colonies can produce still more HA-1 agglutinin.

The validity of this explanation must remain subject to further experimentation, particularly as the assumption that the two populations are genetically isolated is based on a limited knowledge of B. leachii reproductive biology. Nevertheless, the results of this experiment pose interesting questions as to how the genetic constitution of individual ascidians could affect their response to pathogens. If the capacity to respond to a particular pathogen is genetically controlled it is probable that through natural selection, populations of high and low responding ascidians would have evolved in areas of continued high and low pathogen abundance respectively. In fact the "environmental stimuli" referred to in this experiment could be pathogens.

The effect of injecting colonies with erythrocytes was investigated in randomly chosen B. leachii colonies originating from an interbreeding population (Fig 4.2 & Table 4.1). Hence genetic differences between colonies were not likely to markedly influence the HA-2 titres recorded in this experiment and any change can therefore be attributed to a response, be it physiological or "immunological", induced by the injected erythrocytes.

An increase in the HA-2 agglutinating titre of haemolymph was first observed two weeks after the injection of erythrocytes, and was still apparent at four weeks. The titre then fell and was similar at six weeks to that of the BSS-injected controls. A second injection of erythrocytes six weeks after priming produced an apparently identical response with respect to the interval between injection and the onset of the rise in titre and also the magnitude of this rise.

At present there is no information about the duration of the response after the second injection, but the minor change in titre is markedly different from that observed in the secondary antibody

response of higher vertebrates. Representatives of each of the vertebrate classes have been shown to be capable of an anamnestic antibody response, although this ability does not seem to be well developed in the fishes and amphibians and depends on the nature and dose of the immunogen (review: Marchalonis, 1977). The possibility remains, therefore, that weak immunological memory might yet be demonstrated in this ascidian species utilising different immunisation protocols and other antigens.

From work carried out on Ciona intestinalis, an ascidian belonging to an Order different from that occupied by B. leachii, it has been concluded that the inflammatory reaction to a second injection of erythrocytes into the tunic is heightened in comparison with primary reactions (Wright and Cooper, 1975; Parrinello et al., 1977). Wright and Cooper (1975) recorded the number of animals in which the injected erythrocytes were encapsulated by cells. A high concentration of erythrocytes given in a single injection induced encapsulation whilst erythrocytes injected at a lower concentration were phagocytosed, leading the authors to conclude that encapsulation is a more severe response than phagocytosis. Parrinello et al. (1977), on the other hand, measured the time required for elimination of erythrocytes through the tunic. Unfortunately, haemagglutinin levels were not measured in either study and it remains an open question as to whether such humoral factors participate in the direction of these inflammatory reactions. In both studies, the secondary reactions were said to be non-specific since a second injection with erythrocytes from a different species produced reactions similar to those observed in animals given two identical injections. However, if the different erythrocytes carried determinants recognised as cross-reactive by the tunicate (e.g. sheep, chicken and many other types of erythrocytes bind

the B. leachii HA-2 agglutinin), the animal could have responded to the same molecule(s) regardless of the type of erythrocyte injected. This possibility does not appear to have been considered.

Wright (1974) injected human erythrocytes into the tunic of Ciona intestinalis and at selected times thereafter bled the animals and examined the injection sites for erythrocytes. The aim of the study was to correlate changes in the agglutination titre of the haemolymph for human and duck erythrocytes with the clearance kinetics observed for these erythrocytes over a 168 hr period, but no statistically significant change in titre was recorded. The results with B. leachii are consistent with this observation, since it was not until 14 days after injection that any significant increase in titre was recorded.

It is noteworthy that in B. leachii the effect of injecting sheep or chicken erythrocytes appears to be restricted to the HA-2 agglutinin, since the HA-1 titre exhibited no change. This finding, together with the fact that neither sheep nor chicken erythrocytes possess binding sites for the HA-1 agglutinin, suggests that the erythrocytes stimulated production of HA-2 specifically, perhaps with the effect of enhancing elimination of the erythrocytes from the circulation, rather than evoking some non-specific physiological change. The experiments reported in the following chapter were performed to determine whether the HA-2 agglutinin does indeed function as an opsonin in this species. The function of the HA-1 agglutinin is also unknown. It would be of interest, particularly as the HA-1 titre can apparently be altered by external stimuli, to investigate whether injecting B. leachii colonies with guinea pig erythrocytes, which carry ligand sites for both the HA-1 and the HA-2 agglutinins, would induce an increase in the titre of not only the HA-2 but also the HA-1 molecule.

Chapter 5.

THE HA-2 AGGLUTININ AND RECOGNITION OF SHEEP ERYTHROCYTES BY B. leachii HAEMOCYTES.

5.1 Introduction.

Although other authors have reported that opsonic factors exist in the haemolymph of a variety of invertebrates (for example, Stuart, 1968; Prowse & Tait, 1969; Pauley *et al.*, 1971a; Paterson & Stewart, 1974), very little is known about the molecular properties of these opsonins. It has been speculated from as early as 1966 (Boyden, 1966) that agglutinins may have an opsonic role. However, aside from a few studies (Tyson *et al.*, 1974; Harm & Renwrantz, 1980; Renwrantz, *et al.*, 1981) the evidence supporting this notion has been wholly circumstantial (see Chapter 1, Section 1.3.4.). Even in studies where evidence for the opsonic activity of an agglutinin was presented, a number of questions relating to how these molecules interacted with the phagocytic cells remained unanswered, e.g.: Did the opsonin/agglutinin bind to similar sites on the phagocyte and the opsonised particles? Were the opsonins secreted by circulating haemocytes or by other cells? What was the role of agglutinin molecules found on the surface of haemocytes in some species (Amirante, 1976; Amirante & Mazzalai, 1978; van der Knaap *et al.*, 1981a)?

The ascidian system was chosen to address some of these questions for several reasons:

(1) Since the HA-2 haemagglutinating titre of haemolymph could be altered on stimulation (see Chapter 4) it was conceivable that this molecule could function as a recognition factor for foreign particles by *B. leachii* haemocytes.

(2) The availability of purified preparations of these agglutinins and of antisera specific for each, permitted the association of the agglutinins with various cells *in vitro* to be investigated in a precise fashion, and most importantly

(3) the possible functions of ascidian agglutinins had not been previously investigated in any detail.

5.2 The response of B. leachii colonies to an injection of erythrocytes.

Before postulating an opsonic role for the agglutinins it was necessary to determine whether ascidian haemocytes could recognise and ingest particles known to bear agglutinin receptors on their surfaces. For this reason B. leachii colonies were observed for approximately 48 hr after having received an injection of sheep erythrocytes. Both the external appearance of the colonies and the behaviour of the haemocytes were recorded.

Sheep erythrocytes (0.05 ml of 15% (v/v) suspension in Botrylloides salt solution (BSS)) were injected into a portion of the colony visibly ramified with blood vessels. A successful injection could be identified readily as the vessels and ampullae within the immediate vicinity of the injection (within 2 cm^2) were coloured bright red by the erythrocytes. Approximately 15 min after the injection, vessels 2-3 cm away from the injection site contained erythrocytes and by 30 min vessels surrounding zooids situated approximately 4 cm from the injection site were red with erythrocytes. The only erythrocytes still visible after 5 hr were near the injection site where aggregates appeared to be obstructing vessels and ampullae. An examination of haemocytes collected from colonies 5 hr after injection revealed phagocytosed erythrocytes within a specific type of haemocyte (Fig 5.1). A comparison with haemocytes obtained from colonies not injected with erythrocytes verified the assumption that the structures observed were phagocytosed erythrocytes.

Colonies maintained in sea water for 24-48 hr after the injection developed a lesion at the injection site. The lesion

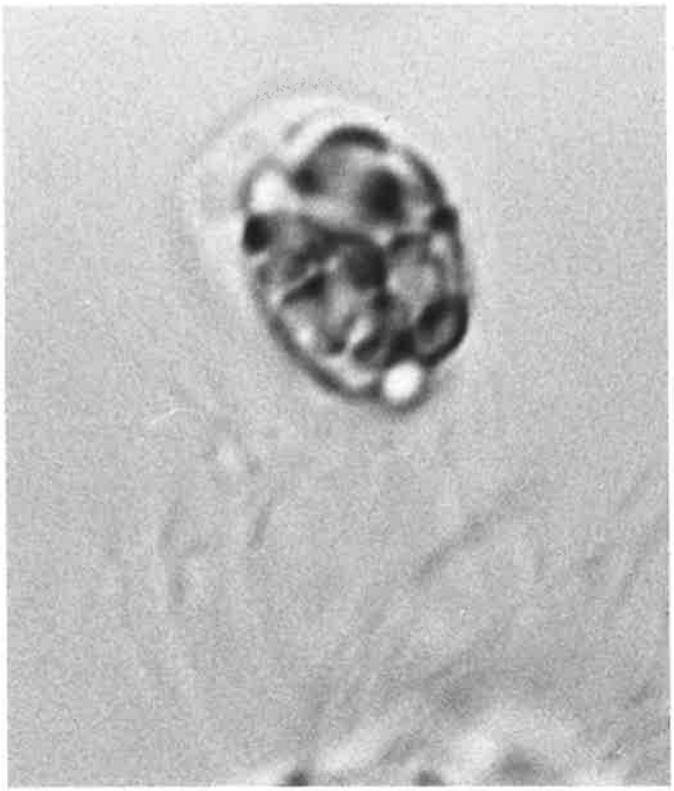


FIGURE 5.1 A B. leachii haemocyte containing phagocytosed erythrocytes. Phagocytosis occurred in vitro. (mag 3,000x).

contained gelatinous material that was slightly more liquid than the surrounding test. Most of the blood vessels in this area appeared either to have been reabsorbed or to have degenerated, leaving only a small number of vessels apparently clogged with erythrocytes. Nevertheless some of the haemocytes collected by piercing the lesion did contain phagocytosed erythrocytes.

These observations are very similar to those of Parrinello et al., (1977) on the tunic reaction of Ciona intestinalis (a solitary ascidian) to erythrocyte injections. In C. intestinalis, animals that developed a "blister", or lesion generally died, whereas B. leachii colonies were able to wall off the affected area. Although this created a hole in the colony, it did not appear to disrupt growth in other directions.

5.3 The adhesion of erythrocytes to B. leachii haemocytes in vitro.

The development and standardization of the in vitro culture of B. leachii haemocytes permitted detailed investigations to be carried out concerning the question of whether or not the HA-2 molecule, the sheep erythrocyte agglutinin, has any role in facilitating recognition of these cells by the haemocytes.

B. leachii haemocytes are very fragile in vitro but with care it was possible to obtain consistently good monolayers. Haemolymph was collected into siliconised tubes on ice; the cells were sedimented by gentle centrifugation and washed with BSS at 4°. A gentle washing procedure was critical for good monolayers. The cells were washed by centrifuging (using a swing out head) at 150 g for 5 min and gently resuspending in BSS. At the final washing the cells were resuspended in Botrylloides cell culture medium (BCCM) and added to wells containing a glass coverslip. The extent of adhesion to the coverslip

varied according the the colony from which the cells were taken, although the addition of 1×10^6 haemocytes was usually sufficient to produce a monolayer of the required density. Although the *in situ* temperature for these animals is around 12 - 17°, all long-term incubations (longer than 15 min) were carried out at 4°. At 4° the cells spread and adhered to the coverslips; raising the temperature to 15° resulted in a loss of cells from the monolayer.

Difficulties arose when erythrocytes were added to monolayers for rosetting experiments. Originally 1.5×10^8 erythrocytes in 1 ml of BCCM were added to each washed monolayer and the cells were incubated together for 1 hr at 4°. This procedure caused massive agglutination of the erythrocytes and attempts to wash away the aggregates resulted in the loss of substantial numbers of ascidian haemocytes. The culture medium alone did not cause agglutination, hence the factor responsible must have been released from the haemocytes. When the number of erythrocytes added to each well was reduced to 3×10^7, the problem of agglutination was overcome and good rosettes were obtained.

The solutions used in the handling of these cells were developed after measuring the concentrations of the major cations in haemolymph and the total osmolarity of haemolymph. *Botrylloides* salt solution (BSS) consisted of a buffered (pH 7.0) solution of salts, the concentration of salts being chosen so that the cation composition and the total osmolarity of the solution mimicked that of haemolymph. The culture medium (BCCM) was modified from that developed by Warr *et al.* (1977) for culturing *Pyura stolonifera* cells. Dulbecco's modified Eagles medium was used as a nutrient source with extra salts being added to adjust the concentration of ions and the osmolarity to that of

B. leachii haemolymph. The compositions of these solutions are given in Chapter 2, Sections 2.17.1 and 2.17.2.

5.3.1 The effect of sugars on the adherence of erythrocytes to haemocytes.

As the HA-2 agglutinin was specific for lactose (Chapter 3 and Schluter et al., 1981), the adhesion of sheep erythrocytes to ascidian cells, if mediated via this molecule, should also have been inhibited by lactose.

Eighteen ascidian cell monolayers, six for each treatment, were prepared as described (Chapter 2, Section 2.18.2). The coverslips with their adherent cells were washed in BSS and incubated in wells containing 0.5 ml of either 0.25M lactose, 0.25M glucose or BCCM. The sugars were dissolved in distilled water to yield solutions iso-osmotic with haemolymph and then diluted in BCCM. The monolayers were incubated for 15 min at room temperature. They were then divided into two groups of nine monolayers, each group containing triplicate monolayers for the three treatments. Monolayers of group 1 received 3×10^7 erythrocytes which had been sensitised with one haemagglutinating unit (1 HAU) of purified HA-2 agglutinin, while monolayers in the second group received 3×10^7 unsensitised erythrocytes. The erythrocytes were added to each well as a cell suspension in 0.5 ml of the incubating solution currently bathing that monolayer i.e. either 0.25 M lactose, 0.25 M glucose or BCCM. All monolayers were then incubated for 1 hr at 4° after which the coverslips were removed, washed in BSS, fixed in methanol and stained with Wright's stain.

For the purpose of scoring the rosettes, the ascidian cells were divided into four categories on the basis of their appearance after

staining. These were: "red stained cells", "dark blue cells", "small blue cells" and "others". The categories "red stained cells" and "small blue cells" represented apparently homogeneous cell populations, but two different types of evenly stained cells were included in the "dark blue cells" category (see Fig 5.2). For each slide, the ascidian cells visible in three randomly chosen fields (mag. 600x) were counted, with cells being scored as positive if they had four or more adherent erythrocytes. This experiment was repeated and the results of both experiments are displayed in Table 5.1.

The statistics for this and similar subsequent experiments were performed on the unpooled data expressed as the percent of positive cells within each cell type. Two of the assumptions that the data should satisfy before they are analysed by an analysis of variance are normality and homogeneity of variances. Percentage data are non-normal a priori; the common procedure for dealing with data of this type is to use the arcsine transformation. This transformation stretches out both tails of a distribution and compresses the middle. If the percentages in the original data are clustered around the centre of the distribution, i.e. between 30 and 70%, then it is generally not necessary to apply the arcsine transformation (Sokal & Rohlf, 1969). The data obtained from the above experiment and similar subsequent experiments, although not confined to the 30 - 70% range, are concentrated around the centre of the distribution. Hence, the effect of an arcsine transformation is likely to be minor. In all experiments the variances of the original data were shown to be homogeneous by the Cochran test (Winer, 1962). The C statistics obtained after the data were subjected to an arcsine transformation were larger than those of the original data, indicating greater heterogeneity of variances after transformation. For this reason and because the sampling distribution

FIGURE 5.2 The effect of sugars on the adhesion of erythrocytes to B. leachii haemocytes. Adhesion was performed in the presence of: no sugar (A), 25 mM lactose (B) or 25 mM glucose (C). Haemocytes of different types were placed in four categories for scoring purposes and representatives are shown. "Red staining" cells (R), "Dark blue cells" (DB), "Small blue cells" (SB). (mag 460x (A), and 480x (B) & (C)).

A. Adhesion of erythrocytes when no sugar was added.

A

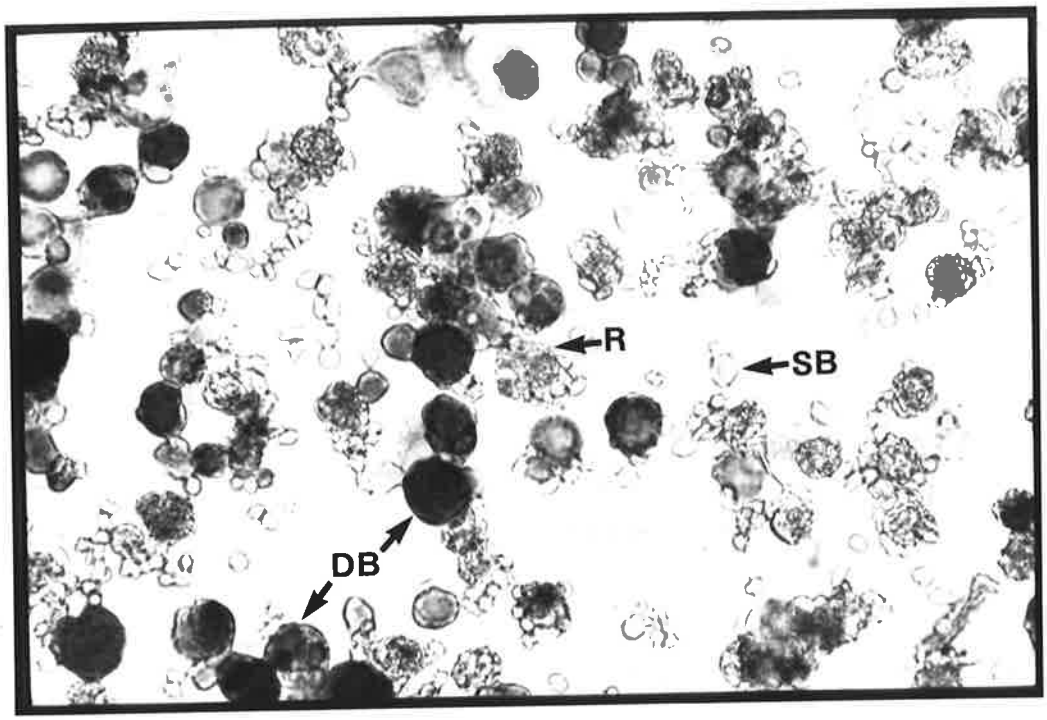
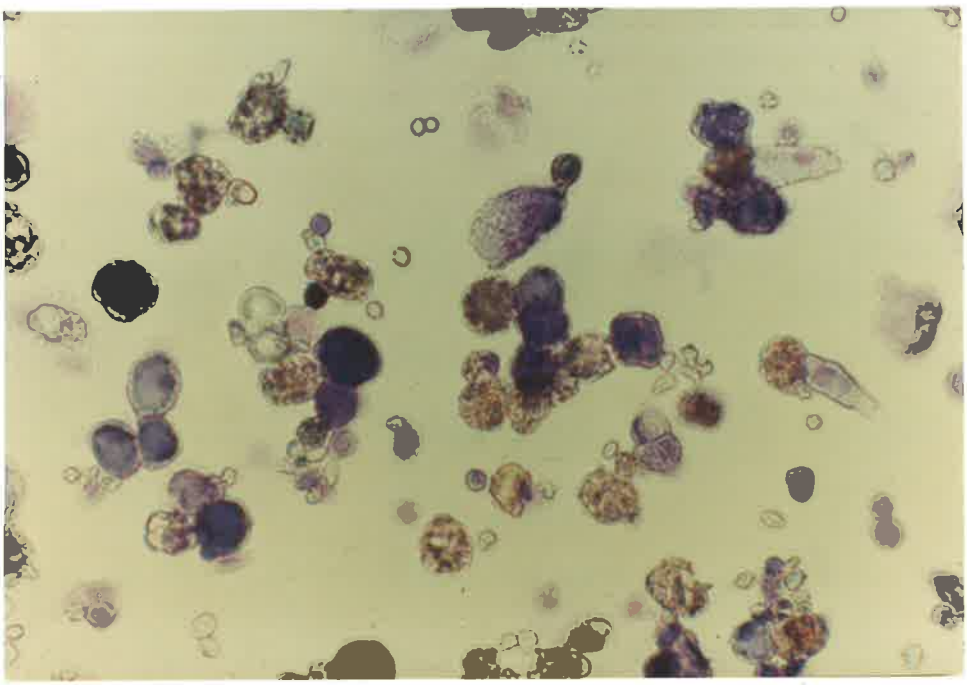


FIGURE 5.2B & C The effect of adhesion of erythrocytes to B. leachii haemocytes.

B. Adhesion in the presence of 25 mM lactose.

C. Adhesion in the presence of 25 mM glucose.

B



C

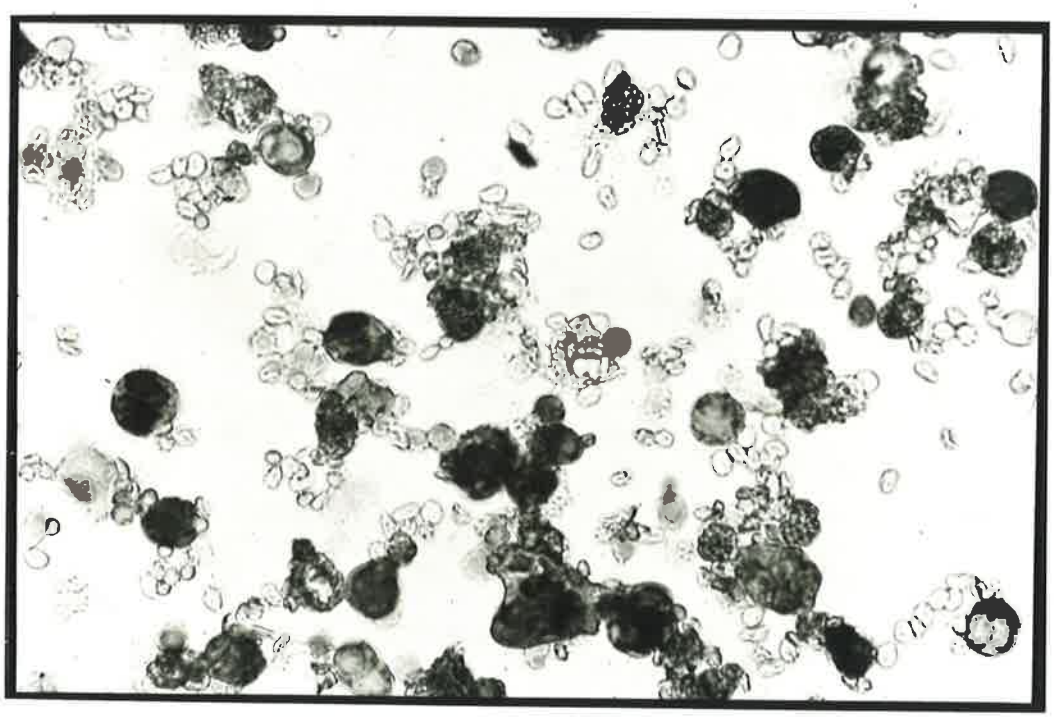


TABLE 5.1 The adhesion of HA-2 sensitised and unsensitised erythrocytes to different types of B. leachii haemocytes in the presence of lactose or glucose.*

UNSENSITISED ERYTHROCYTES							
Incubation conditions		Type of ascidian cell@: Red cells	Dark blue	Small blue	Other	Overall % positive cells#	no. of cells counted
BCCM (control)	I¶	74.31 ± 1.47	43.12 ± 4.9	26.53 ± 2.03	35.18 ± 5.86	48.93 ± 0.77	1300
	II	68.29 ± 0.87	41.30 ± 8.33	38.01 ± 2.69	33.63 ± 4.50	49.22 ± 3.04	1470
BCCM & 25 mM glucose§	I	82.69 ± 0.63	34.71 ± 2.37	24.10 ± 6.97	28.80 ± 1.96	47.01 ± 2.58	1115
	II	72.24 ± 1.21	38.22 ± 5.03	30.25 ± 4.43	35.76 ± 2.49	48.78 ± 1.69	1198
BCCM & 25 mM lactose	I	25.44 ± 1.79	12.49 ± 0.79	12.65 ± 3.95	14.24 ± 5.03	17.69 ± 1.83	1147
	II	25.06 ± 2.13	14.97 ± 2.87	16.32 ± 2.23	12.29 ± 0.53	18.17 ± 0.97	1376

* Figures in the body of the table are mean ± standard error of the percent of positive cells within a cell type from three replicate slides.

@ As determined by their appearance with Wright's stain.

The number of positive cells as a percent of all cells counted.

¶ Duplicate experiments (I & II).

§ All sugar solutions were iso-osmotic with B. leachii haemolymph.

TABLE 5.1 cont. The adhesion of HA-2 sensitised and unsensitised erythrocytes to different types of B. leachii haemocytes in the presence of lactose or glucose.*

Incubation conditions		SENSITISED ERYTHROCYTES[ø] Type of ascidian cell[@]: Red cells	Dark blue	Small blue	Other	Overall % positive cells[#]	no. of cells counted
BCCM (control)	I[¶]	75.93 ± 1.75	39.02 ± 4.21	29.58 ± 2.17	29.35 ± 2.56	46.90 ± 1.03	1225
	II	74.64 ± 1.76	40.55 ± 5.76	32.93 ± 2.67	32.12 ± 1.42	48.15 ± 0.57	1188
BCCM &	I	80.76 ± 1.05	31.81 ± 1.17	21.64 ± 1.67	26.52 ± 1.31	45.39 ± 0.45	1139
25 mM glucose[§]	II	74.34 ± 2.63	42 71 ± 2.75	33.31 ± 1.08	38.79 ± 4.56	50.69 ± 2.03	1152
BCCM &	I	29.21 ± 2.44	8.78 ± 4.49	9.43 ± 0.88	15.47 ± 2.02	18.04 ± 1.04	1125
25 mM lactose	II	27.95 ± 4.23	16.78 ± 4.58	17.14 ± 5.23	15.98 ± 2.33	20.69 ± 3.68	1372

* Figures in the body of the table are mean ± standard error of the percent of positive cells within a cell type from three replicate slides.

ø Erythrocytes were sensitised with 1 HAU of purified HA-2 agglutinin before being added to the ascidian cells.

@ As determined by their appearance with Wright's stain.

The number of positive cells as a percent of all cells counted.

¶ Duplicate experiments (I & II).

§ All sugar solutions were iso-osmotic with B. leachii haemolymph.

of the F ratio is relatively insensitive to moderate departure from normality (Winer, 1962) it was decided to perform factorial analyses of variance on untransformed data.

Since, as indicated above, the variances of the data presented in Table 5.1 were shown to be homogeneous by the Cochran test ($C = 0.124$ where $K = 48$ and $n = 3$) (Winer, 1962), a 4-factor analysis of variance with replication was performed on the untransformed data (Table 5.2). This analysis indicated that the two experiments did not produce identical results. There was a significant interaction between cell types and experiments ($F = 6.2$, $P < 0.01$) indicating that differences between experiments existed in the adhesion of erythrocytes to certain types of cells. As can be seen from Table 5.1, experiment 2 did show a slight increase in the numbers of positive cells in the categories "small blue cells" and "others" with a corresponding decrease in the numbers of positive "red staining cells" relative to that of the first experiment. Nevertheless, as indicated by the non-significance of both the (Experiments x Sugars) and (Experiments x Sensitisation) interaction terms, the overall effect of both the addition of sugars and the sensitisation of erythrocytes was the same for both experiments.

The sensitisation of erythrocytes did not significantly affect the percent of positive cells within any of the cell types (Table 5.2). The factor(s) causing adhesion must therefore have been present on a washed monolayer.

Because the sugars did not uniformly affect the adhesion of erythrocytes to all cell types, shown by the significant (Cell type x Sugars) interaction ($F = 18.01$, $P < 0.01$), the interpretation of the main effect due to the sugars was rather difficult. In an effort to determine how each sugar affected the

TABLE 5.2 Factorial analysis of variance summary table.

(analysis of data from TABLE 5.1)

Source of variation	SS	df	MS	F
Main effects:				
Cell type#	28615.85	3	9538.62	43.8**
Experiments (Exp)§	238.724	1	238.72	6.8**
Sensitisation (Sens)#	1.08	1	1.08	1
Sugars#	23290.46	2	11645.23	357.8**
Interaction terms:				
Cell type x Exp.	653.35	3	217.78	6.2**
Cell type x Sens.	64.63	3	21.54	2.2
Cell type x Sugar	5679.01	6	946.5	18.01**
Exp. x Sens.	88.63	1	88.63	2.5
Exp. x Sugar	65.10	2	32.55	1
Sens x Sugar	18.02	2	9.01	1
Cell type x Exp. x Sens.	29.91	3	9.97	1
Cell type x Exp.x Sugar	316.02	6	52.67	1.5
Cell type x Sens x Sugar	85.97	6	14.33	1.12
Exp. x Sens. x Sugar	30.96	2	15.48	1
Cell type x Exp. x Sens. x Sugar	76.62	6	12.77	1
Error:				
Within cell¶	3355.78	96	32.96	
Total	62610.	143		

Fixed factors.

§ Random factors.

** Probability < 0.01; all other probabilities > 0.05.

¶ n = 3

adhesion of erythrocytes to particular types of cells a Student-Newmans-Keuls test (Sokal & Rohlf, 1969) was performed on the mean percent of positive cells determined for each cell type and each sugar treatment. These means were calculated after pooling results from both experiments I & II and the results of the sensitised and unsensitised erythrocyte treatment. This test indicated (Table 5.3) that within each sugar treatment, significantly more "red staining cells" had adherent erythrocytes than did the other types of cells. Furthermore the numbers of positive "red staining cells" in the control and glucose treatments were significantly greater than those found for any of the other cells. This analysis also revealed that glucose did not alter the numbers of cells with adherent erythrocytes, since within a cell type the means for the glucose and control (no added sugar) treatments were not significantly different. Lactose, however, did significantly reduce the adhesion of erythrocytes to the ascidian cells. Erythrocyte adhesion, in the presence of lactose, was reduced to levels significantly below that observed for the glucose and control treatments for all cells other than the "red staining cells", which nevertheless exibited markedly lower adhesion in the presence of lactose than that observed for the control or glucose treatments.

5.3.2 The effect of anti-HA-2 immunoglobulin on the adhesion of erythrocytes to haemocytes.

Although lactose inhibited the adhesion of erythrocytes to ascidian haemocytes, this result could be explained without involving the HA-2 molecule. For example, adhesion might have been mediated by a lactose-specific molecule other than the HA-2 agglutinin or, alternatively, lactose may have interfered with erythrocyte binding in a non-specific manner, perhaps by altering the properties of the cell

TABLE 5.3 Results of a Student-Newman-Keuls test performed on the data from TABLE 5.1*.

Type of haemocyte	Sugar	Mean of the % of positive cells	Significance
Red cells	Glucose	77.5	
Red cells	None	73.3	
Dark Blue	None	41.0	
Dark Blue	Glucose	36.86	
Other	None	32.57	
Other	Glucose	32.47	
Small blue	None	31.76	
Small blue	Glucose	27.32	
Red cells	Lactose	26.91	
Other	Lactose	14.41	
Small blue	Lactose	13.89	
Dark blue	Lactose	13.26	

* The analysis was performed on the data from Table 5.1 after pooling the results from experiments I & II and the Sensitised and Unsensitised erythrocyte treatments. The groups are arranged according to the descending order of their means. The lines join groups with means not significantly different at the 1% level of significance.

surfaces e.g. hydrophobicity, or by binding to the haemocyte membrane in such a way that although unoccupied, the sites for erythrocyte adhesion were less accessible. In an effort to eliminate such alternative explanations, a rabbit antiserum directed against the HA-2 agglutinin was tested for its capacity to inhibit adhesion.

Monolayers of haemocytes (three replicates for each treatment) were incubated for 30 min at room temperature in 1 ml of a 1:10 dilution in BCCM of a rabbit antiserum specific for either the HA-2 or the HA-1 agglutinin, or for mouse IgG. Also included were monolayers in 1 ml of BCCM without antiserum. The purpose of the HA-1 antiserum was as a specificity control in addition to that provided by the rabbit anti-mouse immunoglobulin, for since the HA-1 agglutinin cannot bind to sheep erythrocytes there seemed little likelihood that this agglutinin was involved in the adhesion of these cells. After incubation, 0.5 ml of medium was removed and 3×10^7 unsensitised erythrocytes were added in 0.5 ml of fresh antiserum, of the same specificity as the previous incubation (diluted 1:10 in BCCM). The monolayers were then incubated for 1 hr at 4°, washed and fixed (Chapter 2, Section 2.18.3). Examination of these monolayers revealed that all the rabbit antiserum irrespective of specificity had inhibited erythrocyte adhesion to about the same extent. It was suspected that this inhibition of adhesion was caused by glycosylated proteins in the rabbit antisera. It was important for this experiment that IgG immunoglobulins, themselves being glycoproteins (Winkelhake, 1978), did not inhibit HA-2 activity by its binding to their oligosaccharide units. Accordingly whole rabbit serum and purified rabbit IgG immunoglobulins were tested for their ability to inhibit the agglutination of sheep erythrocytes by purified HA-2. The results, summarised in Table 5.4, demonstrated that whole rabbit serum did

TABLE 5.4 The effect of rabbit anti-serum and purified rabbit Immunoglobulin on the agglutination of sheep erythrocytes by purified HA-2*.

Sample tested for inhibition			Inhibitory Titre.
Rabbit anti-sera:	anti HA-1		1:16
	anti mouse IgG2a		1:16
IgG preparations¶:	anti LBP-3	(8.5 mg/ml)	NI
	anti mouse IgG2b	(1.4 mg/ml)	NI
	anti mouse IgG	(0.25 mg/ml)	NI
	anti mouse C3	(9.0 mg/ml)	NI

* Serial two-fold dilutions, from neat, of rabbit serum or IgG in a microtitre tray were each mixed with an equal volume (25 µl) of purified HA-2 agglutinin diluted 1:4,000 (4.5 haemagglutinating units) and 50 µl of sheep erythrocytes (0.5% v/v in saline). The diluent was TSA plus 5 mM EDTA and 0.1 mg/ml BSA. Agglutination was assessed after 2 hr at room temperature.

¶ IgG purified from rabbit serum by octanoic acid treatment (Steinbuch & Audran, 1969).

NI = not inhibited

indeed inhibit HA-2 activity whereas purified rabbit IgG, at concentrations similar to those of whole serum (8 - 10 mg/ml), had no effect. IgG preparations purified from rabbit sera by octanoic acid treatment (Steinbuch & Audran, 1969) were therefore used in subsequent experiments.

The earlier experiment was now repeated using IgG preparations from rabbit antisera specific for the HA-2 agglutinin, LBP-3 and mouse IgG2b, each being tested for its capacity to inhibit the adhesion of erythrocytes to B. leachii haemocytes. The anti-LBP-3 antibody used in this experiment reacted strongly with both the LBP-3 molecule and the HA-1 agglutinin, since these share a common subunit (Schluter, 1982; Ey, personal comm.). The experimental procedure was the same as before, with triplicate monolayers for each antibody treatment plus three for the BCCM treatment where no antibody was added. Each IgG preparation was diluted 1:10 in BCCM as before. The results of this experiment (Table 5.5) suggested that both the anti-HA-2 and the anti-LPB-3 immunoglobulins were inhibitory and the experiment was therefore repeated with the following immunoglobulin dilutions: anti-HA-2, 1:10, 1:50 and 1:200; anti-LBP-3, 1:10 and 1:200, and anti-mouse IgG2b, 1:10 and 1:50. Each dilution was tested on triplicate monolayers. These results are included in Table 5.5.

When the protein concentrations of the IgG preparations were determined it was apparent that the anti-mouse IgG2b preparation had less protein than the other preparations. Thus to make a valid comparison between treatments, statistical analyses of experiment II only included data from the immunoglobulin treatments where the protein concentrations were approximately equal (i.e. anti-HA-2, dilution 1:200 (0.059 mg/ml protein); anti-LBP-3, dilution 1:200 (0.043 mg/ml protein) and anti-mouse IgG2b, dilution 1:10 (0.14 mg/ml protein)). As the

TABLE 5.5 The effect of different rabbit immunoglobulin preparations on the adhesion of unsensitised erythrocytes to different types of B. leachii haemocytes.*

Anti-IgG[ø] tested for inhibition of adherence		Type of ascidian cell[@]: Red cells	Dark blue	Small blue	Other	Overall % positive cells[#]	no. of cells counted
No anti-IgG	I[¶]	53.62 ± 3.05	39.90 ± 5.13	29.38 ± 1.59	33.36 ± 4.4	43.32 ± 2.44	745
	II	65.02 ± 2.1	41.83 ± 6.17	36.83 ± 6.01	52.79 ± 3.7	53.04 ± 0.7	800
anti mouse IgG2b (1.4 mg/ml) diluted:							
1:10	I	50.42 ± 2.18	29.43 ± 4.18	29.22 ± 0.84	35.39 ± 0.91	41.32 ± 1.23	873
1:10	II	55.88 ± 0.89	28.47 ± 8.01	31.91 ± 3.5	47.69 ± 3.66	45.89 ± 1.19	836
1:50	II	57.15 ± 1.8	43.70 ± 1.96	43.83 ± 2.88	43.27 ± 5.12	49.25 ± 2.97	851
anti HA-2 (11.7 mg/ml) diluted:							
1:10	I	12.82 ± 2.14	6.00 ± 3.4	7.36 ± 1.22	6.89 ± 1.78	9.77 ± 1.43	862
1:10	II	5.44 ± 0.85	0 ± 0	6.57 ± 2.06	2.72 ± 0.79	4.65 ± 0.52	814
1:50	II	9.33 ± 1.15	2.22 ± 2.22	9.07 ± 1.86	14.57 ± 2.75	10.56 ± 0.55	888
1:200	II	43.63 ± 1.76	35.12 ± 2.87	20.28 ± 2.03	42.57 ± 2.93	36.72 ± 1.54	847
anti LBP-3 (8.5 mg/ml) diluted:							
1:10	I	12.63 ± 0.8	12.18 ± 3.04	4.98 ± 2.08	12.33 ± 0.91	10.90 ± 0.99	935
1:10	II	7.39 ± 0.45	3.90 ± 2.09	3.41 ± 2.02	7.13 ± 1.39	6.07 ± 0.73	918
1:200	II	49.56 ± 2.94	36.11 ± 1.39	23.66 ± 5.02	35.06 ± 1.59	39.02 ± 0.41	749

* Figures in the body of the table are mean ± standard error of the percent of positive cells within a cell type from three replicate slides.

¢ Immunoglobulin prepared from rabbit anti-sera by octanoic acid treatment (Steinbuch & Audran, 1969).

@ As determined by their appearance with Wright's stain.

The number of postive cells as a percent of all the cells counted.

¶ Duplicate experiments (I & II).

variances were shown to be homogeneous by the Cochran test ($C = 0.26$, where $K = 16$ and $n = 3$), the analyses were performed on the untransformed data. A two-factor analysis of variance (with replication) indicated that there were significant differences between both treatments ($F_{(3,32)} = 10.442$, $P < 0.001$) and cell types ($F_{(3,32)} = 31.63$, $P < 0.001$). A Student-Newmans-Keuls test was performed on the means for each IgG treatment and the BCCM treatment, calculated after pooling the data for all cell types. This analysis indicated that the haemocytes not exposed to antibody (BCCM treatment) had significantly more adherent erythrocytes than haemocytes exposed to antibody ($P < 0.01$). None of the immunoglobulin treatments were significantly different from each other. It should be noted that at a 1:10 dilution, the anti-mouse IgG2b preparation contained 2-3 times more protein than a 1:200 dilution of the other two IgG preparations. It was therefore possible that the slight inhibition of erythrocyte adhesion detected with the anti-IgG2b immunoglobulin was the result of a high protein concentration rather than a specific effect. Thus, it would be unwise to make conclusions as to the specificity or otherwise of the inhibitory effects of the immunoglobulins from these data.

The problem regarding the specificity of the inhibitory effects of the immunoglobulins was finally resolved by repeating the experiment using the same experimental protocol, but using immunoglobulin preparations which all had a final concentration of 0.35 mg/ml of protein. This concentration was chosen firstly because it lay within the range where the anti-HA-2 and the anti-LBP-3 preparations appeared to inhibit erythrocyte adhesion (Table 5.5) and secondly because the anti-mouse IgG2b preparation at this concentration did not inhibit the agglutination of sheep erythrocytes by pure HA-2 agglutinin (Table 5.4).

The data from this experiment (Table 5.6) again satisfied the assumption of homogeneity of variances (Crochran test, C = 0.145, K = 16 and n = 3) and were analysed initially by a two-factor analysis of variance but since the (Cell type x Immunoglobulin treatment) interaction was significant ($F_{(9,32)}$ = 5.37, P < 0.001) a Student-Newmans-Keuls test was performed on the means presented in Table 5.6. As with the sugar inhibition experiment (Section 5.3.1), this test indicated that within each treatment there were significantly more "red staining cells" with adherent erythrocytes than was observed for the other types of cells (Table 5.7). The effect of the various immunoglobulin preparations on adherence was now quite clear-cut. The immunoglobluin treatments fell into two significantly different groups, adherence in the presence of anti-HA-2 and anti-LBP-3 antibody being significantly less with all types of cells than that found with either the BCCM treatment or anti-mouse IgG2b antibody. In addition, within a cell type the anti-HA-2 and anti-LBP-3 immunoglobulins inhibited adhesion to a similar extent, while the anti-mouse IgG2b and the BCCM treatment (no immunoglobulin) also produced an equivalent level of adhesion for each type of cell. From these data it was clear that the adhesion of erythrocytes to ascidian cells could be inhibited by antibody directed against either the HA-2 molecule or the LBP-3 molecule. This effect appeared to be specific, since the same concentration of anti-mouse IgG2b immunoglobulin did not inhibit adhesion. The inhibitory effect of the anti-HA-2 immunoglobulin can be seen in Fig 5.3.

FIGURE 5.3 The effect of rabbit immunoglobulins on the adhesion of erythrocytes to B. leachii haemocytes. Adhesion was performed in the presence of IgG preparations containing antibodies of the following specificity; anti-HA-2 (A), anti-LBP-3 (B), anti-mouse IgG2b (C), no immunoglobulin (D). Haemocytes of different types were placed in four categories for scoring purposes and representatives are shown. "Red staining" cells (R), "Dark blue cells" (DB), "Small blue cells" (SB). (mag 280x).

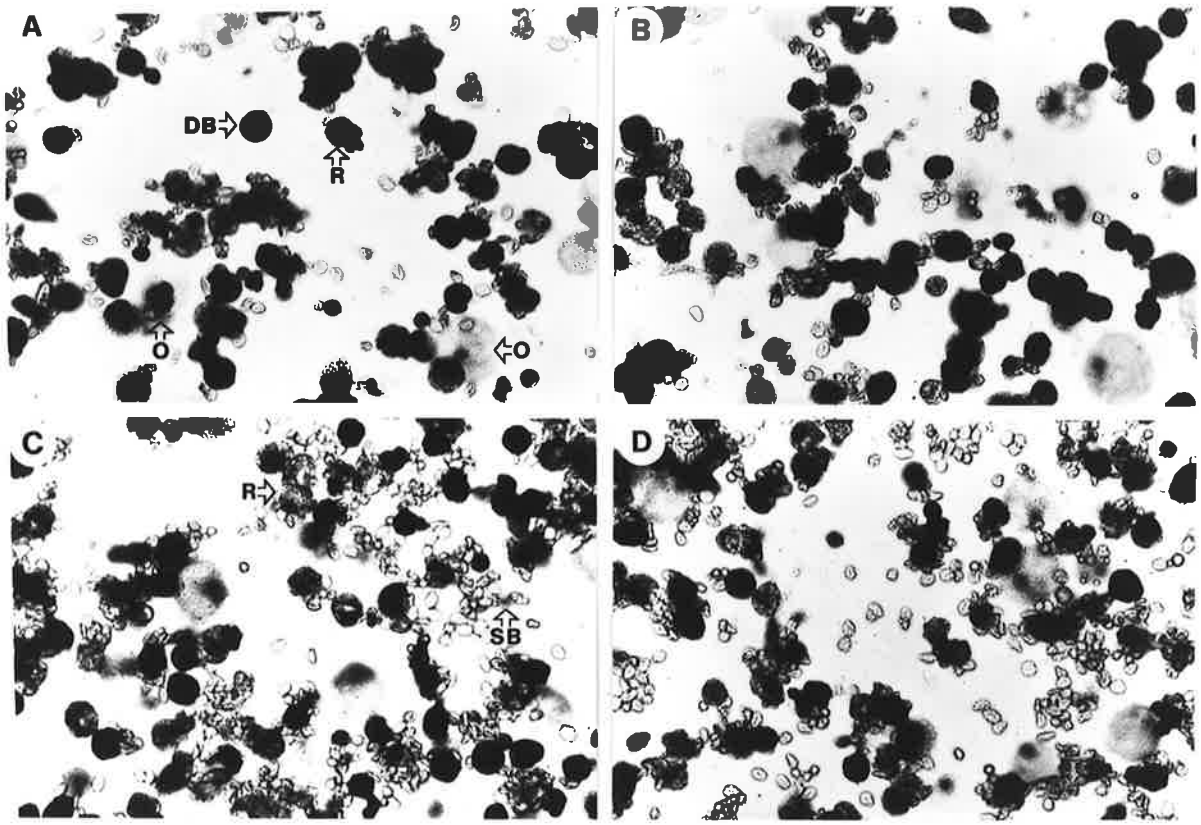

A
DB
R
O
O
B
C
R
SB
D

TABLE 5.6 The effect of different rabbit immunoglobulin preparations on the adhesion of unsensitised erythrocytes to B. leachii haemocytes.*

Anti-IgG¢ tested for inhibition of adherence	Type of ascidian cell@:				Overall % positive cells#	no. of cells counted
	Red cells	Dark blue	Small blue	Other		
No immunoglobulin	75.58 ± 0.71	41.62 ± 4.22	47.79 ± 1.07	51.33 ± 0.31	61.26 ± 0.72	1230
Anti-IgG2b	80.16 ± 0.84	50.51 ± 3.52	49.24 ± 3.08	57.56 ± 2.34	66.86 ± 1.11	1264
Anti-HA-2	22.23 ± 0.81	16.61 ± 1.28	10.01 ± 2.32	13.12 ± 2.69	17.65 ± 1.31	1206
Anti-LBP-3	29.16 ± 3.36	16.44 ± 2.01	16.11 ± 4.20	17.10 ± 2.79	23.15 ± 2.04	1258

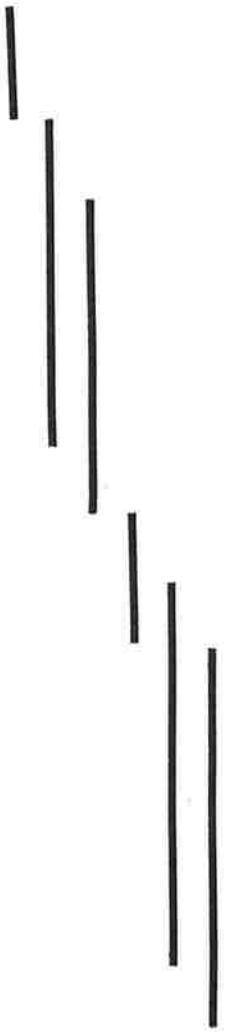
* Figures in the body of the table are mean ± standard error of the percent of positive cells within a cell type from three replicate slides.

¢ Immunoglobulin prepared from rabbit anti-sera by octanoic acid treatment. All immunoglobulins were tested at a final concentration of 0.35 mg/ml.

@ As determined by their appearance with Wright's stain.

The number of positive cells as a percent of all the cells counted.

TABLE 5.7 Results of a Student-Newmans-Keuls test performed on the means displayed in Table 5.6*.

Type of haemocyte	Specificity of the immunoglobulin	Significance
Red cells	anti-mouse IgG2b	
Red cells	no IgG	
Other	anti-mouse IgG2b	
Other	no IgG	
Dark blue	anti-mouse IgG2b	
Small blue	anti-mouse IgG2b	
Small blue	no IgG	
Dark blue	no IgG	
Red cells	anti-LBP-3	
Red cells	anti-HA-2	
Other	anti-LBP-3	
Dark blue	anti-HA-2	
Dark blue	anti-LBP-3	
Small blue	anti-LBP-3	
Other	anti-HA-2	
Small blue	anti-HA-2	

* The groups are arranged in decending order, i.e. the group with the largest mean appears at the top of the list. Lines join groups with means not significantly different at the 5% level of significance.

5.3.3 The specificity of the anti-LBP-3 and anti-HA-2 antibodies.

The ability of the anti-LBP-3 immunoglobulin to inhibit the adhesion of sheep erythrocytes to ascidian cells was somewhat unexpected since sheep erythrocytes do not have binding sites for the LBP-3 molecule (P.L. Ey, personal communication). One possible explanation was that the anti-LBP-3 immunoglobulin preparation reacted with the HA-2 agglutinin. This was examined using an agglutination enhancement assay. The basis of this assay is that erythrocytes coated with sub-agglutinating doses of an agglutinin can be made to agglutinate by the addition of an antibody specific for the coating molecule. The antibody binds to the agglutinin on the erythrocyte surface and so crosslinks the cells. If the erythrocytes are coated with a constant amount of agglutinin then it is possible, by titrating the antibody, to quantitate the extent to which antibodies of different specificity react with the agglutinin.

Sheep erythrocytes were sensitised with 0.25 HAU of purified HA-2 agglutinin, washed three times in saline and resuspended to a concentration of 0.5% (v/v) in saline. The immunoglobulin preparations (anti-HA-2, anti-LBP-3 and anti-mouse IgG2b) were each diluted in serial 2-fold steps in HA-2 diluent (Chapter 2, Section 2.6), commencing with a 1:10 dilution, and mixed with an equal volume (50 µl) of the erythrocyte suspension. Because the cells were sensitised with HA-2, any agglutination would indicate the presence of antibody which had bound and crosslinked the HA-2 molecules. Of the three immunoglobulin preparations tested, only the anti-HA-2 produced any agglutination and this preparation retained its agglutinating activity until diluted beyond 1:160.

A more sensitive enzyme-linked immunosorbent assay (ELISA), carried out by Dr. P.L. Ey, indicated that the activity of the

anti-LBP-3 preparation for the HA-2 molecule was less than 0.02% that of the anti-HA-2 preparation, on a protein weight basis. This finding confirmed the agglutination-enhancement result and showed clearly that the inhibitory activity of the anti-LBP-3 preparation in the erythrocyte adhesion experiment (Table 5.6) could not have been caused by antibodies in the preparation binding to HA-2 molecules. It is of interest to note that the reciprocal activity, i.e. of the anti-HA-2 antibody for the LBP-3 molecule was 0.55% that of the anti-LBP-3 preparation, also measured by ELISA. This slight but nonetheless significant cross-reactivity may have been due to minor contamination of the preparations, rather than genuine antigenic cross-reactivity between the two agglutinins.

It was clear from these data that the anti-LBP-3 antibodies must have inhibited the adhesion of erythrocytes to ascidian cells through means other than by binding to HA-2 molecules which may have been present on the surface of the haemocytes and perhaps also the erythrocytes. Since sheep erythrocytes cannot bind LBP-3 molecules, the effect of the anti-LBP-3 antibody probably occurred by binding to LBP-3 or HA-1 molecules on the ascidian cell surface. The binding of these antibodies to cell-associated LBP-3/HA-1 molecules may have thereby affected the availability of HA-2 molecules.

5.4 An investigation of the surface of B. leachii haemocytes with fluorescent anti-HA-2 and anti-LBP-3 immunoglobulins.

The results of the previous sections supported the notion that the adhesion of sheep erythrocytes to B. leachii haemocytes was mediated via the HA-2 agglutinin. However, nothing was known as to how the agglutinin became bound to the haemocytes. Because unsensitised erythrocytes, as well as those sensitised with HA-2 agglutinin, adhered

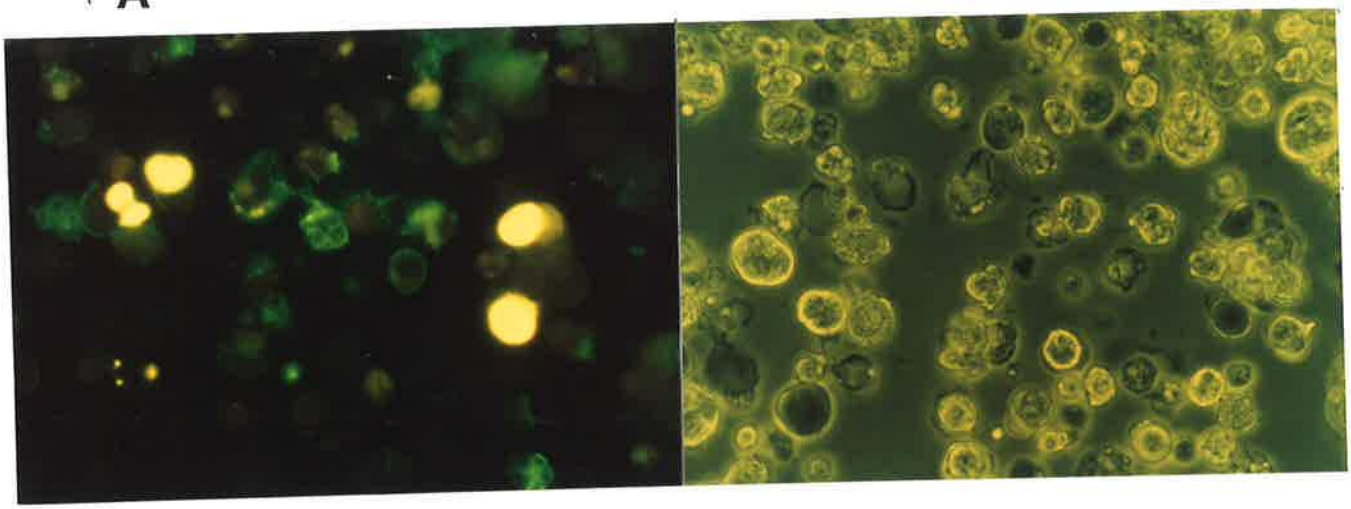
to washed ascidian cell monolayers (Section 5.3.1), it appeared likely that HA-2 molecules were present on the surface of the ascidian cells. It also seemed probable that molecules of LBP-3 or HA-1 were located on the surface of washed ascidian haemocytes, since the inhibition of sheep erythrocyte adhesion by antibodies specific for LBP-3/HA-1 was most readily explained by assuming that these antibodies bound to LBP-3 or HA-1 molecules on the ascidian cells. The occurrence of these molecules on various B. leachii haemocytes was investigated by fluorescence microscopy using fluorescein-labelled IgG from the different rabbit antisera.

Immunofluorescence was performed on monolayers of ascidian haemocytes prepared and washed as described (Chapter, Section 2.18.2). Duplicate monolayers in 0.5 ml of BCCM were incubated with 50 µl of fluorescent immunoglobulin specific for one of the following proteins: LBP-3/HA-1, HA-2, or mouse IgG1. The protein concentration of these immunoglobulin preparations was 1.02 mg/ml, 0.78 mg/ml and 0.72 mg/ml respectively. Incubation was at 4° for 30 min after which the monolayers were washed and mounted in BSS for observation. Both living and fixed cells were used for fluorescent labelling, there being no detectable difference between the labelling patterns for the two methods. When unfixed cells were labelled, it was necessary to fix these cells before observing them under ultra violet light, in order to prevent cell lysis. The fixation procedures and the preparation of the fluorescent immunoglobulins are given in Chapter 2, Section 2.21.2 and 2.21.1.

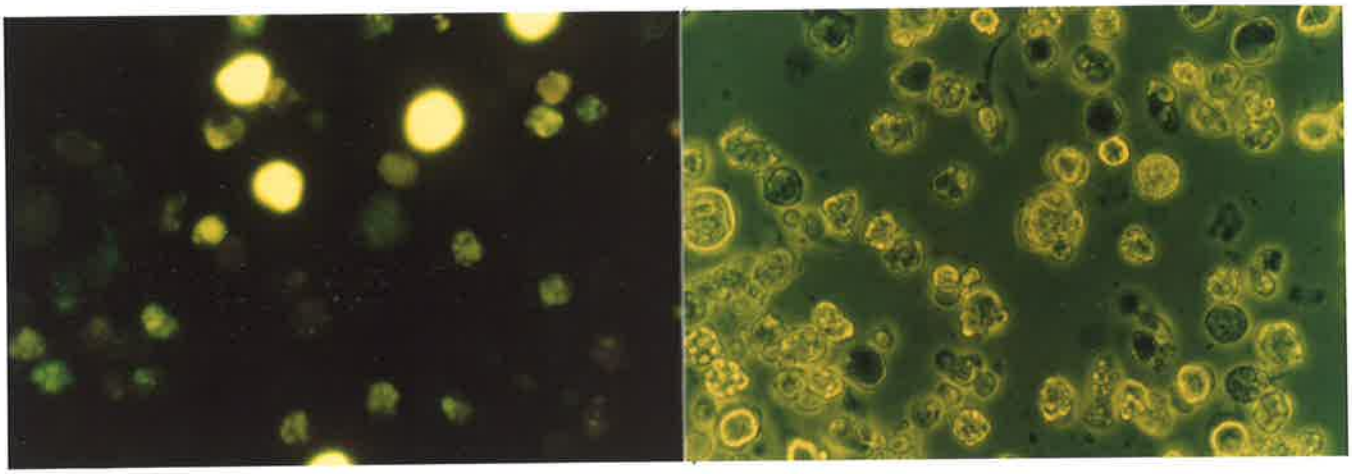
Experiments of this type consistently produced good cell surface fluorescence with the anti-LBP-3 immunoglobulin (Fig 5.4 and Fig 5.5), but no fluorescence was detected using the fluorescent anti-HA-2 or anti-mouse IgG1 immunoglobulins (Fig 5.4). The latter two fluorescent

FIGURE 5.4 B. leachii haemocytes incubated with: fluorescein-labelled anti-LBP-3 immunoglobulin (A); fluorescein-labelled anti-HA-2 immunoglobulin (B); or HA-2 agglutinin followed by a further incubation with fluorescein-labelled anti-HA-2 (C). To indicate the extent of fluorescence, the adjacent photograph shows the same cells as they appeared with white light under phase contrast.
The yellow fluorescing cells were autofluorescent. (mag 320x).

A



B



C

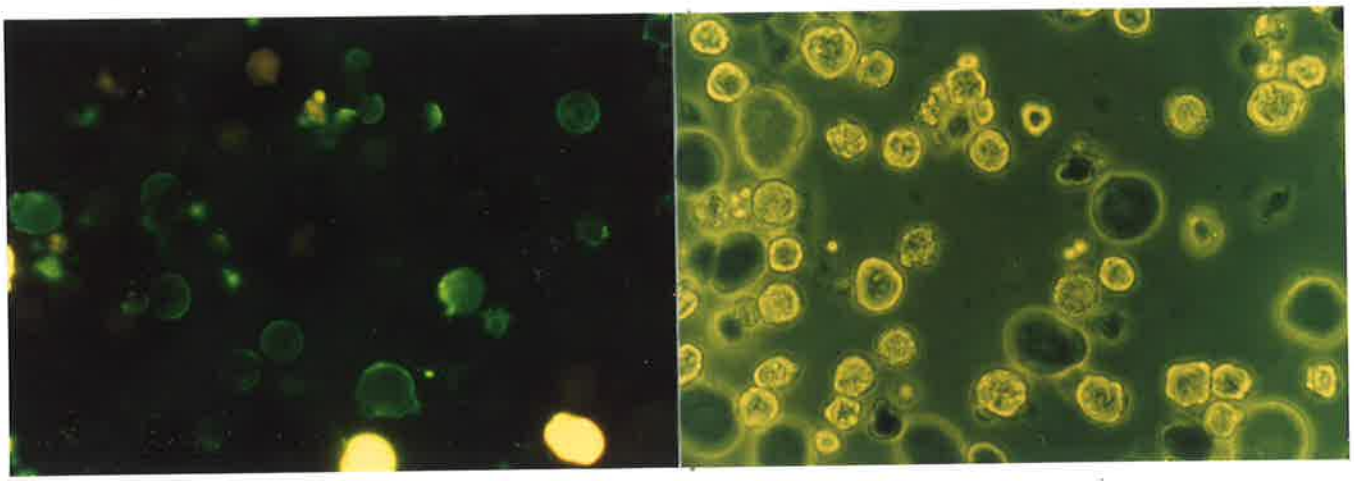
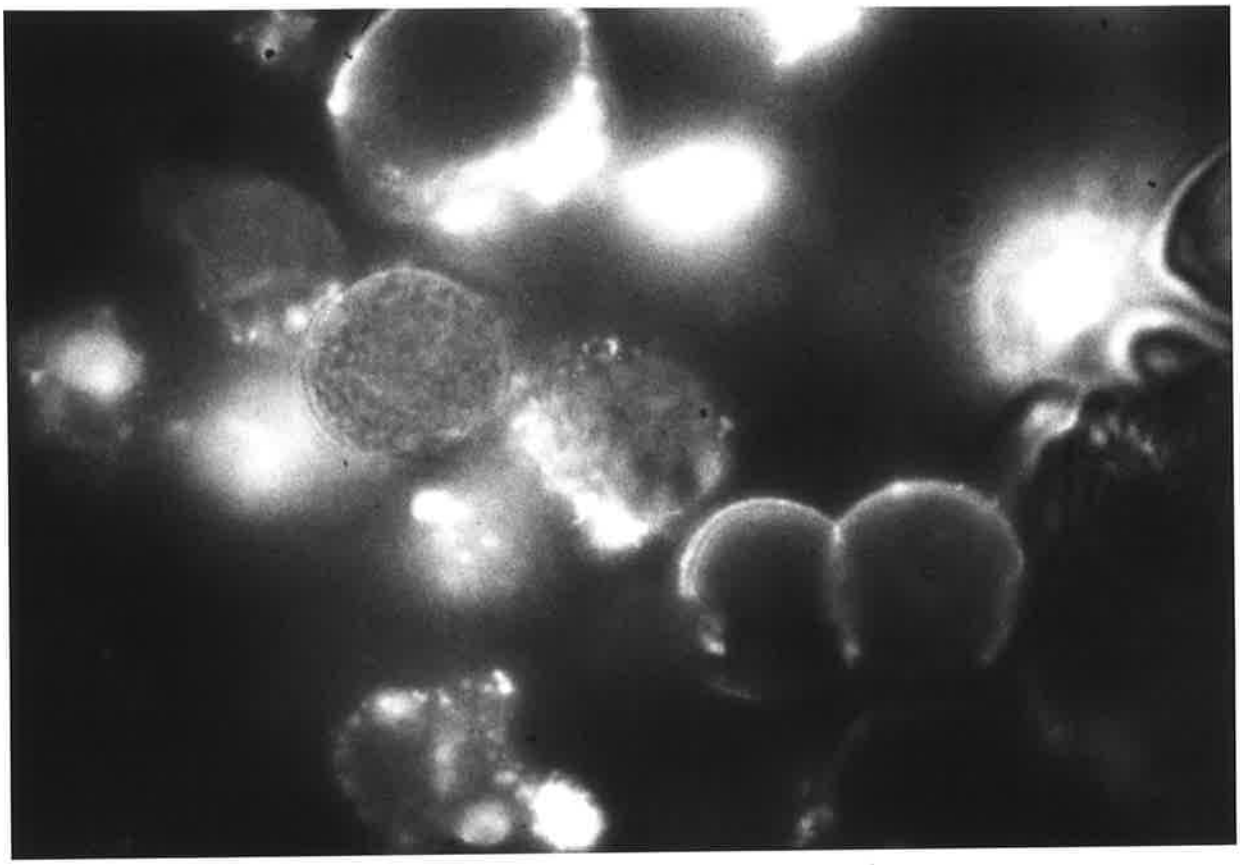
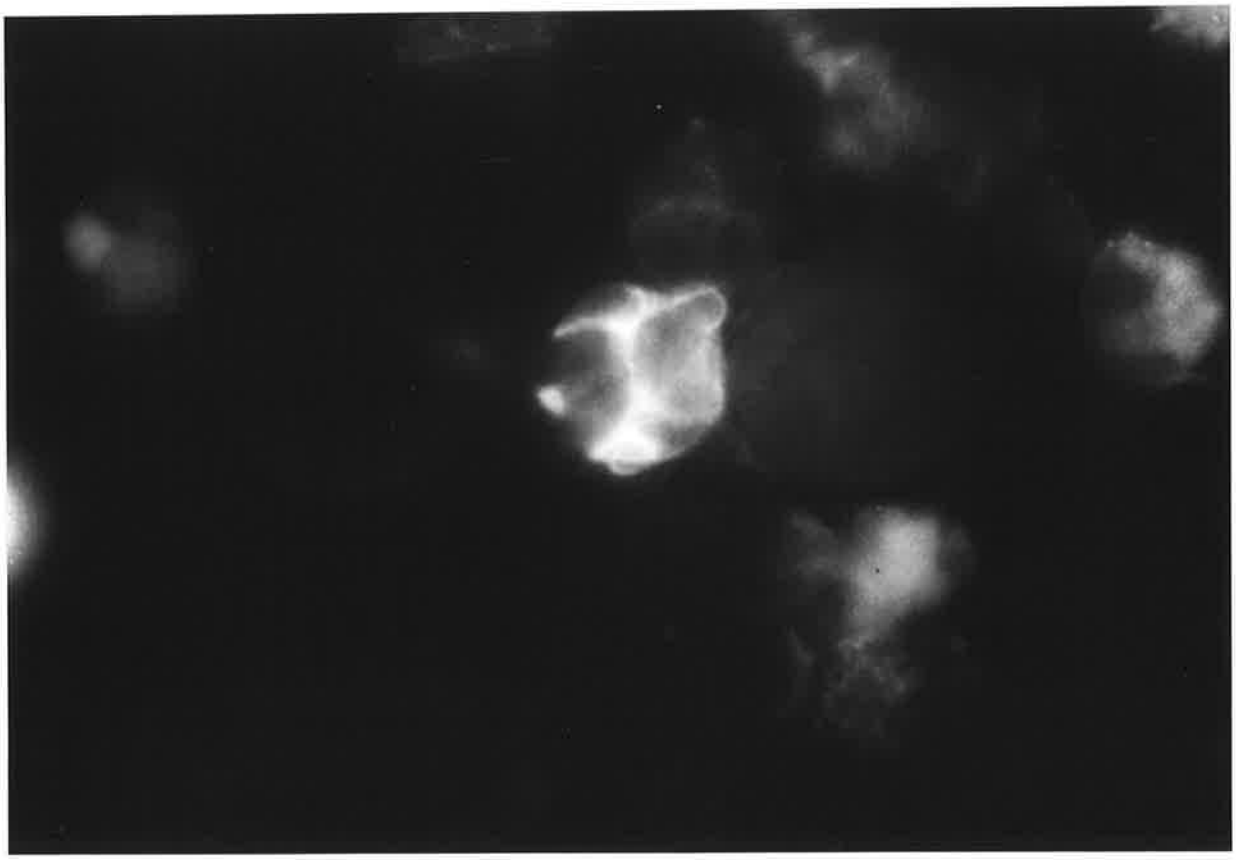


FIGURE 5.5 B. leachii haemocytes incubated with fluorescein-labelled anti-LBP-3 immunoglobulin. Figs (A) and (B) show the fluorescence of a number of different types of cells. (mag 1,500x).

A



B



antibody preparations were shown to be active by adding them to suspensions of erythrocytes sensitised with either the HA-2 agglutinin or mouse IgG1 anti-sheep erythrocyte antibodies. In both cases, the appropriately sensitised erythrocytes became strongly fluorescent while unsensitised cells remained negative.

Photographs of four randomly chosen fields (mag 400x), each field being photographed with both phase contrast or ultra violet light, were used to calculate the percentage of cells showing fluorescence. Of the 529 cells counted, 26.6% were seen to fluoresce with labelled anti-LBP-3 immunoglobulin.

5.5 An examination of the adhesion of erythrocytes to B. leachii haemocytes using immunofluorescence.

The fact that there was no detectable fluorescence with anti-HA-2 immunoglobulin suggested that very little HA-2 agglutinin was present on the surface of ascidian haemocytes. Since the evidence indicated that the HA-2 agglutinin was mediating the adhesion of erythrocytes to ascidian cells, this molecule should have been detectable on monolayers of ascidian cells to which unsensitised erythrocytes had attached. This was examined in the following experiment.

Unsensitised sheep erythrocytes were added to washed monolayers of B. leachii haemocytes and the plates were incubated at 4° for 1 hr to allow time for adhesion to occur. The monolayers were then washed with BSS to remove unbound erythrocytes and incubated with 50 µl of fluorescent anti-HA-2 immunoglobulin in 0.5 ml of BCCM for 30 min at 4°. Control monolayers, which had not been mixed with erythrocytes, were incubated with fluorescent anti-HA-2 or anti-mouse IgG1 immunoglobulins. Following incubation with the fluorescent

immunoglobulins, the cells were washed, fixed in 2.5% (w/v) paraformaldehyde for 30 min at 4° and mounted in BSS for observation.

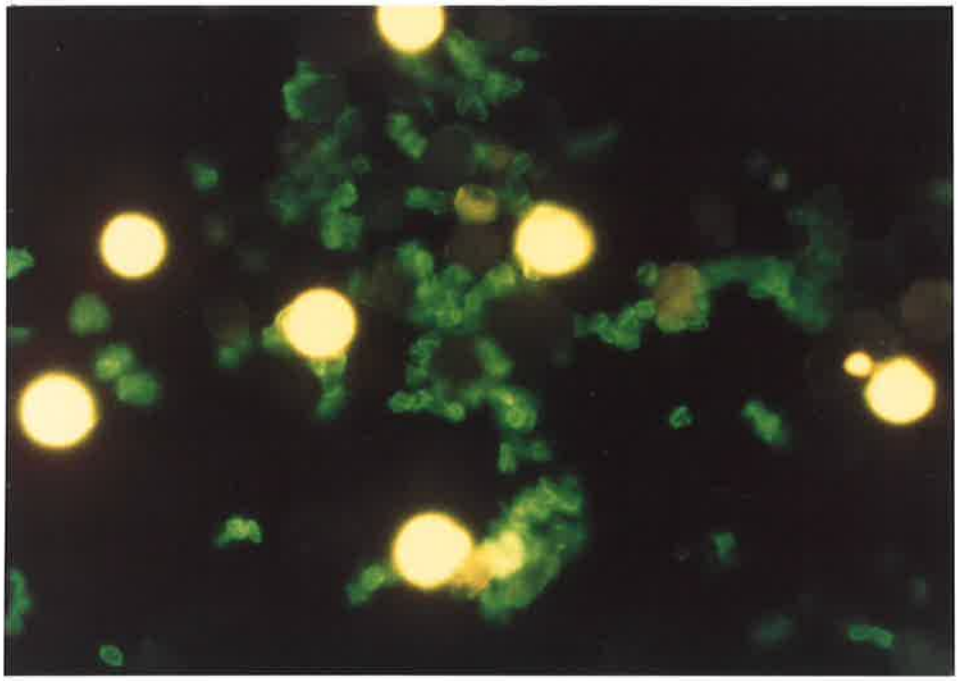
No fluorescence was detectable on the control monolayers but the experimental monolayers, in contrast, had brightly fluorescent erythrocytes adhering to, in most cases, non-fluorescent ascidian cells (Fig 5.6). This fluorescence could only have been due to the labelled anti-HA-2 immunoglobulin binding to HA-2 molecules on the surface of the erythrocytes. As the erythrocytes were not sensitised with agglutinin before adding them to the monolayers and normal sheep erythrocytes did not fluoresce, it was concluded that the ascidian cells were secreting HA-2 molecules which bound to the erythrocytes, sensitising them for adhesion. Accordingly, ascidian haemocytes should have receptors for the HA-2 agglutinin on their surface.

It was particularly important in terms of testing the proposal that the HA-2 agglutinin functions as an opsonin in B. leachii, to determine whether or not receptors for this agglutinin did in fact occur on the surface of some haemocytes. To test this point ascidian cell monolayers were incubated with 0.5 ml of fluorescein-labelled HA-2 agglutinin. This preparation had a protein concentration of 0.12 mg/ml and a sheep erythrocyte agglutinating titre of 1/400, which is approximately 3-4 times greater than normal haemolymph. It was anticipated that if receptors for the HA-2 agglutinin were present on the surface of the cells then fluorescence would be observed. This was not the case. The control monolayers, incubated with fluorescent anti-HA-2 immunoglobulin, were also negative whereas those incubated with fluorescent anti-LBP-3 immunoglobulin exhibited good fluorescence as had been previously observed.

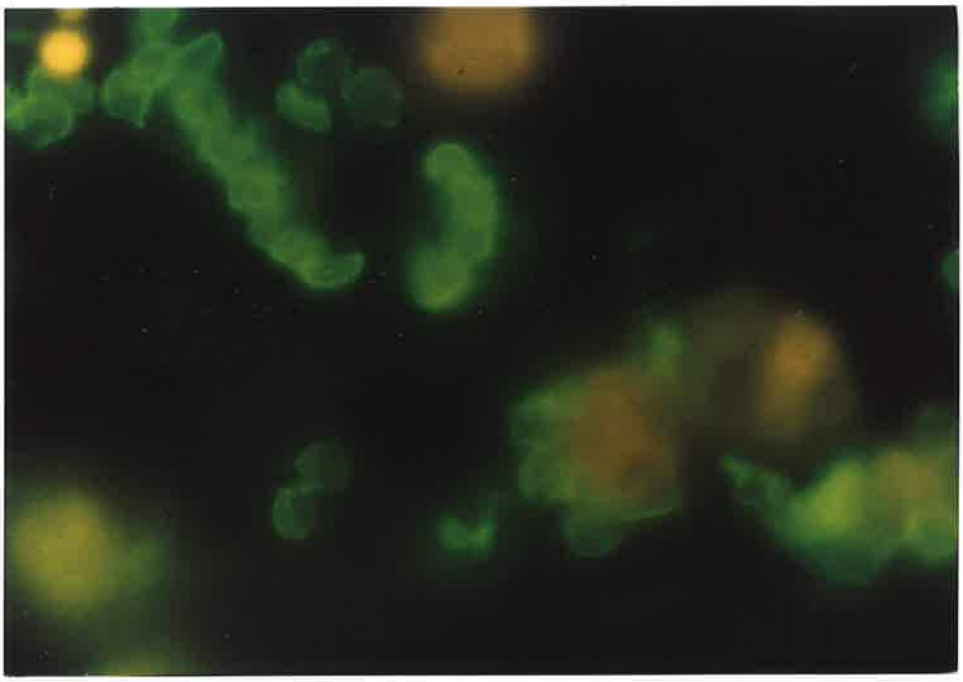
On the basis of these results, it was postulated that if receptors for the HA-2 agglutinin did exist on the surface of certain

FIGURE 5.6 The adherence of fluorescein-labelled anti-HA-2 immunoglobulin to unsensitised sheep erythrocytes on a monolayer of *B. leachii* haemocytes. Magnification 460x (A) and 1,100x (B).

A



B



haemocytes, then they must have either bound free HA-2 molecules with low affinity or been present in such low numbers that the amount of labelled HA-2 which bound to them was insufficient to be detected. It was thought that in monolayers incubated first with HA-2 agglutinin and then with fluorescent anti-HA-2 immunoglobulin, any cell surface fluorescence might be enhanced by a "piggy-back" effect, i.e. since the antibodies in the fluoresceinated preparation are directed against all parts of the HA-2 molecule then it is conceivable that more than one fluorescent anti-HA-2 immunoglobluin molecule would adhere to a single HA-2 molecule.

Duplicate monolayers were first incubated at 4° for 1 hr with 0.5 ml of BCCM containing 0.24 mg or 0.048 mg of purified HA-2 agglutinin corresponding to an agglutinating titre of 1/1,500 and 1/300 respectively. Following this exposure to the HA-2, the monolayers were washed, at room temperature, in three separate 20 ml aliquots of BSS so as to ensure the removal of all unbound HA-2 agglutinin. They were then each immersed in 0.5 ml of a 1:10 dilution of fluorescent anti-HA-2 immunoglobulin in BCCM for a second incubation at 4°. After 30 min the monolayers were washed and fixed in paraformaldehyde. The control monolayers underwent the same treatment but were not exposed to any purified HA-2 agglutinin. Instead, they were incubated with 0.5 ml of BCCM and then, after washing, with 0.5 ml of fluorescent anti-HA-2 or fluorescent anti-mouse IgG1 immunoglobulin (each diluted 1:10) or fluorescent HA-2 agglutinin (0.12 mg/ml). As an additional control, fluorescent anti-HA-2 immunoglobulin (diluted 1:10) was also added to monolayers of ascidian cells which had been pre-incubated with sheep erythrocytes and washed. All the monolayers in this experiment were prepared from a single suspension of ascidian cells so that the results for each treatment could be compared.

The results are summarized in Table 5.8 and the fluorescence of cells exposed to 0.24 mg of HA-2 agglutinin and then to fluorescein-labelled anti-HA-2 immunoglobulin can be seen in Fig 5.4C and Fig 5.7. The percentage of cells showing fluorescence with anti-HA-2 immunoglobulin after exposure to 0.24 mg of HA-2 agglutinin was calculated from photograghs of five randomly chosen fields (mag 400x), each field being photographed with both ultra violet light and under phase contrast. A total of 477 cells were counted and of those 19% were fluorescent. Haemocytes incubated with fluorescent HA-2 agglutinin were again negative. These results therefore indicate that HA-2 receptors are present on the surface of B. leachii haemocytes, however a previous exposure to HA-2 molecules was required for their detection

As had been found previously, haemocytes which had not been pre-incubated with HA-2 agglutinin were not labelled with fluorescent anti-HA-2 immunoglobulin. This concentration of immunoglobulin was nevertheless sufficient to reveal HA-2 molecules on the surface of the adherent erythrocytes. It seems, as was suggested above, that few HA-2 molecules are present on the surface of washed haemocytes.

5.6. Discussion.

Phagocytosis by circulating amoebocytes appears to be an important step in the elimination of sheep erythrocytes from the circulation of B. leachii colonies. From Rowley's description of Ciona intestinalis haemocytes (Rowley, 1981), it is likely that phagocytosis was performed by cells analogous to his "granular amoebocytes". Amoebocytes from a variety of other ascidian species have also been found capable of phagocytosing particles both in vivo and in vitro (Fulton, 1920; Endean, 1960; Smith, 1970; Anderson, 1971;

TABLE 5.8 The fluorescence of B. leachii haemocytes incubated with labelled anti-HA-2 or anti-IgG1 immunoglobulin or labelled HA-2 agglutinin.

Incubation I[#]		Incubation II[§]	Intensity of
Treatment of ascidian cell monolayers			fluorescence
HA-2 agglutinin (0.096 mg/ml)	then	anti-HA-2 IgG*	+
HA-2 agglutinin (0.48 mg/ml)	then	anti-HA-2 IgG*	+ + +
Culture medium[¶]	then	anti-HA-2 IgG*	-
Culture medium[¶]	then	HA-2 agglutinin* (0.12 mg/ml)	-
Culture medium[¶]	then	anti-IgG1 IgG* (control)	-

1 hr at 4°.

§ 30 min at 4° - details of the procedure are given in the text.

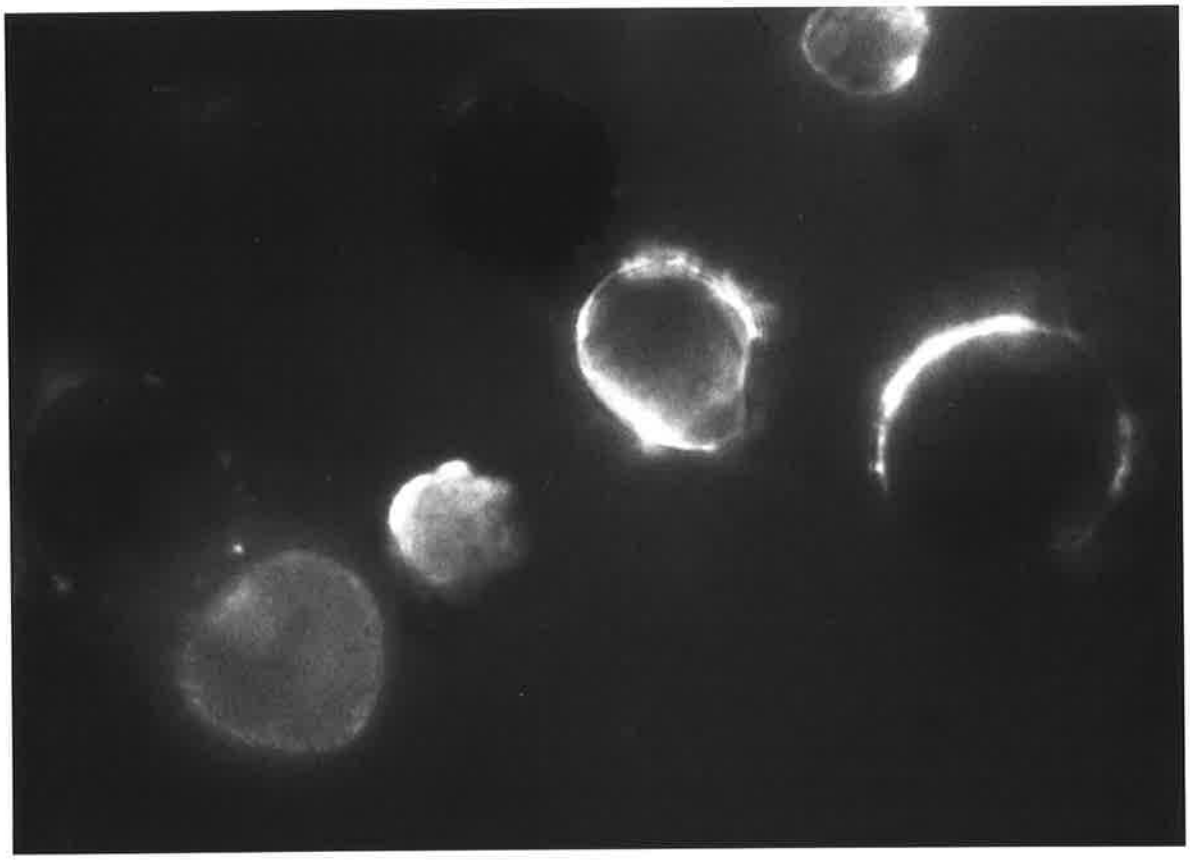
* FITC-cellite labelled preparations

¶ Botrylloides cell culture medium.

\+ Fluorescence just visible.

+++ Marked fluorescence.

FIGURE 5.7 The fluorescence with fluorescein-labelled anti-HA-2 immunoglobulin of B. leachii haemocytes previously treated with HA-2 agglutinin (0.48 mg/ml). (mag 1,500x).



Brown & Davies, 1971; Fuke & Sugai, 1971; Wright, 1974; Rowley, 1981). However, virtually no evidence is available to indicate how the foreign particles are recognised by the phagocytic cells. The *in vitro* culture of *B. leachii* haemocytes, developed as part of this thesis, provided an experimental system whereby one could conceivably test whether or not the sheep erythrocyte agglutinin (the HA-2 agglutinin) could function as a recognition molecule in this species and promote the phagocytosis of particles to which it became bound.

It was found that the adhesion of sheep erythrocytes to *B. leachii* haemocytes could be inhibited by approximately 40% in the presence of lactose, while glucose at the same concentration was ineffective. The HA-2 agglutinin is specifically inhibited by lactose and this result was certainly consistent with the idea that this agglutinin was somehow involved in the adhesion of the erythrocytes to the haemocytes. Rabbit immunoglobulins directed against the HA-2 molecule likewise inhibited erythrocyte adhesion whereas immunoglobulins specific for mouse IgG2b had no effect.

Work with fluorescein-labelled antibodies supported the opsonic role of the HA-2 agglutinin. It was found that sheep erythrocytes not previously exposed to any HA-2 agglutinin not only adhered to ascidian haemocytes but, as revealed by immunofluoresence, they were also coated with HA-2 agglutinin. Furthermore, the results suggested that receptors for the HA-2 molecule exist on the surface of some ascidian cells. From these findings one can conclude that ascidian haemocytes were secreting HA-2 agglutinin which then sensitised the erythrocytes for adhesion via the HA-2 receptor on the surface of ascidian cells. As it was impossible to detect any HA-2 agglutinin on the surface of the haemocytes in the absence of foreign particles, it was possible that the erythrocytes were stimulating the secretion of the HA-2

molecules. Such an idea is not novel for not only have agglutinins been detected within the haemocytes of other invertebrates (Cornick & Stewart, 1973; Amirante, 1976; Amirante & Mazzalai, 1978 and van der Knaap et al., 1981a), but it was suggested from an observation by van der Knaap et al. (1981a) that amoebocytes from the pond snail, Lymnaea stagnalis, may also release agglutinin upon contact with foreign material. They observed that sheep erythrocytes incubated with snail amoebocytes in the absence of serum were agglutinated. However, it was not made clear whether or not the agglutinin was released irrespective of the presence of the sheep erythrocytes. It is possible that the snail agglutinin, like the HA-2 molecule, could be an opsonin, since the opsonising and the agglutinating activities of haemolymph were positively correlated (van der Knaap et al., 1982). However until the snail agglutinin is purified, its function cannot be reliably ascertained.

It is interesting to note that although adherent erythrocytes fluoresced brightly with anti-HA-2 immunoglobulin, the majority of the ascidian cells to which they were attached showed no fluorescence. This indicates that there must have been considerably more binding sites for the HA-2 agglutinin on the surface of the erythrocytes than on the ascidian cells and/or that the HA-2 molecule bound to the ascidian cells with lower affinity. It is possible that when HA-2 molecules are aligned on a particle, such as an erythrocyte, they are presented in a manner more favourable for binding to the ascidian cell HA-2 receptors than when in an uncomplexed soluble state. There are precedents for this type of phenomenon, an example being that of antibody binding to macrophage Fc receptors (Karush, 1976). In this instance, the preferential binding of aggregated immunoglobulin or of antibody-antigen complexes has been demonstrated to involve multi-point

attachment to Fc receptors on the surface of the macrophage (Karush, 1976; Knutson et al., 1977). Thus, a high binding affinity of the agglutinin-particle complex for the ascidian cells relative to that of free agglutinin may be expected without necessitating any conformational change in the agglutinin upon binding to its substrate.

Alternatively, the binding of the agglutinin to a foreign particle (or its oligosaccharide ligand) may generate or expose (by allosteric effects) another binding site for a receptor present on the surface of haemocytes. If such a site had a higher affinity for the haemocyte receptor than uncomplexed HA-2 then this could also explain why HA-2 molecules were not detected on the surface of haemocytes. One way of investigating this possibility would be to incubate haemocytes with HA-2 molecules in the presence of lactose and determine the extent of HA-2 adherence by then incubating the haemocytes with fluorescein-labelled anti-HA-2 immunoglubulin. The concentrations of both the lactose and the agglutinin would be critical in such an experiment. The lactose must saturate sufficient HA-2 molecules to cause a noticable change in the level of fluorescence, yet the saturation of all the lactose binding sites on the agglutinin molecule might inhibit its attachment to the haemocytes. A similar type of experiment could be performed with HA-2:anti-HA-2 complexes. However, it could not be assumed that the binding of antibodies to various determinants on the HA-2 agglutinin would necessarily have the same effect on the HA-2 molecule as the binding of a foreign particle (e.g. sheep erythrocytes) to the lactose-specific sites.

The role of the LBP-3 molecule and the HA-1 agglutinin in the ascidian system is not clear. It is important to note that the anti-LBP-3 immunoglobulin preparation used here did not differentiate

between the LBP-3 and the HA-1 agglutinin; in fact it reacted to approximately the same extent with each protein.

The adhesion of sheep erythrocytes to ascidian haemocytes was inhibited not only by anti-HA-2 immunoglobulin but also by anti-LBP-3 immunoglobulin. Since no reactivity could be detected between the anti-LBP-3 immunoglobulin and the HA-2 agglutinin and as sheep erythrocytes do not have receptors for LBP-3 molecules it is reasonable to conclude that the inhibition by anti-LBP-3 immunoglobulin must have occurred at the ascidian cell surface. It was shown by immunofluoresence that there are indeed LBP-3 (or HA-1) molecules on the surface of a variety of ascidian haemocytes. The binding of antibody to LBP-3 molecules on the ascidian cell surface may cause these complexes to cap in a way similar to that observed with the binding of concanavalin A to the surface of both oyster haemocytes (Yoshino et al., 1979) and earthworm leucocytes (Roch & Valembois, 1978). If, as seems likely from Fig 5.4, that the HA-2 receptors occur on cells with surface LBP-3/HA-1 molecules, then capping of the LBP-3 molecules might affect the distribution and availability of HA-2 receptors such that sheep erythrocytes can no longer adhere.

It has been reported that some purification of haemocytes from the blood of the blowfly, Calliphora vicina, could be obtained by equilibrium density centrifugation on a discontinuous gradient of Ficoll (Peake, 1979). Similar attempts to purify and separate the various types of B. leachii haemocytes using discontinuous Percoll gradients proved unsuccessful. It was possible, but not consistently so, to obtain reasonable purity of some cell types, e.g. bands were found to contain 76% "lymphocyte-like" cells, 81% compartmental cells or 74% amoeboid cells. However the extreme fragility of the cells collected off a Percoll gradient made it impossible to perform assays

with the purified cell preparations. This occurred even though all Percoll concentrations had been made iso-osmotic with haemolymph. In addition, the extent of purification varied depending on the colony from which the cells were collected, thus subtle modifications of the gradient, to obtain greater purity, were impossible. For these reasons the question of whether the cells that rosette and phagocytose erythrocytes possess LBP-3 molecules and HA-2 receptors on their surfaces must be addressed by a different means. The technique of double labelling, i.e. incubating haemocytes with both fluorescein-labelled anti-LBP-3 immunoglobulin and rhodamine-labelled anti-HA-2 immunoglobulin molecules, could be useful for this purpose. This method would alleviate the problems of clearly identifying the various cell types. Nevertheless, a comparison of cell morphology is the only means by which this question can be discussed at the present time. Using the Rowley classification of ascidian haemocytes (Rowley, 1981), cells with HA-2 receptors appear to be a subset of those cells possessing surface LBP-3 molecules. Other types of cells which were labelled with fluorescein-labelled anti-LBP-3 immunoglobulin included compartmental cells and signet ring cells. Although HA-2 receptors were found predominantly on the granular amoebocytes (or "red staining cells"), the cells that most frequently formed erythrocyte rosettes, the percentage of rosette-forming cells in the total haemocyte population was higher than the percentage of cells calculated to have HA-2 receptors. The immunofluorescence technique used may not have revealed all the cells with HA-2 receptors, because it may be neccessary for a large number of fluorescent antibody molecules to bind to the surface of a cell before fluoresence is observed. It should also be noted that the proportion contributed by each cell type to the

total haemocyte population varied between colonies. This was particularly the case for the granular amoebocytes.

Not only did the proportion of each cell type vary between colonies but the extent to which erythrocytes bound to the ascidian cells also varied. A similar problem was noted when amoebocytes from the gastropod, Biomphalaria glabrata were cultured in vitro (Abdul-Salam & Michelson, 1980). The rate of phagocytosis measured with amoebocytes from individual snails varied markedly, but in this case the problem could be overcome by preparing amoebocyte monolayers from pooled haemolymph. Because it had been reported that a cytotoxic reaction occurred when cells from allogeneic individuals of the ascidian Halocynthia roretzi were mixed (Fuke, 1980), haemocytes from different B. leachii colonies were not pooled for the experiments described in this thesis. Erythrocyte adhesion was observed in all experiments, although the degree of adhesion varied between experiments (occasionally by as much as 10 - 15%). However, since comparisons between treatment and control monolayers were always made within an experiment where all monolayers had been prepared from the same haemocyte suspension, the variation observed between experiments had no bearing on the overall result.

It is not yet known whether the cells secreting the HA-2 agglutinin are identical to those with HA-2 agglutinin receptors on their surfaces. Nevertheless, it is clear that the HA-2 agglutinin can mediate the adhesion of sheep erythrocytes to B. leachii haemocytes. In addition, preliminary data (from experiments not presented in this thesis) have indicated that of a variety of bacteria which are agglutinated by B. leachii haemolymph, Streptococcus faecalis, the only type tested which was also agglutinated by purified HA-2 agglutinin,

adhered to B. leachii haemocytes. This suggests that the HA-2 agglutinin could indeed function as an opsonin in this species, binding to any particle bearing the appropriate sugar residues. The HA-1 agglutinin and the LBP-3 molecule may function in an analogous manner, but this remains to be shown by further investigation.

Chapter 6.

THE OPSONIC ACTIVITY OF THE HA-2 AGGLUTININ WITH MOUSE MACROPHAGES

6.1 Introduction.

Other studies have revealed that haemagglutinins are found in a variety of invertebrates (review: Ey & Jenkin, 1892). Investigations indicate that at least some of these molecules may have a protective function (Tyson & Jenkin, 1973 and 1974; Renwrantz & Mohr, 1978 and Harm & Renwrantz, 1980) since they are required for the recognition and elimination of foreign particles by the animals' phagocytic cells (see Chapter 1). However, none of the agglutinins characterised so far show any structural homology with the immunoglobulins of vertebrates (Gold & Balding, 1975).

Ascidians are protochordates, which are believed to occupy a phylogenetic position between the vertebrates and the invertebrates. Both this study (see Chapter 3) and other investigations have revealed the presence of agglutinins for vertebrate erythrocytes in the haemolymph of various ascidians (e.g. Fuke & Sugai, 1972; Wright, 1974; Anderson & Good, 1975; Parrinello & Patricolo, 1975; Wright & Cooper, 1975) but in most cases there is a lack of good data on the structure and function of these molecules. As immunoglobulin molecules have been identified in all the classes of vertebrate (review: Marchalonis, 1977) including two cyclostomes, the hagfish (Raison et al., 1978) and the lamprey (Marchalonis & Edelman, 1968), it is probable that a modified form of the gene coding for the precursor form of immunoglobulin exists today in advanced invertebrates. The phylogenetic position of the ascidians makes these animals potentially important in investigations on the evolutionary origin of immunoglobulins and it is not unreasonable to propose the erythrocyte agglutinins of protochordates as prime candidates to exhibit some homology with immunoglobulin.

The results of the previous chapters demonstrated that B. leachii haemolymph contained two haemagglutinins, HA-1 and HA-2, which exhibit different binding properties. A third, non-agglutinating protein (termed LBP-3), having a specificity apparently identical to that of the HA-1 agglutinin, has also been identified (Ey & Jenkin, 1982; Schluter, 1982).

It was shown in Chapter 5 that sheep erythrocytes adhered to and were phagocytosed by B. leachii haemocytes. The results of in vitro experiments (Chapter 5, Section 5.5) indicated that the HA-2 agglutinin was responsible for the adhesion of sheep erythrocytes to the B. leachii cells. As a bacterium, which was agglutinated by purified HA-2 agglutinin, also adhered to B. leachii haemocytes (results not shown) it is possible that this agglutinin functions as an opsonin in this species, for foreign particles bearing the appropriate carbohydrate moieties. The functions of the HA-1 agglutinin and LBP-3 are not known. In view of the close affinity of the protochordates to the vertebrates, it was of considerable interest to determine whether or not the opsonic factor(s) in the haemolymph of B. leachii could act as recognition factor(s) for phagocytic cells from a vertebrate. Certainly within the vertebrate classes, immunoglobulin molecules from one class will function as recognition factors for phagocytes from another class (Jenkin, unpublished observations). If factors present in the haemolymph of a protochordate functioned as opsonins not only for this animal but also for vertebrate phagocytes, then some homology between the protochordate opsonin and vertebrate immunoglobulin might be indicated. With such a possibility in mind, B. leachii haemolymph was tested for its ability to induce firstly the adhesion of sheep erythrocytes to mouse macrophages (Section 6.2) and secondly the phagocytosis of any adherent erythrocytes (Section 6.3). Both aspects

(adherence and phagocytosis) were investigated together, but for clarity in presentation each will be discussed separately in order to emphasize different points.

6.2 The role of the B. leachii haemagglutinins in the adherence of sheep erythrocytes to mouse macrophages.

6.2.1 The adherence to mouse macrophages of sheep erythrocytes sensitised with B. leachii haemolymph.

To determine if factor(s) in B. leachii haemolymph opsonic for vertebrate macrophages were present, sheep erythrocytes were sensitised with various dilutions of B. leachii haemolymph and assayed for their ability to adhere to mouse macrophages. The erythrocytes (1% v/v in physiological saline) were sensitised with an equal volume of haemolymph diluted 1:64, 1:256 and 1:512 in saline, these dilutions being equivalent to 1, 1/4 and 1/8 haemagglutinating units (HAU) respectively. Cells sensitised with 1 HAU (a 1:512 dilution) of a rabbit antiserum specific for sheep erythrocytes (rabbit antibody) were used as positive controls. After sensitisation, the cells were washed, resuspended in medium 199 supplemented with 10% foetal calf serum (M199-FCS) and 1 ml aliquots containing 1.5×10^8 erythrocytes were added to Leighton tubes containing a monolayer of mouse macrophages on a flying coverslip. The monolayers were incubated overnight at 4°. The coverslips were then removed, washed, fixed and stained with Wright's stain. Macrophage monolayers which had been incubated with an equivalent number of unsensitised erythrocytes served as negative controls. Two to three repeat experiments consisting of three macrophage monolayers for each dilution of haemolymph and the unsensitised control were set up.

The significance of differences in the adherence of sensitised and unsensitised erythrocytes to macrophages was determined using a two-way analysis of variance (ANOVA) (Sokal & Rohlf, 1969). All analyses were carried out on the mean number of erythrocytes adhering to each macrophage. This value was calculated from a frequency distribution of macrophages having specific numbers of adherent red cells (from 0 to $\geqslant 6$). For ease of display in the tables, however, the numbers of erythrocytes adhering to macrophages were placed in the categories 0, 1-2, 3-5, and 6 or more. The majority of macrophages in the latter category were completely surrounded by erythrocytes. The tables show pooled data from a number of repeat experiments, since in each case the interaction term was non-significant.

The data presented in Table 6.1 show that erythrocytes treated with the two highest concentrations of haemolymph adhered strongly to macrophages. However, the adhesion of cells treated with a 1:512 dilution of haemolymph was not significantly different from that of unsensitised erythrocytes. The adherence of sensitised compared to that of unsensitised cells is depicted in Fig 6.1.

Although haemolymph promoted the adherence of erythrocytes to macrophages it was feasible that a variety of erythrocyte agglutinins could also induce the binding of these cells to macrophages. If this was found to be the case for erythrocyte agglutinins known to differ structurally from immunoglobulin, then it was possible that the B. leachii sensitising factors could also have caused adhesion in a manner that may not suggest any binding site homology with immunoglobulin. On the other hand, if the promotion of adherence by agglutinins were a rare event then the probability that the ascidian factors were similar to immunoglobulin would be increased.

TABLE 6.1 The adhesion of sheep erythrocytes to mouse peritoneal macrophages after treatment with haemolymph from Botrylloides leachii.

Treatment of erythrocytes	Number of macrophages counted	% of macrophages[@] with adherent erythrocytes				Mean number[¶] of erythrocytes per macrophage
		0	1-2	3-5	⩾6	
None (control)[¢]	2,068	57.2 ± 2.7	35.3 ± 2.3	7.40 ± 0.87	0.35 ± 0.16	0.73 ± 0.05
Haemolymph[¢] (1:64 dilution)	2,241	7.80 ± 0.87	15.6 ± 1.2	20.1 ± 2.0	56.3 ± 2.8	4.40 ± 0.11
None (control)[*]	1,652	65.0 ± 1.7	30.5 ± 0.81	4.40 ± 0.85	0.12 ± 0.12	0.54 ± 0.03
Haemolymph[*] (1:256 dilution)	2,722	37.2 ± 2.6	37.7 ± 1.5	19.4 ± 1.5	6.2 ± 1.2	1.60 ± 0.13
None (control)[§]	1,507	66.0 ± 2.0	30.2 ± 1.6	3.65 ± 0.66	0.22 ± 0.34	0.52 ± 0.04
Haemolymph[§] (1:512 dilution)	1,600	60.7 ± 2.9	31.7 ± 2.2	7.13 ± 0.82	0.50 ± 0.17	0.69 ± 0.06

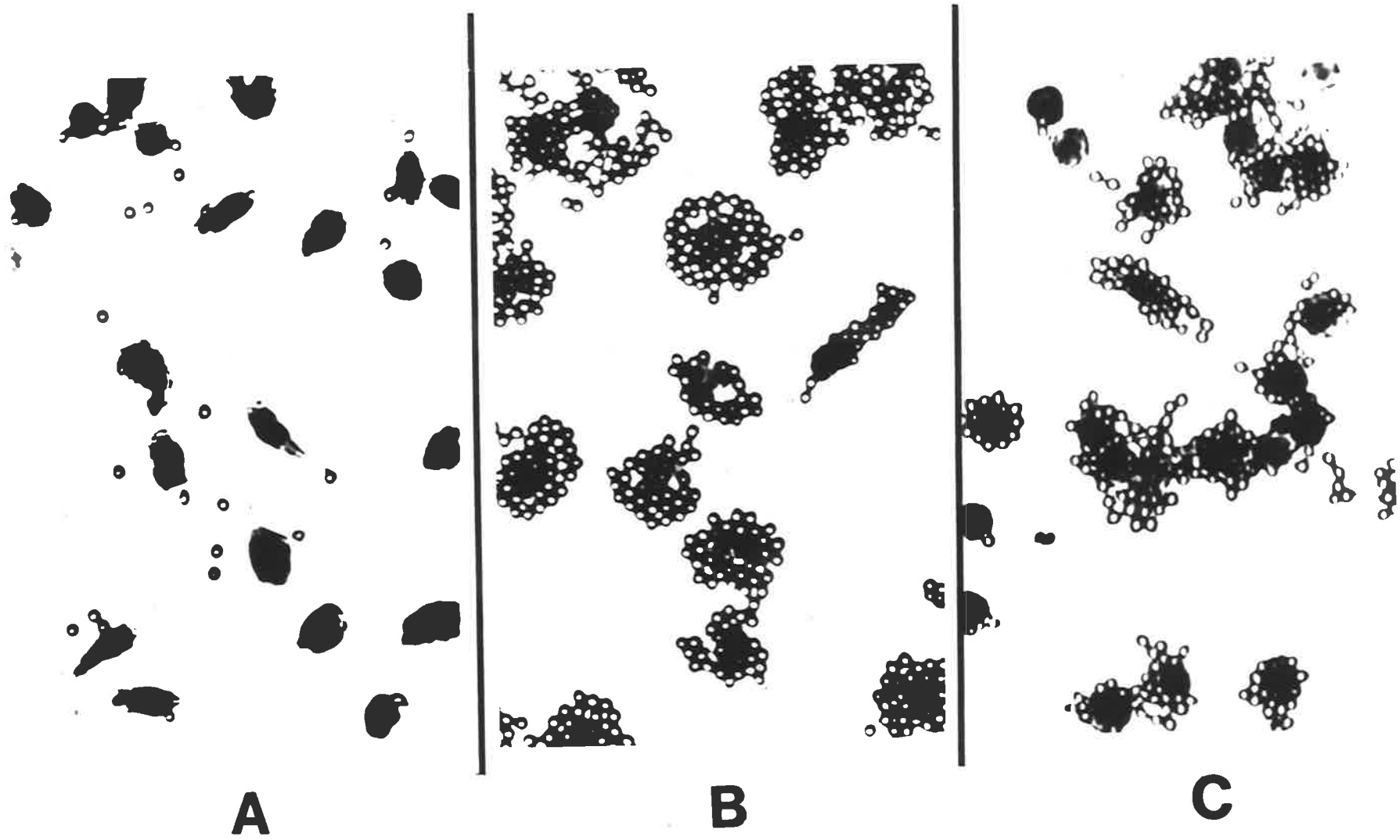
@ Mean percentage (± S.E.) of all macrophages counted.

¶ Overall mean number (± S.E.) of erythrocytes adhering to each macrophage.

¢,* The results were derived from three separate experiments. Statistical analysis (2-way ANOVA on unpooled means) indicated the experiments to be homogeneous ($F_{(2,9)} = 1.719$, $P > 0.1$ (¢), $F_{(2,9)} = 2.917$, $P > 0.1$, (*)), but the differences between sensitised and non-sensitised erythrocytes were significant ($F_{(1,9)} = 703.5$, $P < 0.001$ (¢), $F_{(1,9)} = 69.9$, $P < 0.001$ (*)). Experiments ¢ and * were performed on different days.

§ The results were derived from two separate experiments. Statistical analysis (2-way ANOVA) indicated the experiments to be homogeneous ($F_{(1,8)} = 0.28$, $P > 0.5$) and the differences between the sensitised and the non-sensitised erythrocytes were also not significant ($F_{(1,8)} = 4.99$, $P > 0.05$).

FIGURE 6.1 Adherence of sheep erythrocytes to mouse macrophages. The erythrocytes were pretreated with saline (A), or with 1 HAU of either rabbit anti-sheep erythrocyte serum (B) or *B. leachii* haemolymph (C).


A
B
C

Accordingly, sheep erythrocytes were incubated with haemolymph from another invertebrate and with agglutinins such as plant lectins to determine whether these molecules could also cause erythrocytes to bind to macrophages. Erythrocytes were sensitised with one haemagglutinating unit (1 HAU) of haemolymph from the fresh water crayfish Cherax destructor (diluted 1:8) or with 1 HAU of the lectins concanavalin A (5 - 2.5 µg/ml), phytohaemagglutinin (2.5 - 1.3 µg/ml) or wheat germ lectin (5 µg/ml) and added to mouse macrophage monolayers to test for adhesion. Unsensitised erythrocytes and erythrocytes sensitised with 1 HAU of B. leachii haemolymph served as controls.

None of these treatments resulted in the large numbers of adherent erythrocytes that were observed with 1 HAU of B. leachii haemolymph (Tables 6.2 & 6.3), although 1 HAU of wheat germ agglutinin did produce adhesion significantly greater than that of the unsensitised control (Table 6.3). The inability of haemolymph from C. destructor (formerly Parachaeraps bicarinatus) to sensitise erythrocytes for adhesion to macrophages was consistent with the data obtained by McKay & Jenkin (Jenkin pers. comm.). These results indicated that erythrocytes incubated with various agglutinins did not necessarily become sensitised for adherence to macrophages. The factor(s) in B. leachii haemolymph appeared to be unusual in promoting adhesion between erythrocytes and mouse macrophages, hence further study was justified to determine whether any homology did exist between the ascidian opsonins and immunoglobulin.

TABLE 6.2 The capacity of sheep erythrocytes sensitised with haemolymph from *Cherax destructor* or *Botrylloides leachii* to adhere to mouse peritoneal macrophages.

Treatment of erythrocytes	Number of macrophages counted	% of macrophages[@] with adherent erythrocytes				Mean number[¶] of erythrocytes per macrophage
		0	1-2	3-5	≥6	
None (control)[¢]	2,648	57.5 ± 2.0	35.1 ± 2.3	7.0 ± 0.61	0.36 ± 0.13	0.72 ± 0.04
C. destructor Haemolymph[¢] (1:8 dilution)	2,713	57.0 ± 4.9	34.1 ± 3.2	8.3 ± 1.7	0.61 ± 0.26	0.78 ± 0.11
B. leachii Haemolymph[*] (1:64 dilution)	1,633	7.70 ± 0.94	16.8 ± 1.2	20.6 ± 2.5	54.6 ± 3.1	4.3 ± 0.12

@ Mean percentage (± S.E.) of all macrophages counted.

¶ Overall mean number (± S.E.) of erythrocytes adhering to each macrophage.

¢ Three separate experiments with 3 slides within each. The difference between sensitised (*C. destructor*) and non-sensitised cells (2-way ANOVA on unpooled means) was non-significant ($F_{(1,12)} = 0.678$, $P > 0.25$).

* Two separate experiments with 3 slides within each. The difference between sensitised (*B. leachii*) and non-sensitised (control) cells was highly significant ($F_{(1,8)} = 346.6$, $P < 0.001$).

TABLE 6.3 The capacity of sheep erythrocytes sensitised with various plant lectins to adhere to mouse peritoneal macrophages.

Treatment of erythrocytes	Number of macrophages counted	% of macrophages[@] with 0 adherent erythrocytes	1-2	3-5	≥6	Mean number of erythrocytes per macrophage[¶]
None (control)[ɸ]	1698	66.90 ± 1.2	29.6 ± 1.0	3.40 ± 0.5	0.17 ± 0.1	0.50 ± 0.03
Concanavalin-A[ɸ] (1 HAU)	2596	72.20 ± 1.6	22.8 ± 1.3	4.70 ± 0.6	0.37 ± 0.12	0.47 ± 0.04
None (control)[*]	1379	63.30 ± 1.5	32.2 ± 1.0	4.20 ± 0.54	0.36 ± 0.14	0.58 ± 0.04
Phytohaemagglutinin[*] (1 HAU)	2081	69.08 ± 1.9	25.6 ± 1.7	5.20 ± 0.85	0.19 ± 0.09	0.51 ± 0.03
None (control)[§]	1939	62.80 ± 1.2	32.3 ± 0.8	4.5 ± 0.41	0.36 ± 0.08	0.59 ± 0.03
Wheat Germ Lectin[§] (1 HAU)	3299	27.10 ± 3.4	34.5 ± 1.5	25.2 ± 2.3	13.2 ± 2.5	2.20 ± 0.26

@ Mean percentage (± S.E.) of all macrophages counted.

¶ Overall mean number (± S.E.) of erythrocytes adhering to each macrophage

¢,§ Three separate experiments with 3 slides for each treatment and 2 for the controls. Statistical analysis (2-way ANOVA) indicated the experiments to be homogeneous ($F_{(2,9)} = 1.71$ $P > 0.1$ (¢) and $F_{(2,9)} = 0.77$, $P > 0.25$ (§)).

* Two separate experiments with 3 slides for each treatment and 2 for the controls. Although these experiments were not homogeneous. ($F_{(1,6)} = 8.02$, $P < 0.05$) the interaction term was not significant ($F_{(1,6)} = 0.14$, $P > 0.5$).

¢,*,§ Difference between sensitised and non-sensitised erythrocytes (2-way ANOVA on unpooled means): $F_{(1,9)} = 0.32$, $P > 0.25$ (¢); $F_{(1,6)} = 3.09$, $P > 0.1$ (*); $F_{(1,9)} = 14.1$, $P < 0.005$ (§).

6.2.2 Nature of the factors promoting adhesion of erythrocytes to macrophages.

One can envisage several ways in which cells may become "sticky" for other types of cells. For example, enzymes present in the haemolymph may irreversibly alter the erythrocyte membrane causing the cells to become adhesive for macrophages. Alternatively, adhesion could be mediated through specific opsonic or agglutinating molecules which bind to the erythrocytes. In the latter case, the elution of these factors from sensitised cells should return the cells to their original non-adhesive condition. Furthermore, providing the factors are not inactivated by this procedure, they should be recovered in the eluate.

Because of the possibility that the sheep erythrocyte agglutinin (HA-2) and the opsonic factor binding to vertebrate macrophages were one and the same, and since the agglutinating activity of the HA-2 molecule was lactose specific (Chapter 3, Section 3.1 and Schluter et al, 1981), lactose was tested for its capacity to elute the hypothetical opsonic factor(s) from sensitised cells. Sucrose and maltose acted as controls. In order to measure any reduction in the binding of the sensitised erythrocytes to macrophages following treatment with lactose, it was first necessary to construct a standard curve relating the degree of adhesion to the concentration of haemolymph used to sensitise the erythrocytes.

The experiment involved sensitising batches (30 ml) of 1% erythrocytes with an equal volume of haemolymph diluted to 1, 1/2, 1/4, 1/8 or 1/16 of an HAU at 37° for 30 min. Two and four batches of erythrocytes were sensitised with 1 HAU and 1/2 HAU of haemolymph respectively, the other haemolymph dilutions were each used to sensitise only one batch of red cells. All the cells were sedimented

by centrifugation and one batch of cells from each haemolymph treatment was resuspended in 2 ml of saline containing 1 mg/ml of bovine serum albumin (BSA) as a carrier protein. These erythrocytes were used to construct the standard curve. The additional batches of cells sensitised with 1 HAU and 1/2 HAU were used to test the ability of sugars to elute the agglutinin. The cells sensitised with 1 HAU were resuspended in 2 ml of 0.135 M (iso-osmotic) lactose and those sensitised with 1/2 HAU in 0.135 M lactose, maltose or sucrose, each containing 1 mg/ml BSA to minimize adsorptive loss of any eluted proteins during the subsequent steps. The cell suspensions were incubated for 1 hr at room temperature and then centrifuged at 400 g for 5 min. The supernatants were dialysed overnight at 4° against PBS and tested for their haemagglutinating and sensitising activity (Table 6.4). All cells were resuspended in a further 10 ml of saline or sugar solution and incubated for an additional 15 min at room temperature. They were then washed in saline (10 ml aliquots, 3x), resuspended in M199-FCS to a final concentration of 1% and tested for their ability to adhere to macrophages. The results of this adhesion assay are displayed in Fig 6.2.

It is clear from Fig 6.2 that the degree of adhesion was dependent on the sensitising dose of haemolymph. The results show that the adhesiveness of sensitised erythrocytes was substantially reduced after exposure to lactose but not after exposure to maltose or sucrose. Moreover, as is evident from the data in Table 6.4, the lactose-derived supernatants were unique in possessing haemagglutinating activity and in their capacity to sensitise fresh erythrocytes for adhesion to macrophages. Thus, treatment of sensitised erythrocytes with lactose specifically caused the cells to again become non-adherent and led to the recovery of the sensitising

TABLE 6.4 The activity of supernatants derived by saline or disaccharide treatment of sheep erythrocytes sensitised with *B. leachii* haemolymph.

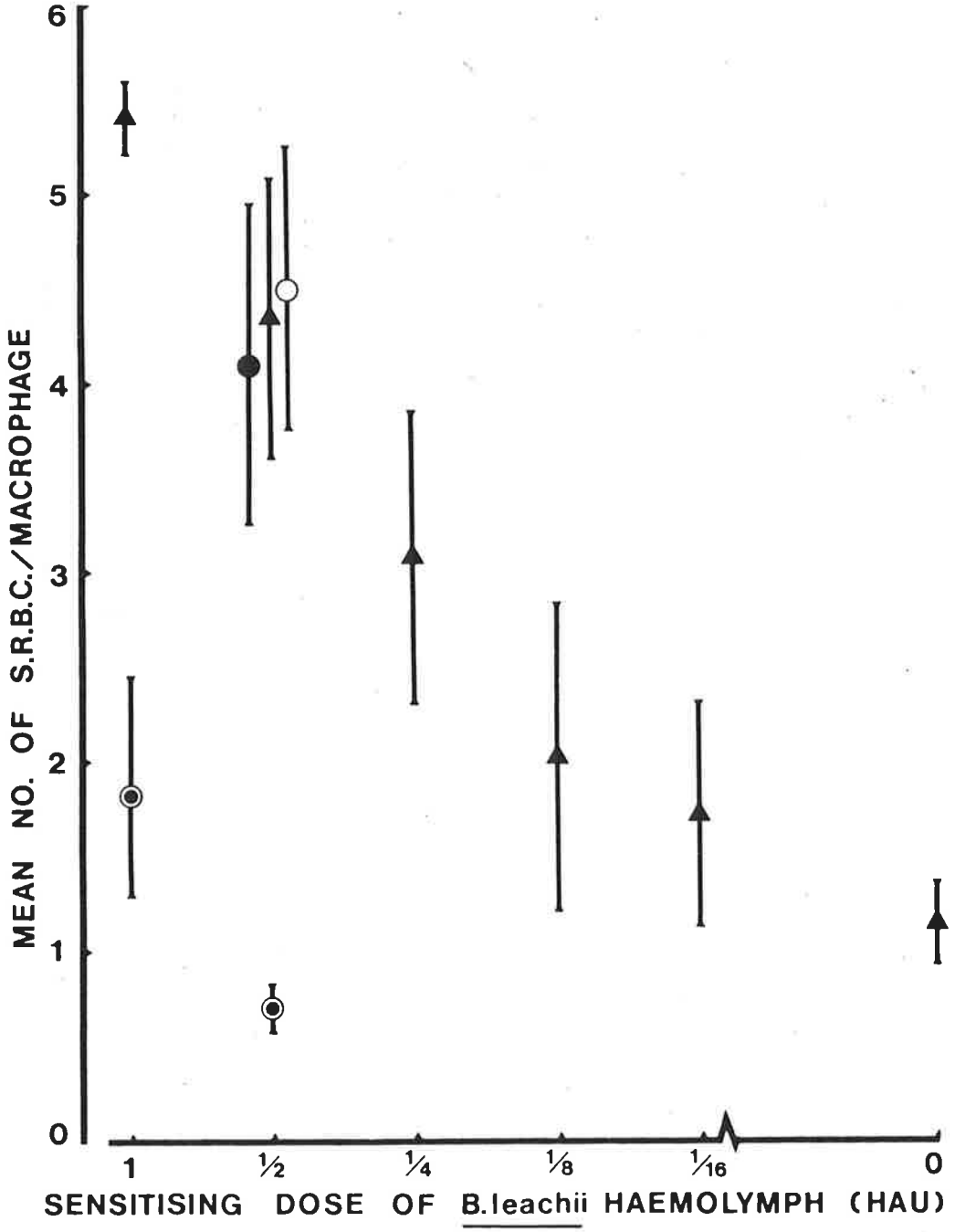
Treatment*	Activity of supernatant collected from erythrocytes sensitised with haemolymph of dose:			
	0.5 HAU		1 HAU	
	Agglutinating activity¶	Macrophage adhesive activity¢	Agglutinating activity¶	Macrophage adhesive activity¢
Saline	0	0.87 ± 0.07	0	1.20 ± 0.08
Lactose	1-2	4.52 ± 0.1	2	4.77 ± 0.07
Maltose	0	0.87 ± 0.09	-	-
Sucrose	0	0.70 ± 0.1	-	-

* The experiment is described in the text. Supernatants were dialysed against PBS and then tested both for agglutinating activity with sheep erythrocytes and for their capacity to sensitise erythrocytes for attachment to macrophages. For the latter, fresh erythrocytes were sensitised with 0.5 HAU of each lactose supernatant, this being equivalent to a dilution of 1:2 and 1:4 of the supernatants from cells originally sensitised with 0.5 and 1 HAU of haemolymph respectively. Erythrocytes incubated with 1:2 dilution of each of the other supernatants (from cells sensitised with 0.5 HAU) were also tested.

¶ Reciprocal titre.

¢ Mean number (± S.E.) of erythrocytes bound per macrophage (5 replicates per group). Controls were unsensitised erythrocytes and erythrocytes sensitised with 0.5 HAU of *B. leachii* haemolymph giving mean numbers of erythrocytes binding per macrophage of 0.74 ± 0.05 and 4.71 ± 0.13 respectively.

FIGURE 6.2 The adhesion of sheep erythrocytes to mouse macrophages: the effect of incubating sensitised erythrocytes in 0.135 M lactose (⊙), maltose (O) or sucrose (●). The adherence of sensitised erythrocytes treated with sugar solutions is compared to that of cells sensitised with various haemagglutinating doses of haemolymph but incubated in saline (▲). The mean ± standard deviation of 5 replicate monolayers is given.


MEAN NO. OF S.R.B.C./MACROPHAGE
6
5
4
3
2
1
0
1
½
¼
⅛
1/16
0
SENSITISING DOSE OF B.leachii HAEMOLYMPH (HAU)

factors in the cell supernatant. As the erythrocyte membranes were not irreversibly altered by the sensitisation process it was clear that the factor(s) inducing adhesion were not enzymes.

6.2.3 The specificity of the binding of sensitised erythrocytes to macrophages.

Compared to C. destructor haemolymph and to the plant lectins mentioned above, B. leachii haemolymph seemed unusual in its ability to promote the adhesion of erythrocytes to mouse macrophages. Several experiments were therefore undertaken to assess whether the factor(s) responsible for this phenomenon would bind directly to macrophages and if so, whether the binding was of a different specificity from that for erythrocytes (i.e. not lactose specific).

Peritoneal exudate cells were collected from several mice, pooled, washed with M199-FCS and resuspended in tris-buffered saline containing EDTA (0.1 mM), sodium azide (0.1%) and 2 mg/ml of BSA (BSA-TESA) to a concentration of 5×10^7 cells/ml. Serial 2-fold dilutions of B. leachii haemolymph in BSA-TESA were added to siliconised glass tubes and each dilution was mixed with an equal volume (0.2 ml) of a suspension of either sheep erythrocytes or mouse peritoneal exudate cells. Microscopic examination revealed that the peritoneal exudate cells immediately became agglutinated into large aggregates, even at low dilutions of haemolymph. The agglutinating titre for both sheep erythrocytes and peritoneal exudate cells is shown in Table 6.5.

The specificity of the peritoneal exudate cell agglutinin was examined by suspending these cells at a concentration of 2.8×10^7 cells/ml in haemolymph diluted 1:3 in BSA-TESA containing no sugar, 30 mM lactose, or 30 mM D-galactose. The tubes were kept at 4° for

TABLE 6.5 The agglutination of mouse peritoneal cells and sheep erythrocytes by *B. leachii* haemolymph.*

Cell type	Final cell concentration (cells/ml)	Agglutination titre^{-1}
Erythrocytes	7.5×10^7	128
Peritoneal exudate cells	9.9×10^6	32

* Serial 2-fold dilutions of haemolymph in siliconised glass tubes were each mixed with an equal volume (0.2 ml) of a suspension of either sheep erythrocytes or mouse peritoneal exudate cells. The diluent was BSA-TESA. Agglutination was assessed from the microscopic appearance of the suspensions.

1 hr, after which time the degree of agglutination was assessed microscopically. Marked agglutination was observed in the tubes containing no sugar or galactose, but not in those containing lactose. These results indicated that the agglutinin for peritoneal exudate cells was specific for a lactose-like determinant and that it was possibly identical to the sheep erythrocyte agglutinin. This possibility was strengthened when it was found that the sheep erythrocyte agglutinin could be adsorbed by both peritoneal exudate cells and erythrocytes (Fig 6.3). On a cell-for-cell basis, peritoneal exudate cells appeared to possess considerably more binding sites than erythrocytes.

6.2.4 Sensitisation of macrophages.

The findings presented above suggested that a single, lactose-specific agglutinin (probably HA-2) was responsible for the agglutination of both peritoneal exudate cells and sheep erythrocytes. The sensitisation of erythrocytes for adherence to macrophages was also inhibited by lactose and it therefore seemed likely that the adherence-sensitising factor was identical to the agglutinin. If so, the adherence of erythrocytes to macrophages was simply a hetero-agglutination reaction. To test this hypothesis, unsensitised erythrocytes were tested for their ability to adhere to macrophages which had been incubated with haemolymph and then washed. The results of this experiment (Table 6.6) showed clearly that macrophages could indeed be sensitised for the adherence of erythrocytes and furthermore that this sensitisation was lactose-specific.

Because the sensitisation of either type of cell could be prevented in the presence of lactose, it was inferred that the erythrocytes were binding to the macrophages not via an immunoglobulin

FIGURE 6.3 Adsorption of B. leachii agglutinins to peritoneal exudate cells and sheep erythrocytes. Seventy-five microlitre aliquots of undiluted haemolymph (which had been dialysed overnight against PBS) were mixed with 25 µl samples containing either washed mouse peritoneal exudate cells (ranging in concentration from 1×10^7 to 1×10^9 cells/ml) or sheep erythrocytes (1.3×10^8 to 2×10^9 cells/ml) in BSA-TESA. The samples were incubated overnight at 4° and then centrifuged at 400g for 5 min. The supernatants were assayed for residual agglutinating activity by titration against sheep erythrocytes. The unadsorbed control (no cells) supernatant had a titre of 1:32. Peritoneal exudate cells (●——●); erythrocytes (▲——▲).

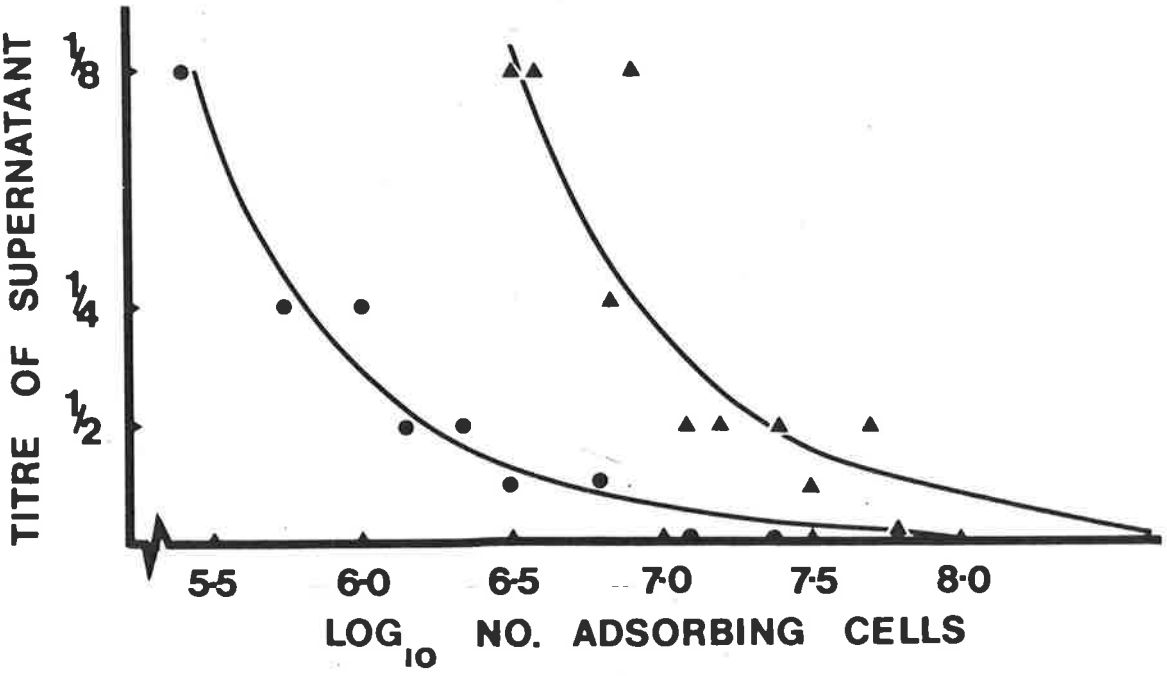

TITRE OF SUPERNATANT
1/8
1/4
1/2
5·5
6·0
6·5
7·0
7·5
8·0
LOG10 NO. ADSORBING CELLS

TABLE 6.6 Sensitisation of macrophages with B. leachii haemolymph for adherence of normal sheep erythrocytes.*

Treatment of macrophages	Number of macrophages counted	% of macrophages¶ with adherent erythrocytes				Mean number of erythrocytes per macrophage¢
		0	1-2	3-5	≥6	
None (control)	1,524	85.0 ± 0.50	14.6 ± 0.52	0.40 ± 0.06	0	0.20 ± 0.005
Haemolymph (1:20)§	1,433	28.6 ± 0.67	42.4 ± 0.53	21.2 ± 0.57	7.80 ± 0.44	1.80 ± 0.04
Haemolymph (1:20) plus lactose@	1,643	79.7 ± 0.92	17.8 ± 0.84	2.31 ± 0.12	0.13 ± 0.04	0.32 ± 0.02
Haemolymph (1:20) plus galactose§	1,325	19.2 ± 0.80	40.8 ± 0.36	22.0 ± 0.42	18.0 ± 0.79	2.50 ± 0.06

* Monolayers of approximately 4.9×10^6 peritoneal exudate cells were incubated at 4° for 2 hr in 1 ml of a 1:20 dilution of haemolymph in M199-FCS containing no sugar, or 0.135 M lactose or galactose. The sugars were made up as iso-osmotic solutions and diluted in M199-FCS. Control monolayers were incubated in M199-FCS. The cells were washed twice with 2.5 ml of M199-FCS before being incubated overnight at 4° with 1 ml of 0.5% unsensitised sheep erythrocytes and treated as described in Section 2.14.3.

¶ Mean percentage (± S.E) of all macrophages counted.

¢ Overall mean number (± S.E) of erythrocytes adhering to each macrophage.

§ $P < 0.01$ compared with unsensitised controls (ANOVA, followed by a Range Simultaneous Test Procedure)

@ $P > 0.05$ compared with unsensitised controls.

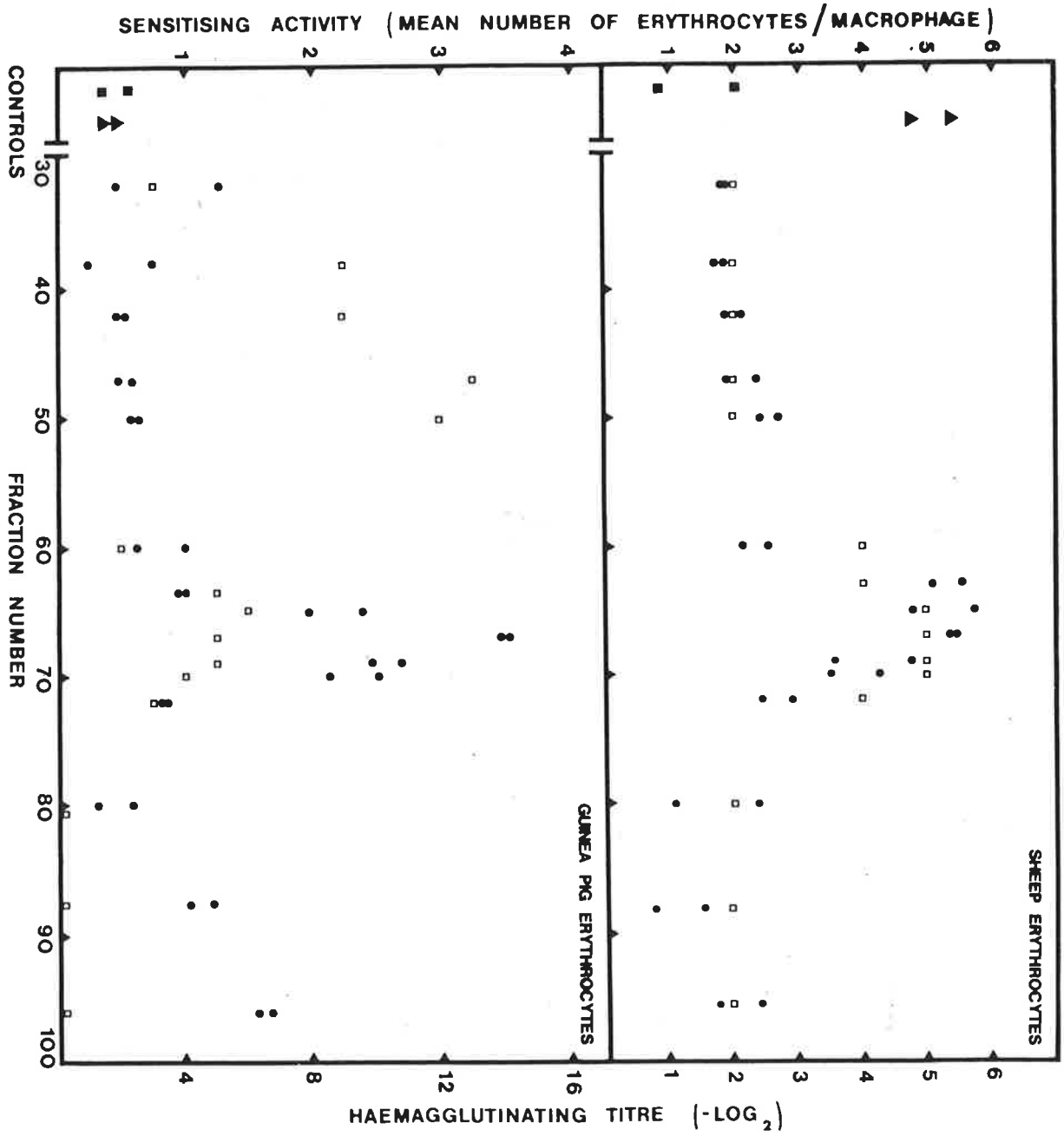
Fc-like site but rather by the cross-linking of similar lactose-like receptors on both cells. The results were consistent with the hypothesis that the erythrocyte-macrophage adhesion was mediated by the HA-2 agglutinin. However the possibility that another as yet uncharacterised lactose-specific factor was responsible could not be excluded and the following experiments were performed to resolve this issue.

6.2.5 Identification of the erythrocyte-macrophage adherence factor.

To investigate the homogeneity and size of the adherence factor, a sample of B. leachii haemolymph was fractionated on Sephadex G-200. In order to prevent sugar-specific retardation effects, both lactose and galactose were included in the eluent. Fractions were dialysed overnight against PBS plus 5 mM $CaCl_2$ before being tested for haemagglutinating activity and for the ability to mediate erythrocyte-macrophage adhesion with both sheep and guinea pig erythrocytes. Erythrocytes to be tested for adhesion were incubated with one HAU of each fraction (or with undiluted samples in the case of fractions having no haemagglutinating activity) prior to addition to macrophage monolayers.

The results showed that both types of erythrocyte could be sensitised for adhesion using those fractions containing HA-2 agglutinating activity (Fig 6.4). It is noteworthy that fractions 40-50, which contained the guinea pig erythrocyte-specific HA-1 agglutinin, were unable at one HAU to sensitise guinea pig erythrocytes for adhesion to macrophages. Unfractionated haemolymph at one HAU was also unable to sensitise guinea pig erythrocytes. The haemagglutinating activity of unfractionated haemolymph for guinea pig erythrocytes would be predominantly due to the HA-1 agglutinin and

FIGURE 6.4 Fractionation of haemolymph on Sephadex G-200. A 5 ml sample of haemolymph containing 0.18 gm of dissolved lactose was applied to a 98 cm x 2.5 cm column of Sephadex G-200 and eluted with PBS containing 50 mM lactose and 100 mM galactose. The column was run at 4° at a flow rate of 16 ml per hr. Fractions of 4.9 ml were collected. Fractions were dialysed overnight against PBS plus 5 mM $CaCl_2$ and then titrated by dilution into tris-buffered saline, pH 7.5 supplemented with 5 mM $CaCl_2$ and 0.01 mg/ml bovine serum albumin to measure agglutinating activity (□) for both sheep and guinea pig erythrocytes and for their capacity to sensitise the erythrocytes for adhesion to macrophages (●). Activity against sheep erythrocytes (TOP) and guinea pig erythrocytes (BOTTOM). The haemagglutinating titre of the unfractionated haemolymph was $1/2^7$ (sheep erythrocytes) and $1/2^{15}$ (guinea pig erythrocytes). The degree of adhesion of unsensitised erythrocytes (■) and of erythrocytes sensitised with 1 HAU of unfractionated haemolymph (▲) is indicated.


SENSITISING ACTIVITY (MEAN NUMBER OF ERYTHROCYTES / MACROPHAGE)
CONTROLS
FRACTION NUMBER
HAEMAGGLUTINATING TITRE (-LOG2)
GUINEA PIG ERYTHROCYTES
SHEEP ERYTHROCYTES

since this agglutinin lacked the ability to mediate guinea pig erythrocyte adhesion, it was not surprising that unfractionated haemolymph shared this inability.

To investigate whether a single factor mediated the adhesion of sheep and guinea pig erythrocytes to macrophages, fractions 66-68 (containing the HA-2 agglutinin) were pooled and 2 ml aliquots were adsorbed twice with approximately 1×10^{10} sheep or guinea pig erythrocytes. Each adsorption was for 30 min at room temperature. The adsorbed supernatants were then compared with the original unadsorbed pool for their capacity to sensitise both kinds of erythrocyte for adherence to macrophages. The results presented in Table 6.7 indicated that the adhesive and haemagglutinating activity for both types of erythrocyte was removed by adsorption to either type of erythrocyte.

These data were consistent with the hypothesis that it is the HA-2 agglutinin which is solely responsible for mediating the adhesion of both kinds of erythrocyte to macrophages. This conclusion was subsequently confirmed when purified HA-2 agglutinin became available (see Chapter 2, Section 2.11). Sheep erythrocytes were sensitised with one HAU of purified HA-2 protein, washed and added to macrophage monolayers. The degree of adhesion of these sensitised erythrocytes was comparable to that of cells sensitised with one HAU of unfractionated haemolymph (Table 6.8).

6.2.6 Macrophages and the HA-1 Receptor.

As reported in Section 6.1.7, the HA-1 containing fractions from the Sephadex G-200 column were unable to sensitise guinea pig erythrocytes for adhesion to macrophages. This could have been due to the absence of HA-1 receptor (ligand) sites on the macrophage surface.

TABLE 6.7 The capacity of partially purified HA-2 agglutinin to sensitise sheep and guinea pig erythrocytes for adhesion to macrophages and the ability of each type of erythrocyte to adsorb this activity.

Pre-treatment of erythrocytes	Type of erythrocytes	Number of macrophages counted	% of macrophages* with adherent erythrocytes				Mean number of erythrocytes per macrophage§
			0	1-2	3-5	≥6	
None (control)	Sheep	952	64.4 ± 0.74	30.0 ± 0.63	4.80 ± 0.70	0.81 ± 0.66	0.59 ± 0.06
	Guinea pig	1054	44.7 ± 8.38	35.0 ± 2.93	17.4 ± 4.14	2.80 ± 1.36	1.27 ± 0.28
HA-2 pool@ (1:32 dilution)¶	Sheep	1006	0.69 ± 0.07	5.10 ± 0.98	11.8 ± 1.46	82.4 ± 1.63	5.49 ± 0.05
	Guinea pig	894	2.40 ± 1.69	11.6 ± 6.22	23.3 ± 3.61	62.7 ± 11.3	4.79 ± 0.48
SRBC-adsorbed HA-2 pool (1:2 dilution)¢	Sheep	999	65.0 ± 4.36	31.1 ± 3.72	3.50 ± 1.50	0.46 ± 0.24	0.53 ± 0.08
	Guinea pig	869	76.3 ± 6.40	20.7 ± 4.71	2.20 ± 1.44	0.75 ± 0.48	0.40 ± 0.15
GpRBC-adsorbed HA-2 pool (1:2 dilution)¢	Sheep	907	75.0 ± 8.86	22.5 ± 7.04	2.46 ± 1.91	0	0.37 ± 0.16
	Guinea pig	930	82.0 ± 2.93	16.1 ± 2.63	1.72 ± 0.42	0.12 ± 0.12	0.27 ± 0.05

@ Fractions 66-68 inclusive from the Sephadex G-200 fractionation of haemolymph (Fig 4.4).

¶ One haemagglutinating dose.

¢ No detectable haemagglutinating activity. SRBC = Sheep erythrocytes; GpRBC = Guinea pig erythrocytes.

* Mean percentage (± S.E.) of all macrophages counted.

§ Overall mean number (± S.E.) of erythrocytes adhering to each macrophage.

TABLE 6.8 Adherence to mouse peritoneal macrophages of sheep erythrocytes sensitised with dialysed haemolymph or with purified HA-2 agglutinin[#].

Treatment of erythrocytes	Number of macrophages counted	% of macrophages* with adherent erythrocytes				Mean no. of erythrocytes per macrophage[¶]
		0	1-2	3-5	≥6	
None (control)	2452	67.3 ± 2.84	26.5 ± 1.83	5.0 ± 0.88	1.10 ± 0.33	0.57 ± 0.07
Haemolymph (1:64 dilution)[¢]	2055	10.7 ± 1.99	17.8 ± 0.22	14.3 ± 1.00	57.1 ± 2.32	4.20 ± 0.16
HA-2 agglutinin (0.23 g/ml)[§]	2000	9.60 ± 1.29	23.9 ± 1.72	17.7 ± 2.04	48.8 ± 0.83	3.90 ± 0.07

This experiment was performed using 5 monolayers for each treatment. Statistical analysis (ANOVA followed by a Student-Newmans-Keuls test) indicated that both the HA-2 sensitised and haemolymph sensitised cells were significantly different from the control $P < 0.01$ and furthermore the HA-2 sensitised and haemolymph sensitised cells adhered to macrophages to the same extent $P > 0.05$.

* Mean percentage (± S.E.) of all macrophages counted.

¶ Overall mean number (± S.E.) of erythrocytes adhering to each macrophage

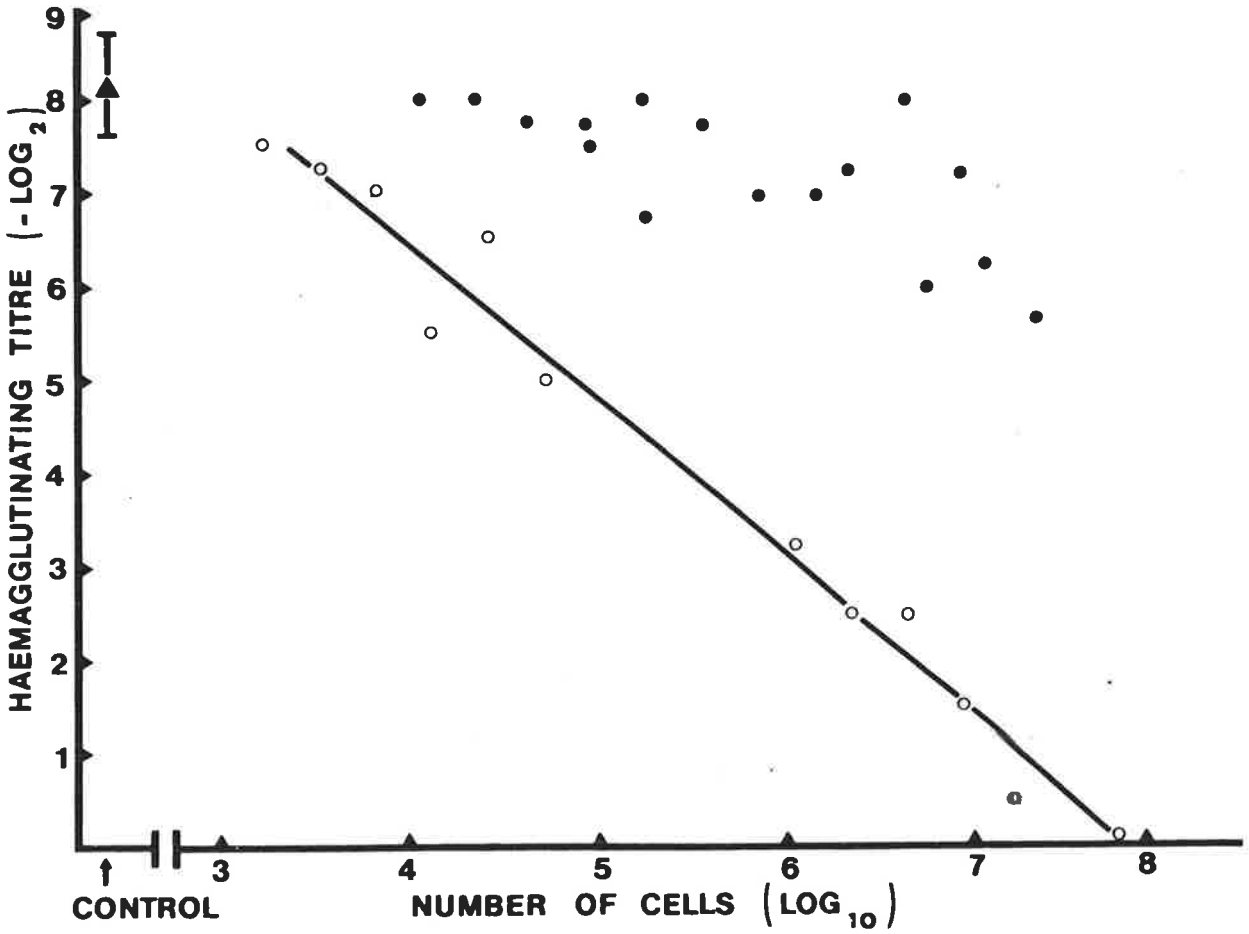
¢ One haemagglutinating dose of haemolymph previously dialysed overnight against PBS.

§ One haemagglutinating dose.

To test this possibility an agglutination experiment was performed with mouse peritoneal exudate cells. A suspension of mouse peritoneal cells (6.5×10^5 cells per 50 µl) was serially diluted in tris-buffered saline (pH 7.5) supplemented with 5 mM $CaCl_2$ and 0.01 mg/ml bovine serum albumin (HA-1 diluent) and to each aliquot was added 50 µl of purified HA-1 (2.5 µg protein/ml) having a titre of 1/512 with 0.5% guinea pig erythrocytes. All tubes were incubated at 4^o for 4 hr. The cells were then examined microscopically for agglutination. No agglutination was found even when using as few as 8×10^4 peritoneal cells.

To exclude the possibility that HA-1 molecules bound to the macrophages without agglutinating them, an adsorption test was carried out. Samples of the HA-1 agglutinin were incubated with mouse peritoneal exudate cells or with guinea pig erythrocytes, the cells being suspended in HA-1 diluent at a final concentration of approximately 2×10^8 and 2.8×10^9 cells/ml, respectively. The assay was performed by making 2-fold serial dilutions of each cell suspension (50 µl) in HA-1 diluent in siliconised tubes. Each aliquot was mixed with 50 µl of HA-1 agglutinin (2.5 µg protein/ml) and incubated overnight at 4°. The samples were then centrifuged (400g, 5 min). The supernatants were removed and titrated for residual haemagglutinating activity, adding 40 µl of 0.5% guinea pig erythrocytes to 40 µl of each dilution of adsorbed haemolymph in microtitre trays. The results (Fig 6.5) showed that the agglutinin was adsorbed completely by the erythrocytes but not at all by the peritoneal cells. It seems therefore that few if any receptor sites for the HA-1 agglutinin occur on the surface of mouse macrophages.

FIGURE 6.5 The capacity of macrophages (●) and guinea pig erythrocytes (O) to adsorb the HA-1 agglutinin. Experimental details are given in the text. The unadsorbed (control) titre (± 95% confidence limits) is indicated. The line drawn for the erythrocytes ($y = -1.66x + 13.08$; $r^2 = 0.97$) was calculated by linear regression analysis. The macrophage data yielded a line ($y = -0.42x + 9.73$; $r^2 = 0.41$) with a slope not significantly different from zero, as determined using the t-test (Sokal & Rohlf, 1969).


HAEMAGGLUTINATING TITRE (-LOG2)
9
8
7
6
5
4
3
2
1
CONTROL
3
4
5
6
7
8
NUMBER OF CELLS (LOG10)

6.2.7 Discussion.

These results showed that sheep erythrocytes sensitised with B. leachii haemolymph could adhere to mouse macrophages. Previous efforts to sensitise erythrocytes with haemolymph from another invertebrate, the fresh water crayfish Cherax destructor, had proved ineffective (Jenkin, pers. comm.) and in view of the affinity of the ascidians to vertebrates, it seemed possible that the sensitising capacity of B. leachii haemolymph was due to a factor which had some functional and structural similarity to immunoglobulin and bound to Fc receptors on macrophages. However, since the haemolymph from both B. leachii and C. destructor was known to contain sheep erythrocyte agglutinins which, depending on their structure and specificity, might have been responsible for promoting adhesion between erythrocytes and macrophages, it was considered important firstly to confirm the inability of C. destructor haemolymph to promote adhesion and secondly to determine whether or not haemagglutinins such as plant lectins could mediate adherence under the same conditions as used with B. leachii haemolymph.

Although Cherax destructor haemolymph, phytohaemagglutinin and concanavalin A were inactive in these experiments, wheat germ lectin produced significantly more adherent erythrocytes than the unsensitised control. The positive result with wheat germ lectin was not unexpected since other workers had shown that mouse macrophages sensitised with this lectin bound and phagocytosed mouse erythrocytes (Goldman & Bursker, 1976). Nevertheless, in the above experiments, the number of adherent wheat germ lectin sensitised cells was lower than that detected when B. leachii haemolymph, with an equivalent haemagglutinating activity, was used to sensitise the erythrocytes.

The ability of haemagglutinins and lectins, to promote adherence of erythrocytes to macrophages clearly depends on the availability of receptors for the haemagglutinins or lectins on the macrophage surface. Although this was not investigated in the case of the plant lectins used here, it has been reported elsewhere that concanavalin A binds to macrophages (Lutton, 1975) and that it can mediate the attachment of bacteria (Allen et al., 1971), yeast cells (Bar-Shavit & Goldman, 1976), mouse erythrocytes (Goldman & Cooper, 1975; Goldman & Bursuker, 1976) and sheep erythrocytes (Capo et al., 1978). However, concanavalin A-sensitised mouse erythrocytes were found to bind strongly to mouse macrophages under experimental conditions different from those used in this study, viz. incubation of erythrocytes with macrophages at 22° and no serum supplement in the culture medium (Goldman & Cooper, 1975), compared with incubation at 4° in 199 medium supplemented with 10% foetal calf serum (this study). Capo et al. (1978) reported low levels of adhesion (1.8 sheep erythrocytes per macrophage) after a 10 min incubation at 4° although a seemingly high concentration of concanavalin A (0.32 mg/ml) was used to sensitise the erythrocytes (cf. Section 6.2.1). Unfortunately, neither the concentration of erythrocytes used during sensitisation nor the amount of lectin required to agglutinate the cells was mentioned. In the present study, erythrocytes were sensitised with one agglutinating dose of lectin or haemolymph and the sensitised cells were incubated with macrophages at 4°. Under these conditions, the B. leachii factor appeared unusual in its capacity to mediate the adhesion of large numbers of erythrocytes.

Since the data showed conclusively that the adhesiveness of sensitised erythrocytes was not due to enzymic alteration of the erythrocyte surface (Fig 6.2 & Table 6.4), it remained to be shown

whether one of the previously recognised agglutinins (HA-1 or HA-2) or some other factor(s) in B. leachii haemolymph were responsible for the adherence phenomenon. The HA-1 agglutinin (the guinea pig erythrocyte agglutinin) is inhibited equally well by lactose and galactose while the HA-2 agglutinin, which binds to a variety of erythrocyte types including those from the sheep and guinea pig, is inhibited only by lactose. Thus, when the agglutination of mouse peritoneal exudate cells by haemolymph was found to be inhibited by lactose but not by galactose, it seemed likely that the HA-2 molecule was agglutinating these cells. This conclusion was supported by the finding that the sheep erythrocyte agglutinin (HA-2) could be adsorbed by both mouse peritoneal cells and sheep erythrocytes (Fig 6.3). Moreover, since either erythrocytes or macrophages could be sensitised by haemolymph for adhesion to the other and because the sensitisation of both types of cell could be reversed by lactose but not by galactose or other sugars, it appeared likely that the HA-2 agglutinin was also responsible for the adhesion between the two types of cell. A purified homogeneous preparation of the HA-2 agglutinin was later shown to sensitise sheep erythrocytes for adhesion to macrophages. Furthermore, comparisons with unfractionated haemolymph indicated that the adhesive activity of the haemolymph could be accounted for entirely by its content of the HA-2 agglutinin. This conclusion was supported by experiments with fractions obtained from chromatography of haemolymph on Sephadex G-200. Fractions which contained HA-2 activity were able to sensitise both sheep and guinea pig erythrocytes for adhesion to macrophages and the adsorption of these fractions with either sheep or guinea pig erythrocytes removed both the haemagglutinating and adhesive activity for each type of red cell.

Although the HA-1 and HA-2 agglutinins were both specific for lactose they were shown to bind to different sites on vertebrate cells. The HA-1 molecule caused agglutination of guinea pig erythrocytes but, unlike the HA-2 agglutinin, it was unable to mediate the binding of these cells to mouse macrophages. Peritoneal exudate cells were not agglutinated by purified HA-1 protein nor were they able to adsorb it. It therefore seems that receptors for the HA-1 agglutinin are not present on the macrophage surface in detectable amounts.

It is important to note that the fractionation and purification procedures employed in this chapter did not separate the HA-1 agglutinin from LBP-3. Nevertheless, it is unlikely that the presence of LBP-3 would have altered any of the conclusions regarding the HA-1 molecule. Firstly, the agglutinating activity of the LBP-3 per $O.D._{280}$ unit is several orders of magnitude lower than that of the HA-1, hence the agglutination titres reflect the HA-1 concentration and not that of the LBP-3. Moreover, the characteristics of the LBP-3 and HA-1 binding sites appear to be identical (Schluter, 1982) and the LBP-3 would not be expected to bind to macrophages.

In conclusion, it appears that the adhesion between erythrocytes and macrophages promoted by B. leachii haemolymph is due to the crosslinking by the HA-2 agglutinin of similar lactose-like determinants on both cell types. It is therefore distinct from the binding of antibody-coated particles to the Fc receptors of macrophages and there is no reason to believe that the HA-2 molecule has any structural similarity with vertebrate immunoglobulin.

6.3 The phagocytosis of HA-2 sensitised erythrocytes by mouse macrophages.

It was established in the previous section that the HA-2 agglutinin, the factor in B. leachii haemolymph responsible for mediating the adhesion of erythrocytes to macrophages,binds to oligosaccharide moieties on the macrophage surface. Although this binding is probably distinct from the binding of immunoglobulins to Fc receptors, it was nevertheless possible that the HA-2 molecule was functionally analogous to immunoglobulin and capable of initiating the ingestion of particles not only by ascidian cells but also by vertebrate macrophages.

The binding of antibody-coated particles to immunoglobulin Fc receptors on the macrophage surface has been recognised for some time to be an effective trigger for ingestion (Berken & Benacerraf, 1966). However a number of recent studies have suggested that particles devoid of immuoglobulin may adhere strongly to macrophages and be efficiently phagocytosed. For example, protein-carbohydrate interactions at the macrophage surface can, under some circumstances, induce ingestion. The adhesion of erythrocytes sensitised with the plant lectins concanavalin A (Bar-Shavit & Goldman, 1976) or wheat germ agglutinin (Goldman & Bursker, 1976) to carbohydrate moieties on the macrophage surface is apparently sufficient to initiate phagocytosis. Adhesion through hydrophobic attractions may also result in the ingestion of particles, e.g. the phagocytosis of aldehyde-treated erythrocytes (Rabinovitch, 1968 & 1970; Capo et al., 1979) and hydrophic bacteria (van Oss & Gillman, 1972). In addition, complement (C3) receptors on the macrophage surface have been shown to bind C3-coated particles and to mediate their phagocytosis in the absence of antibody (Shurin &

Stossel, 1978; Bar-Shavit et al., 1979; Newman et al., 1980). There is however some controversy over the interpretation of the data, since the presence of C3 on antibody-coated particles appears to have strong synergistic effects on Fc-receptor mediated phagocytosis (Ehlenberger & Nussenzweig, 1977). It was suggested by Bar-Shavit et al., 1979 that it was possible that a non-specific serum IgG may have bound to the particles and in the presence of $C3_b$ initiated significant phagocytosis. The possibility that cytophylic antibody may also have been involved in "C3 mediated" phagocytosis should not be overlooked. Nevertheless, from the other studies cited it appears that phagocytosis does not always require the presence of immunoglobulin. Hence it was feasible that the adhesion of particles to the macrophage membrane via the HA-2 agglutinin may also induce phagocytosis.

Very little is understood about the initiation of phagocytosis. Although adherence seems to be a prerequisite for the phagocytic ingestion of particles, adherent particles are not always phagocytosed (e.g. Griffin & Silverstein, 1974). This suggests that a "trigger" is necessary to initiate phagocytosis, but the nature of the "trigger" is not known. Thus, a study that has the potential to provide information that would lead to a better understanding of the "triggering" mechanism(s) is justified. The ability of the HA-2 agglutinin to mediate phagocytosis was addressed both in vivo by measuring the clearance of erythrocytes from the circulation of mice and in vitro using mouse macrophage monolayers.

6.3.1 The clearance of sensitised erythrocytes from the circulation of mice.

If mice are injected intravenously with foreign particles, the particles are cleared from the circulation at an exponential rate by

the phagocytic cells of the liver and spleen. The clearance rate, denoted by the phagocytic index K, varies with the injected particle and is related to the titre of the circulating antibody (Benacerraf et al., 1959; Jenkin, 1964). If a large primary dose of particles has just been eliminated from an animal's circulation then a second dose, given soon after, is removed more slowly and the animal is said to have been blockaded by the initial dose. However, if the particles in the second dose are first opsonised, then their rate of clearance may be returned to that of unblockaded animals. This type of experiment was used to assay for the effectiveness of opsonisation.

The assay system involved injecting mice intravenously with 0.2 ml of a 2% (v/v) suspension, in saline, of Cr^{51}-labelled erythrocytes; at 1, 2, 5, 10 and 15 min after injection, 0.1 ml samples of blood were collected from each mouse. The blood samples were assayed immediately for radioactivity. The elimination from the circulation of Cr^{51}-labelled erythrocytes was indicated by a decline, with time, in the amount of radioactivity detected. In order to obtain an accurate estimate of the rate of clearance, at least 9 mice were injected with either opsonised or normal erythrocytes. Statistical analyses, analysis of variance and t-test, were performed on log (K) values calculated for each mouse. The log transformation was necessary in order to satisfy the assumption of homogeneity of variances.

As unsensitised Cr^{51}-labelled sheep erythrocytes injected into mice intravenously were cleared at a rapid rate (Table 6.9), it was necessary to inject a blockading dose prior to the injection of Cr^{51}-labelled erythrocytes in order to reduce the rate of clearance of the labelled cells. A blockading dose of 0.2 ml of a 10% erythrocyte suspension administered 20 min before the labelled erythrocytes was found to reduce the rate of clearance to a level where

TABLE 6.9 Clearance of Cr^{51}-labelled sheep erythrocytes from the circulation of mice.

Blockaded¶		Unblockaded (control)
Phagocytic index (K)@ after injection with:		
Unsensitised Cr^{51}-erythrocytes (control)¢	Cr^{51}-erythrocytes sensitised with *B. leachii* haemolymph (1HAU)¢	Unsensitised Cr^{51}-erythrocytes¢
0.023*	0.099	0.095
0.018	0.041	0.066
0.033	0.055	0.097
0.03	0.043	0.078
0.02	0.094	0.059
0.027	0.083	0.099
0.03	0.035	0.063
0.021	0.066	0.051
0.037	0.072	0.063
0.038	0.052	0.079
		0.083
0.028 ± 0.0022§	0.064 ± 0.0071#	0.076 ± 0.005

@ K = slope of the clearance curve, Log_{10}(no. counts) = K(Time) + a, where time is 1, 2, 5, 10, or 15 min after the injection of labelled erythrocytes.

¶ 0.2 ml 10% (v/v) unlabelled sheep erythrocytes injected 20 min prior to the injection of Cr^{51}-erythrocytes.

¢ 0.2 ml 2% (v/v) Cr^{51}-erythrocytes

* The K value calculated for each mouse using regression analysis.

§ Mean ± S.E.

# $P < 0.001$ ($F_{(1,28)} = 40.65$) compared with unsensitised controls and $P > 0.1$ ($F_{(1,28)} = 2.71$) compared with the unblockaded rate of clearance (analysis of variance followed by *a priori* comparisons among means, on log transformed data. The log transformation was necessary to satisfy the assumption of homogeneity of variances).

any effect of opsonisation should have been detected. Accordingly, the effect of sensitising the sheep erythrocytes with haemolymph was examined using two groups of 10 blockaded mice. The mice in one group received 0.2 ml of a 2% (v/v) suspension of Cr^{51}-labelled erythrocytes which had previously been sensitised with one HAU of haemolymph (1:64 dilution), while mice in the other group received 0.2 ml of a 2% (v/v) suspension of untreated Cr^{51}-labelled cells. To ensure that the sensitised erythrocytes were injected as a suspension of single cells, the suspension was vigorously pipetted and examined microscopically prior to injection. It can be seen from the data presented in Table 6.9 that the sensitised erythrocytes were cleared at a rate significantly faster than that of the untreated cells. Furthermore, a sensitising dose of one HAU was sufficient to restore clearance to a rate not significantly different from that obtained with unblockaded animals.

Mouse erythrocytes also are agglutinated by the HA-2 molecule (Table 3.1 & 3.2; Schluter et al., 1981) and so it was possible to check the above result with clearance experiments that did not require a blockade. The rate of clearance of Cr^{51}-labelled mouse erythrocytes (0.2 ml of a 2% (v/v) suspension) sensitised with one HAU of haemolymph (a 1:32 dilution) was compared to that of unsensitised erythrocytes. As was expected from the previous experiment, the sensitised cells were cleared at significantly faster rates than were the unsensitised control erythrocytes ($P < 0.001$, $t_{18} = 10.28$; Table 6.10). Hence the HA-2 agglutinin was able to facilitate the removal of erythrocytes from the peripheral circulation of mice. There are two possible explanations for these results. The removal of sensitised erythrocytes could have been due to their adherence and/or phagocytosis by cells in the liver and spleen, or alternatively they

TABLE 6.10 Clearance of Cr^{51}-labelled mouse erythrocytes from the circulation of mice.

Phagocytic index (K)@ after injection with:	
Cr^{51}-erythrocytes¶ sensitised with *B. leachii* haemolymph (1 HAU)	Unsensitised Cr^{51}-erythrocytes¶
0.013*	0.0065
0.013	0.0069
0.017	0.0089
0.019	0.0073
0.015	0.0078
0.017	0.0062
0.019	0.0039
0.02	0.0059
0.013	0.0062
0.02	
0.017 ± 0.0009§	0.0066 ± 0.0005

@ K = slope of the clearance curve, Log_{10}(no. counts) = K(Time) + a, where time is 1, 2, 5, 10, or 15 min after the injection of labelled erythrocytes.

¶ 0.2 ml 2% (v/v) Cr^{51}-labelled erythrocytes.

* The K value calculated for each mouse using regression analysis.

§ Mean ± S.E. $P < 0.0005$ as determined by a t-test performed on log transformed data ($t_{(15)} = 8.802$). The log transformation was necessary to satisify the assumption of homogeneity of variance.

could have become attached via the HA-2 molecule to fixed, non-phagocytic (e.g. epithelial) cells. To determine unambiguously whether macrophages can indeed phagocytose erythrocytes sensitised with B. leachii haemolymph more efficiently than unsensitised cells, an in vitro system using peritoneal exudate cells was utilised.

6.3.2 The in vitro phagocytosis of sheep erythrocytes sensitised with haemolymph or antibody.

Phagocytosis was measured in vitro by adding Cr^{51}-labelled sheep erythrocytes to monolayers of mouse macrophages. The erythrocytes were sensitised with one HAU of either B. leachii haemolymph (1:64 dilution) or rabbit antibody specific for sheep erythrocytes (1:640 dilution). After the cells had been washed, a 1 ml suspension (1.5×10^8 cells) was added to Leighton tubes containing flying coverslips with adherent monolayers of spread macrophages. An equal number of unsensitised erythrocytes added to other monolayers served as the controls. All monolayers were incubated for 1 hr at 4° to allow erythrocyte-macrophage adhesion. They were then transferred to 37° and the ingestion of erythrocytes was recorded at specific time points over a two hour period, 2-3 coverslips with their adherent monolayers being sampled at each time point. Ingestion was measured as the radioactivity associated with the coverslips after they had been incubated in Tris-buffered ammonium chloride solution (see Chapter 2, Section 2.14.4) to lyse non-phagocytosed cells and extensively washed.

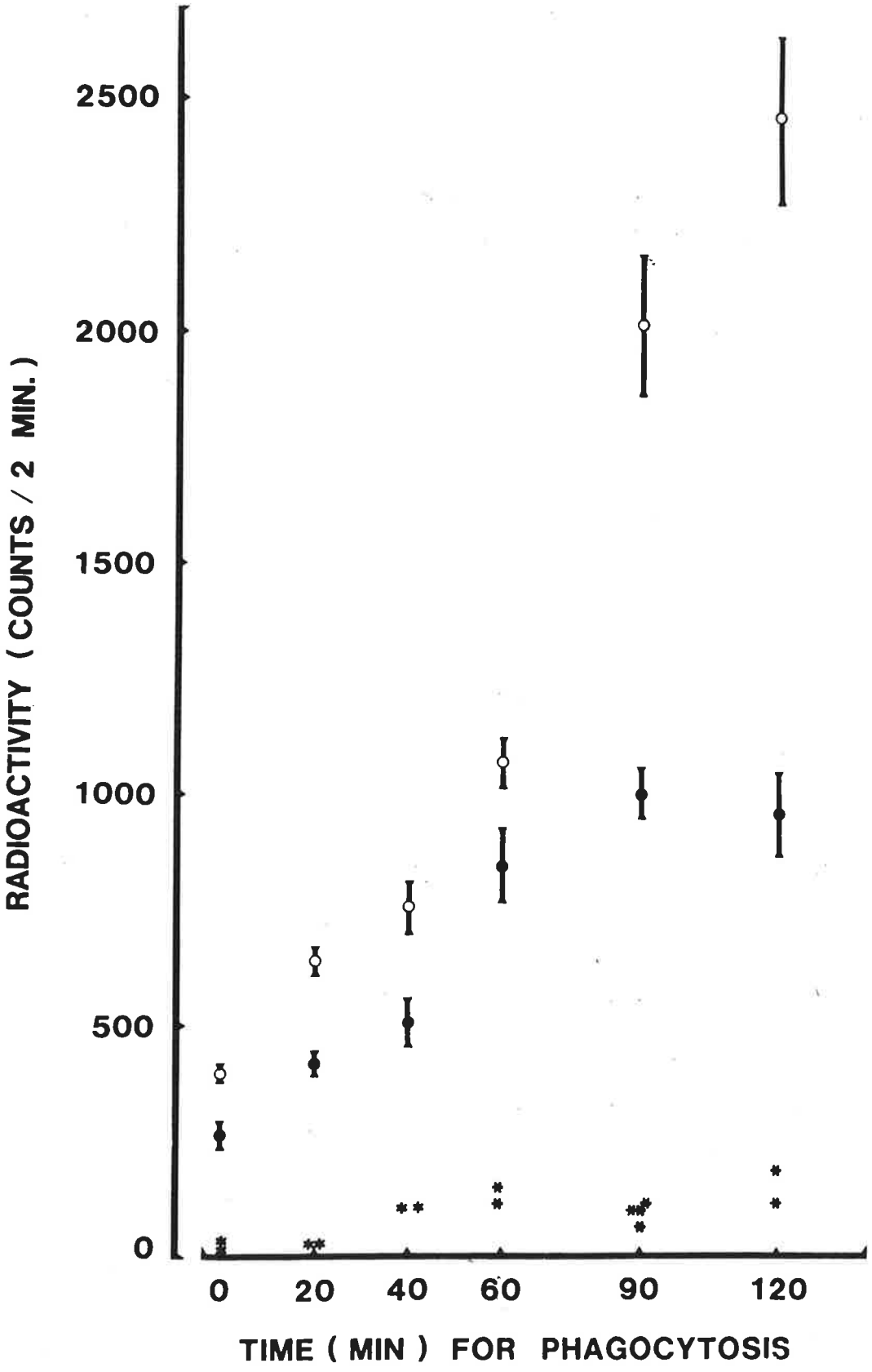
It is evident from Fig 6.6 that erythrocytes sensitised with haemolymph were phagocytosed at a substantially greater rate than were unsensitised cells, although fewer B. leachii sensitised cells were phagocytosed during a two hour period than was found with antibody sensitised cells. It is interesting to note that a comparable rate of

phagocytosis was observed for both types of sensitised cells during the first 60 min of the experiment, after which time the ingestion rate of B. leachii sensitised cells decreased.

In order to check whether the radioactivity associated with the coverslips was a true representation of the cells phagocytosed, an additional experiment was performed. It was considered possible that the macrophages might have digested some of the phagocytosed erythrocytes and released a fraction of the Cr^{51} label into the culture medium. If so, then the culture medium from those tubes in which phagocytosis occurred should contain more radioactivity than medium from comparable tubes where phagocytosis was not possible.

Two sets of 24 tubes were set up. One set consisted of 12 monolayers incubated as before with 1 ml of 0.5% Cr^{51}-erythrocytes sensitised with haemolymph, while the other 12 monolayers were incubated with antibody sensitised erythrocytes. The second set of 24 tubes contained a flying coverslip with no adherent macrophages and 1 ml of 0.5% erythrocytes, 12 tubes having antibody-sensitised cells and 12 tubes having haemolymph-sensitised cells. The tubes were incubated at 37° for 0, 20, 40, 60, 90 or 120 min. A 0.4 ml aliquot of culture medium was removed at the above times from each tube, centrifuged immediately at 400 g for 5 min and the supernatant was assayed for radioactivity. In the tubes with macrophages, the monolayers were also removed at the times specified and assayed for phagocytosis. All treatments had duplicate tubes for each time point. This experiment was repeated using haemolymph-sensitised erythrocytes and unsensitised erythrocytes incubated with and without macrophages as described. The rate of phagocytosis obtained in each group was similar to that measured previously, these and the previous data were therefore pooled and the combined results are displayed in Fig 6.6. The

FIGURE 6.6 The phagocytosis of sensitised and unsensitised sheep erythrocytes by mouse macrophages. Cr^{51}-labelled erythrocytes were added to mnonolayers of mouse macrophages and incubated at 37° for the specified times. Phagocytosis, measured as the radioactivity remaining after lysis of adherent erythrocytes, is shown for erythrocytes sensitised with one HAU of either B. leachii haemolymph (1:32 dilution, ●) or rabbit antibody (1:640 dilution, O) and unsensitised erythrocytes (✱). Mean values ± one standard error are shown for samples for which 3-7 monolayers were counted.


2500
2000
1500
1000
500
0
RADIOACTIVITY (COUNTS / 2 MIN.)
0
20
40
60
90
120
TIME (MIN) FOR PHAGOCYTOSIS

radioactivity detected in the supernatant, expressed as a percent of the total added, ranged from an average of 1.8% for the unsensitised erythrocytes to 2.7% for the haemolymph-sensitised cells. Macrophages had no effect on the quantity of radioactivity in the supernatant in either group, even in tubes where substantial phagocytosis had occurred. It appeared, therefore, that the label associated with the ingested erythrocytes remained within the macrophages for the duration of the experiment and since no radioactivity was found associated with coverslips that did not have adherent macrophages, the radioactivity associated with coverslips following NH_4Cl lysis was taken as a valid indicator of phagocytosis.

6.3.3 The relationship between adhesion and phagocytosis.

Macrophages clearly phagocytosed erythrocytes which had been sensitised with B. leachii haemolymph and the rate of phagocytosis of the cells during the first hour of incubation was comparable to that of antibody-sensitised cells (Fig 6.6). Upon further incubation, the antibody-sensitised cells continued to be ingested at an unchanged rate, whereas the ingestion of haemolymph-sensitised cells ceased, i.e. the number of haemolymph-sensitised cells which had been ingested after two hours was the same as that observed after one hour. Because adherence is a pre-requisite for phagocytosis, it was conceivable that this cessation of phagocytosis of haemolymph-sensitised cells was due to a loss of adherent erythrocytes.

To determine whether the number of antibody-sensitised and haemolymph-sensitised erythrocytes initially adhering to macrophages was similar and whether the number of adherent cells decreased, by dissociation, with time, the following experiment was performed. Two groups of 24 macrophages monolayers were set up to monitor the extent

of adherence and phagocytosis over a two hour period. Phagocytosis was monitored to ensure that the erythrocytes used in this adherence assay followed the same ingestion kinetics as that observed earlier with other batches of erythrocytes. Both groups of monolayers consisted of three sets of eight tubes: one set was incubated with unsensitised erythrocytes and the other two with erythrocytes sensitised with either antibody or haemolymph (each at one HAU). To measure phagocytosis, one group (24 monolayers) was incubated at 4° for 1 hr to allow the erythrocytes to adhere to the surface of the macrophages. The monolayers were then incubated at 37° for various lengths of time. The second group of 24 monolayers, used to measure any alteration in erythrocyte adhesion, was kept at 4°, since phagocytosis does not occur at this temperature (Rabinovitch, 1967). Two monolayers from each set of erythrocyte treatments within both the phagocytosis and the adherence groups were sampled at 0, 30, 60 and 120 min. These coverslips, with their adherent monolayers, were immediately washed in saline, then incubated in either Tris-NH_4Cl (phagocytosis group) or saline (adherence group), washed and assayed for radioactivity.

From Table 6.11 it is apparent that, as in the previous experiment (Fig 6.6), phagocytosis of the haemolymph-sensitised cells virtually ceased after 60 min whereas the antibody-sensitised cells continued to be ingested. At 120 min the number of phagocytosed antibody-sensitised cells more than doubled that of the haemolymph-sensitised cells. In contrast, after 2 hr incubation at 4° the number of adherent erythrocytes per coverslip was similar for both the haemolymph and antibody-sensitised cells (7.8×10^5 and 9.6×10^5 respectively). Although very few unsensitised cells bound to the macrophages, a rather high proportion of these were phagocytosed. The

TABLE 6.11 The adhesion and phagocytosis of sensitised and unsensitised sheep erythrocytes by mouse macrophages *in vitro*.

Treatment of erythrocytes	Sampling time (min) for assaying phagocytosis and rosette formation							
	0		30		60		120	
	Phag.	Rosettes#	Phag.	Rosettes	Phag.	Rosettes	Phag.	Rosettes
B. leachii haemolymph@	211¶ 1.8×10^4*	7366 6.4×10^5	308 2.6×10^4	7952 6.9×10^5	598 5.2×10^4	10344 9.0×10^5	632 5.5×10^4	8969 7.8×10^5
Anti-SRBC Antibody§	87 7.5×10^3	3456 3.0×10^5	631 5.5×10^4	8359 7.3×10^5	993 8.6×10^4	10567 9.2×10^5	1666 1.4×10^5	11023 9.6×10^5
Unsensitised (control)	29 2.5×10^3	104 9.0×10^3	57 4.9×10^3	258 2.2×10^4	92 8.0×10^3	301 2.6×10^4	122 1.1×10^4	330 2.8×10^4

Adherent erythrocytes.

@ One haemagglutinating dose = 1:32.

§ Anti-sheep erythrocyte antibody, one haemagglutinating dose = 1:1280.

¶ Radioactivity (counts/5 min) of the retained sheep erythrocytes - mean of duplicate monolayers.

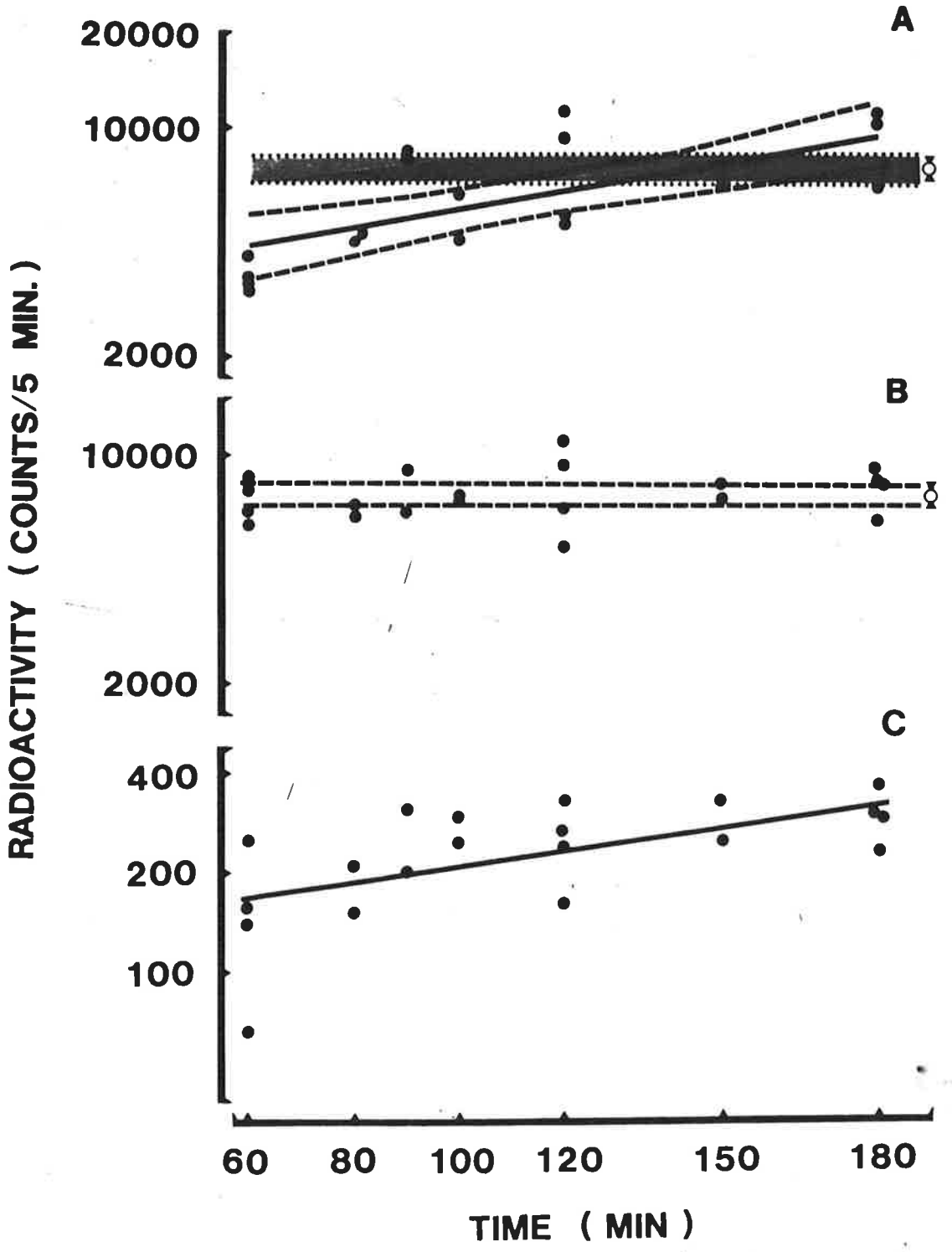
* Numbers of erythrocytes as calculated from their specific activity.

adhesion of these unsensitised cells may have been mediated by cytophylic antibody on the macrophage surface.

If, as was suggested by the previous preliminary experiment, one HAU of haemolymph or antibody can sensitise erythrocytes for adhesion to macrophages to the same extent, then the lack of phagocytosis of haemolymph-sensitised cells after one hour incubation was not explicable through a lack of adherent cells and alternative explanations were necessary to account for the phenomenon. It was therefore important to assess more accurately the relative extent of adherence. Accordingly, erythrocyte adhesion was re-examined with a larger number of monolayers and extra time points. In this case, monolayers were incubated with unsensitised erythrocytes or with cells sensitised with one HAU of either haemolymph or antibody (12 monolayers per treatment). The monolayers were incubated as before for 1 hr at 4° before beginning the adhesion measurements and incubation was continued at 4° for all groups. Two monolayers from each group were sampled after 60, 80, 120, 150, and 180 min of incubation (total elapsed time). The data obtained were homogenous with the adhesion data from the previous experiment (Table 6.11); consequently they were pooled and are displayed in Fig 6.7.

Regression lines were calculated from the erythrocyte adhesion data for each group and the amount of variation explained by these lines was determined by an analysis of variance (Sokal & Rohlf, 1969). The variance explained by the regression line for the haemolymph-sensitised cells was not significant i.e. the slope of the regression line did not differ significantly from zero. This indicated that the maximum number of haemolymph-sensitised erythrocytes adhering to the macrophages was attained before sampling began and remained static subsequently (Fig 6.7B). These data could therefore be

FIGURE 6.7 The adherence of erythrocytes sensitised with either antibody or haemolymph. Cr^{51}-labelled erythrocytes, sensitised with 1 HAU of rabbit antibody (A) or B. leachii haemolymph (B), or unsensitised (C), were added to monolayers of mouse macrophages and incubated at 4° for the time specified. Adherence was measured from the radioactivity associated with monolayers after washing. Regression lines, calculated for both antibody-sensitised erythrocytes ($\log y = 0.0028x + 3.6$) and unsensitised erythrocytes ($\log y = 0.0024x + 2.2$) are shown. The regression line calculated for haemolymph-sensitised erythrocytes did not explain a significant proportion of the variance and these data are therefore summarized as a mean $\pm$ 95% confidence limits (plot B). The kinetics of adhesion of haemolymph-sensitised and antibody-sensitised erythrocytes are compared together in (A), the stippled area representing the 95% confidence limits around the mean for the haemolymph sensitised cells (⧖). Confidence limits (95%) are also given for the regression line of the antibody treated cells (- - -).


A
B
C
20000
10000
2000
10000
2000
400
200
100
RADIOACTIVITY (COUNTS/5 MIN.)
60
80
100
120
150
180
TIME (MIN)

summarized by a mean with 95% confidence limits calculated after pooling across all time points. Regression lines calculated for the adherence of unsensitised and antibody sensitised erythrocytes did explain a significant amount of the variation (Fig 6.7A & C, $P < 0.001$ and $P < 0.01$ respectively) and the slopes were significantly greater than zero. This indicates that the numbers of these cells adhering to the macrophages increased during the experiment. The regression coefficients (i.e. slopes) of these two lines were not significantly different (t-test, $P > 0.5$; Sokal & Rohlf, 1969), although the absolute number of adherent erythrocytes was quite different between the two groups. The unsensitised cells may have bound through cytophilic antibody on the macrophage surface.

It was shown by the overlapping 95% confidence limits, calculated for the mean of the haemolymph sensitised cell adherence data and the antibody treatment regression line, that over the last 60 min of the experiment the number of adherent antibody and haemolymph-sensitised cells did not differ significantly. This occurred despite the different binding kinetics of these two cell preparations (Fig 6.7A). It is possible therefore, that the low numbers of haemolymph-sensitised erythrocytes phagocytosed were due not to a reduction in the number of adherent erythrocytes but rather to differences in the triggering of phagocytosis.

6.3.4 Discussion.

Other workers have previously reported that the plant lectins, concanavalin A and wheat germ lectin can, under some conditions, induce phagocytosis of erythrocytes (Bar-Shavit & Goldman, 1976; Goldman & Bursker, 1976 and Goldman & Cooper, 1975) but to my knowledge there have been no similar attempts with invertebrate lectins. The results

presented earlier in this chapter (Section 6.2) showed that the HA-2 agglutinin was responsible for promoting the adhesion of haemolymph-sensitised sheep erythrocytes to mouse macrophages, and the data presented in this Section (6.3) have indicated that *in vitro* (and probably *in vivo*), the HA-2 agglutinin can also induce mouse macrophages to phagocytose a significant number of erythrocytes.

Although adhesion through the HA-2 agglutinin induced significant phagocytosis, the number of HA-2-sensitised erythrocytes phagocytosed after 1 hr *in vitro* was substantially lower than that of antibody-sensitised cells. This finding might have been explained if sensitisation with one HAU of antibody had caused more erythrocytes to bind to the macrophages than did sensitisation with one HAU of haemolymph. In fact, after 1 hr of incubation at 4° the reverse was true - there were more adherent haemolymph-sensitised cells than antibody-sensitised cells. If incubation at 4° was continued for a further 2 hr then the number of adherent antibody-sensitised cells increased to be not significantly different from that of the haemolymph-sensitised cells, which had not altered significantly from the number recorded after 1 hr at 4° (Fig 6.7). This result also indicated that adherent cells did not dissociate from the macrophages to any appreciable extent over this time period at 4°.

The difference in the phagocytic rates may have been due to differences in the number and/or distribution of Fc and HA-2 receptors on the macrophage surface and in the density and distribution of HA-2 or antibody on the erythrocyte surface. The number and distribution of ligand molecules on adherent particles has been shown by other workers to be a critical parameter in the triggering of ingestion. For example, the efficiency with which sheep erythrocytes are phagocytosed by macrophages depends on the number of IgG molecules coating the

erythrocytes (Ehlenberger & Nussenzweig, 1977). Also, Griffin et al., (1976) found that mouse lymphocytes, previously capped at 37° with rabbit anti-mouse IgG, adhered to macrophages via the immunoglobulin cap but were not phagocytosed, whereas lymphocytes treated with antibody at 4°, to prevent capping, were phagocytosed. Thus, it seems that for ingestion to occur the opsonins must be distributed over a considerable proportion of the surface of the bound particle. This appeared to be necessary not only to guide the macrophage membrane over the surface of the particle but also to generate repeated "triggers" for phagocytosis. This conclusion was derived from experiments where erythrocytes, attached to normal macrophages through Fc and C3 receptors, were not phagocytosed, if the excess (unoccupied) Fc receptors were blocked after attachment by anti-macrophage antibody. Macrophages which were not treated with antibody ingested the adherent erythrocytes (Shaw & Griffin, 1981). The anti-macrophage antibody had been previously shown to block IgG-mediated adherence and phagocytosis but not the phagocytosis of formaldehyde-treated erythrocytes or latex particles (Holland et al., 1972; Bianco et al., 1975) or adherence through the C3 receptor (Bianco et al., 1975). In the system described, although the attachment of the macrophage membrane to the surface of a large proportion of the particle was possible through C3 receptors, this was not sufficient to induce phagocytosis. Ingestion appeared to require that repeated "triggers" for phagocytosis, generated through the binding of antibody to Fc receptors, be distributed over the particle's surface.

Since multipoint binding is essential for phagocytosis (Griffin et al., 1976; Shaw & Griffin, 1981) the ingestion kinetics exhibited by particles opsonised with HA-2 agglutinin or antibody could be explained by differences in both the numbers of Fc and HA-2 receptors on the

surface of macrophages and the numbers of agglutinin or antibody molecules on the erythrocyte surfaces. The numbers of HA-2 receptors on the macrophage surface may mean that after 60 min at 37° the remaining adherent agglutinin coated erythrocytes do not bind through sufficient HA-2 receptors to trigger phagocytosis. The relative numbers of HA-2 and Fc receptors on the macrophage surface has yet to be determined. The mean numbers of HA-2 or antibody molecules bound per sheep erythrocyte at one haemagglutinating dose have been calculated to be within the same order of magnitude, approximately 22,000 HA-2 molecules (P.L. Ey personal communication) and 4,000 - 20,000 molecules of IgG antibody (Greenbury et al., 1963; Ey et al., 1980). However, the number of molecules required to trigger phagocytosis may well be different. If more HA-2 molecules, than antibody molecules, are required to generate each phagocytic signal then this would also account for the cessation of HA-2 mediated ingestion while antibody mediated phagocytosis continued.

Another aspect of the difference in phagocytosis of HA-2 and antibody-sensitised erythrocytes to be considered is the relative affinity of antibody and the HA-2 agglutinin for their erythrocyte receptor sites. Although the mean number of bound antibody and HA-2 molecules was probably comparable immediately after sensitisation (see above), after 1 hr at 37° this may no longer have been true. It has been shown that the monomeric immunoglobulin molecules on the macrophage surface are in rapid equilibrium with the immunoglobulins in the cell's immediate environment (Unkeless & Eisen, 1975) and it is likely that this is also the case for HA-2 molecules binding to both erythrocytes and macrophages. Thus, one could reasonably expect some transfer of HA-2 molecules from both adherent and non-adherent erythrocytes to the macrophages, since upon dissociation from an

erythrocyte an HA-2 molecule could rebind to either an erythrocyte or a macrophage. The rate and extent of transfer would clearly depend on the rate of dissociation and on the relative availability of receptor sites on the erythrocytes and macrophages. However, macrophages have a larger number of receptor sites for the HA-2 agglutinin (see Section 6.2.3; Fig 6.3) and the transfer may, ultimately be of sufficient magnitude that the erythrocytes are insufficiently sensitised to trigger phagocytosis. In contrast, any dissociated antibody would preferentially rebind to an erythrocyte, since macrophage Fc receptors have a low affinity for uncomplexed IgG molecules. As a result of these processes, it is probable that, after prolonged incubation with macrophage monolayers at 37°, antibody-sensitised erythrocytes remain fully sensitised but HA-2-sensitised erythrocytes become partly or wholly de-sensitised. Thus, if the number of HA-2 molecules which bind to the macrophages is insufficient to sensitise these cells, one might expect to record a decrease in the number of adherent HA-2 sensitised erythrocytes during the incubation. However, the decrease in the number of HA-2 molecules coating the erythrocytes may have been sufficient to affect phagocytosis, but insufficient to cause a loss of adhesion. The adhesion experiments were performed at 4° and any dissociation of agglutinin molecules would have occurred at a slower rate than at 37°.

It remains to be determined whether or not the macrophage HA-2 receptor is associated with either Fc or C3 receptors. It is quite possible that the HA-2 agglutinin binds to both Fc receptors and C3 receptors since these are glycoproteins (Mellman & Ukeless, 1980; Schneider et al., 1981). However, it would be surprising if these two receptors constituted more than a small fraction of the total binding

sites available to the agglutinin on the macrophage surface. Such information would be valuable in characterising the events involved in the initiation of the phagocytic process. Nevertheless, this study, along with others (see Section 6.2.1), has indicated clearly that the phagocytosis of foreign particles can be induced by molecules other than antibody. Moreover, the present investigation is novel in that an invertebrate lectin has been shown to induce a phagocytic response in vertebrate cells.

Chapter 7.

GENERAL DISCUSSION.

This study was initiated with the aim to obtain information not only on the recognition of foreign particles by the phagocytes of an invertebrate but also on the evolution of vertebrate immunoglobulin. A protochordate and in particular Botrylloides leachii was chosen for this investigation because:-

(1) the protochordates are considered to have diverged from the immediate ancestor of the vertebrates (Berrill, 1955) and hence are likely to be invaluable in a study of the evolution of the vertebrate immune response;

(2) virtually nothing was known about the mechanism utilised by protochordate phagocytes in the recognition of foreign particles;

(3) observations in the field suggested that B. leachii is able to recognise as foreign not only other species but also certain con-specifics, a capacity that has been well documented for a closely related species, Botryllus primigenus (Oka, 1970; Katow & Watanabe, 1980) and

(4) it had been reported from histological work with colonies of B. leachii from Italy that during aestivation, degenerating zooids were phagocytosed by an enlarged population of amoeboid cells (Burighel et al., 1976).

There is a number of ways of approaching an investigation of invertebrate defence responses to foreign particles. In this case, because a study on the recognition of con-specifics by Botryllus primigenus produced evidence that this phenomenon was controlled by either cellular or molecular components of the haemolymph (Mukai, 1967), it was decided as the first approach to investigate the haemolymph of B. leachii for possible "recognition" molecules. In Chapter 1, the possibility that invertebrate haemagglutinins may function as opsonins was examined. However, it was noted that the

evidence for this suggestion was largely circumstantial save for the demonstration by Renwrantz and his colleagues that the opsonising and agglutinating activities of Helix pomatia haemolymph can be inhibited by the same sugar (Renwrantz, 1979; Harm & Renwrantz, 1980). If haemagglutinins did perform an essential function in ascidians then it might be expected that they would be found in the haemolymph not only of B. leachii but also of a wide variety of ascidians. This was found to be the case, although considerable variation in titres was observed and haemagglutinins of different specificity were evident in different species (Chapter 3). It was considered that these results warranted a detailed investigation aimed at determining unambiguously whether or not the haemagglutinins from B. leachii function as recognition factors for this species.

The possibility that the mechanism by which B. leachii cells recognise foreign material may represent an early ancestral form of the mechanism used by vertebrate macrophages was another aspect of this study. It was proposed that if a molecule with an opsonic function in B. leachii colonies also functioned as an opsonin for vertebrate macrophages then some structural homology between the invertebrate opsonin and antibody might underly this functional homology. Accordingly, the capacity of the B. leachii agglutinins to opsonise particles for phagocytosis by vertebrate macrophages was examined.

Investigations by Sam Schluter, performed in parallel with this study (Schluter et al., 1981; Schluter, 1982), resulted in the purification and characterisation of two haemagglutinins (HA-1 and HA-2) originally detected in B. leachii haemolymph. As the physico-chemical properties of the B. leachii agglutinins became evident, the haemolymph of other ascidians was examined not only for

the presence of haemagglutinins *per se* but also for any structural similarities of the haemagglutinins with the HA-1 and HA-2 molecules from *B. leachii*. Haemolymph samples from 22 different species of ascidians were tested in this regard and samples from four species appeared to possess agglutinins resembling, at least superficially, the HA-1 agglutinin. One of the samples (from *Botryllus* sp.) also contained an "HA-2 like" molecule. It is noteworthy that the four ascidians possessing the "HA-1 like" molecule all belong to the same order as *B. leachii*; three belong to the same family and of these *Botryllus* sp. is the most closely related to *B. leachii*, being classified as a member of the sub-family Botryllinae.

A detailed comparison of the *B. leachii* and *Botryllus* sp. agglutinins revealed some differences. The *Botryllus* "HA-1 like" molecule eluted at the same position as the *B. leachii* HA-1 after chromatography on Sephacryl S-300, but analysis by velocity sedimentation in sucrose density gradients indicated that it was slightly smaller than the *B. leachii* HA-1. These results suggest that the *Botryllus* sp. agglutinin may be more aglobular than the HA-1 agglutinin which is known to be an asymmetrical molecule (Schluter, 1982). The two molecules exhibited identical sugar binding specificities and a dependence on divalent cations (Ca^{++}; Schluter *et al.*, 1981) for binding activity. No antigenic cross-reactivity was observed. A preliminary SDS-PAGE analysis suggested that the *Botryllus* sp. agglutinin was structurally quite distinct from the *B. leachii* agglutinin or LBP-3 since it did not appear to share any subunits with the *B. leachii* proteins. The *Botryllus* sp. sheep erythrocyte agglutinin was identical to the *B. leachii* HA-2 agglutinin in size and subunit structure. These two agglutinins were also antigenically cross-reactive but not identical and interestingly there

was a slight difference in their binding site specificity as lactose, melibiose and D-galactose were all more effective inhibitors of the Botryllus sp. agglutinin than of the HA-2 molecule. These findings strongly indicate that the genes coding for these two agglutinins have evolved independently from a common ancestral gene.

The initial screening of the haemolymph samples from the different ascidians was based on identifying molecules with similar binding site specificities and cation requirements to those of either the HA-1 or HA-2 agglutinins. Hence, it is feasible that other undetected agglutinins, possibly structurally related to the HA-1 or HA-2 but with different binding site specificities, could exist in the haemolymph of B. leachii and/or other species. Because of the lack of published physico-chemical data on the agglutinins detected by other workers, the identification of any agglutinins structurally related to the HA-1 or HA-2 from the published information is not possible.

The possibility that the B. leachii agglutinins were opsonins was investigated both in vivo and in vitro. It was reasoned that if the response mounted by B. leachii colonies as a defence against foreign particles is analogous to that of vertebrates, then one might expect that the concentration of a recognition molecule (an agglutinin?) in the haemolymph may reflect the extent of previous exposures to stimulating substances. If environmental factors were stimulating the production of the agglutinins, then altering the environment may be expected to cause a change in titre. This was tested experimentally by measuring the change in the HA-1 titre after colonies were relocated, from a region where the animals exhibited a high titre to a region where the HA-1 titre was low and vice versa (Chapter 4, Section 4.2). Transfer to a new environment did alter the

HA-1 titre. A drop in titre was recorded when colonies, originally from the region where HA-1 titres were low, were moved to the region where the animals exhibited high titres, while the reciprocal transfer resulted in an increase in the HA-1 titre. The direction in which the titres changed suggested that environmental stimuli alone were unlikely to have accounted for the quantity of HA-1 agglutinin in B. leachii haemolymph but other factors, possibly genetic, were also involved (Chapter 4, Section 4.4).

In the experimental system described above neither the specificity nor the nature of the stimulus inducing the change in HA-1 titre could be investigated. In another experiment B. leachii colonies were injected with erythrocytes known to have HA-2 but not HA-1 receptors on their surface. It was possible in this system to determine not only whether or not the HA-2 titre could be altered but also the specificity of any change induced. Colonies were injected with sheep or chicken erythrocytes and the haemolymph was monitored for an alteration in the level of the HA-2 agglutinin. The results from this work (Chapter 4, Section 4.3) indicated that it was possible to produce a small but significant increase in the HA-2 agglutinating titre of haemolymph following injection of erythrocytes and furthermore as the HA-1 titre was not altered by injection of sheep or chicken erythrocytes this increase appeared to be specific. There was, however, no indication of an anamnestic response similar to that observed in the vertebrates.

The notion that either of the B. leachii haemagglutinins and in particular, the HA-2, was acting as an opsonin was supported by in vitro work with B. leachii haemocytes. The haemocytes, in vitro, were found to bind untreated sheep erythrocytes. As sheep erythrocytes possess receptors for the HA-2 molecule the possibility that this agglutinin was involved in recognition was investigated. The binding

of sheep erythrocytes to B. leachii haemocytes could be specifically inhibited by a sugar (lactose) which also inhibited the HA-2 agglutinin and by IgG antibodies specific for the HA-2 molecule (Chapter 5, Section 5.3); hence it was concluded that adhesion occurred via the HA-2 agglutinin. The finding that B. leachii haemocytes ingested sheep erythrocytes in vivo was in accordance with the suggestion that the HA-2 is an opsonin.

The method by which the HA-2 agglutinin mediated the adhesion of unsensitised erythrocytes to haemocytes was investigated by immunofluorescent studies. This work indicated that unsensitised erythrocytes became coated with HA-2 agglutinin and that receptors for the HA-2 molecule appeared to exist on the surface of some B. leachii haemocytes. Presumably, therefore, the haemocytes were secreting HA-2 molecules which then sensitised the erythrocytes for adhesion, via the HA-2 receptor, to the haemocyte surface. It is noteworthy that no HA-2 agglutinin could be detected on the surface of washed haemocytes in the absence of erythrocytes. It is possible that the sheep erythrocytes stimulated the release of the HA-2 and/or that uncomplexed HA-2 bound with very low affinity to the surface of the haemocytes. The latter hypothesis accomodates the finding that in the majority of cases HA-2 molecules could not be detected on the surface of haemocytes shown to possess HA-2 receptors (i.e. they had erythrocytes attached), even though the erythrocytes on the same monolayer appeared coated with agglutinin. If, in vivo, foreign particles bearing the appropriate carbohydrate moieties stimulate haemocytes to secrete HA-2 agglutinin, or other recognition molecules, as part of the phagocytic process then it is possible that the efficient phagocytosis of a particle can be

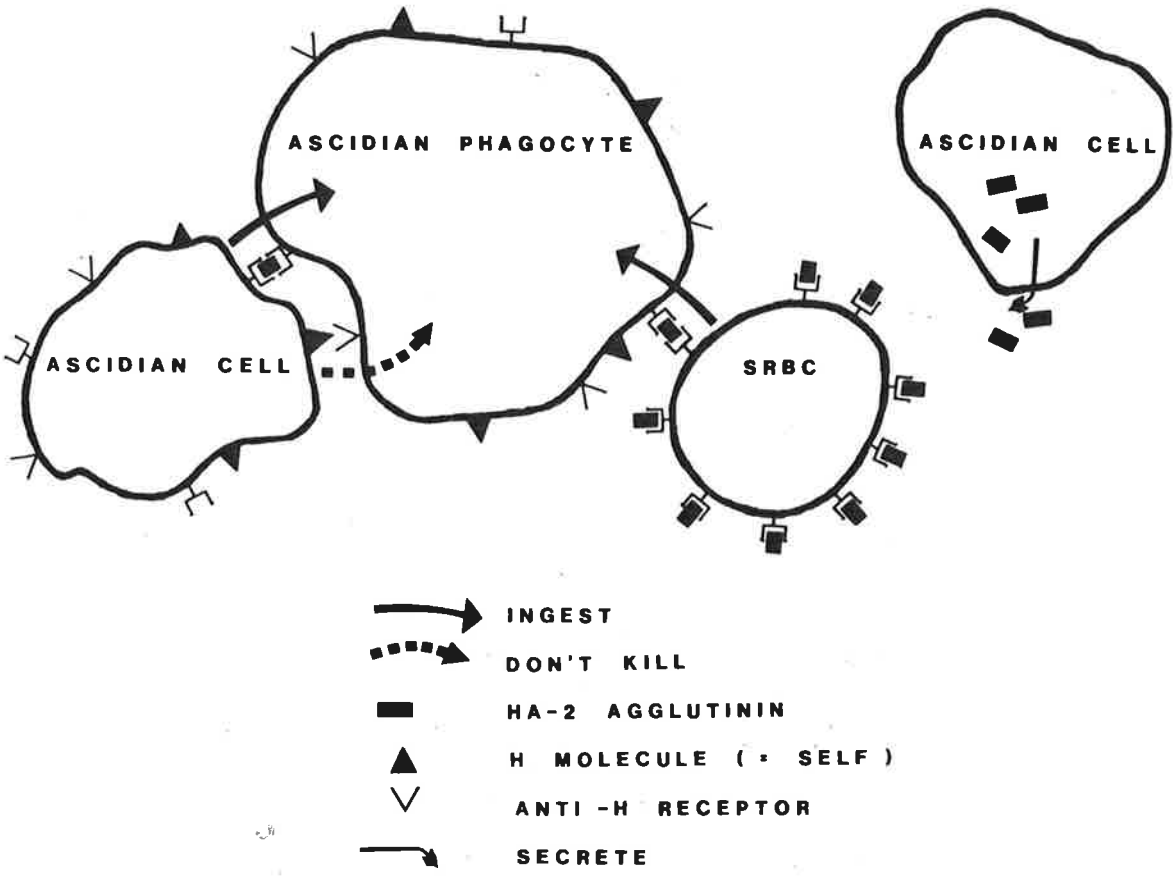
maintained for long periods in the absence of a vertebrate type anamnestic response. Thus, any selective advantage afforded by an anamnestic response could well be minor.

I would like now to discuss these results within the framework of the model proposed in Chapter 1. The model predicts that phagocytosis is controlled by two signals. A signal triggering phagocytosis is generated by the binding of a particle to the phagocytic cell; binding can be via a cell surface receptor or an opsonin, or an attraction caused by the physical characteristics of the particle and the phagocyte (e.g. charge or hydrophobicity). A second signal controls self-reactivity. A "don't kill" signal is generated by the binding of a self recognition molecule (H) to its complementary receptor (anti-H) on the surface of the phagocyte. Thus, phagocytosis may proceed unless self is recognised and the "don't kill" signal over-rides the trigger for phagocytosis.

The adhesion of erythrocytes to B. leachii phagocytes via the HA-2 agglutinin (the opsonin) may be expected to produce an ingestion signal. However, as ascidian cells possess receptors for uncomplexed HA-2 then the adhesion of two ascidian cells via the HA-2 molecule is also feasible. If one of these cells were a phagocyte then presumably ingestion would be triggered unless, according to the model, a "don't kill" signal generated by the recognition of self determinants (H binding to anti-H) were to over-ride the signal for ingestion (Fig 7.1).

It is not known which haemocytes (Fig 7.1) secrete the HA-2 molecules. If only phagocytic cells secrete HA-2, it may be that the physical contact of a particle (e.g. a sheep erythrocyte) with a phagocyte is sufficient to stimulate the release of HA-2. One could

FIGURE 7.1 The model proposed in Chapter 1 to explain phagocytosis by invertebrate cells is here applied to the situation envisaged for B. leachii haemocytes.


ASCIDIAN PHAGOCYTE
ASCIDIAN CELL
ASCIDIAN CELL
SRBC
INGEST
DON'T KILL
HA-2 AGGLUTININ
H MOLECULE (= SELF)
ANTI-H RECEPTOR
SECRETE

argue that the agglutinin would then be in high concentration only in close proximity to the phagocyte. The stimulating particle would rapidly become coated with agglutinin, bind to the haemocyte and so trigger phagocytosis. The necessity for a second signal is not eliminated by assuming that the opsonin is concentrated around the phagocyte. In the absence of a self/non-self recognition method other than via the opsonin, a self cell possessing HA-2 receptors could stimulate the release of HA-2 agglutinin through contact with a phagocyte and so become phagocytosed. In this variation of the model, the "don't kill" signal could be manifested as the inhibition of HA-2 release. However, this is considered to be unlikely because in the absence of a positive "don't kill" signal some self cells bearing HA-2 receptors may be sensitised by the HA-2 molecules in haemolymph and so become phagocytosed.

The proposal that the foreign particle stimulated the immediate release of agglutinin from haemocytes (not necessarily the phagocytes) is in accordance with the in vitro data and could explain why no HA-2 agglutinin could be detected on the surface of washed haemocytes in the absence of sheep erythrocytes. It is however a little more difficult to account for the in vivo data. Sheep erythrocytes were shown to stimulate the production and/or release of the HA-2 agglutinin in vivo, but as this occurred two weeks after erythrocytes had been injected it is unlikely to have been caused by the erythrocytes triggering HA-2 release as an immediate part of the phagocytosis event. It is possible that the phagocytosis of erythrocytes, probably involving a restricted number of haemocytes, may have triggered a systemic response which resulted in an increase in the number of HA-2 secreting cells and/or an increase in the synthesis of HA-2 agglutinin by the haemocytes. Any HA-2 released immediately after injection as part of the process of

phagocytosis may not have been detected because of the insensitivity of the titration assay performed on haemolymph collected from the whole colony. If the model is correct and erythrocytes stimulated HA-2 release by contact with haemocytes, then this would most likely have been a local event and thus undetectable when diluted by the total volume of haemolymph in that colony. Another factor to be considered is that the sheep erythrocyte agglutinating titre of haemolymph from unstimulated colonies is such that erythrocytes are likely to be coated with agglutinin immediately upon entering the circulation. It may be that opsonised particles do not stimulate the release of more agglutinin.

An interesting finding was that uncomplexed HA-2 agglutinin did not appear to bind haemocytes. Cunningham (1980), in a discussion of self-tolerance within the vertebrate immune system, took up the point made by Cohn (1972) that it is the diversity of self-determinants which sets the necessary size of the immune repertoire. He argued that antibodies must be present in enough diversity to ensure that at least some of the antibodies to a foreign antigen bind self determinants with low affinity and so escape the mechanisms which suppress anti-self antibodies. The probability that all invertebrate opsonins will, like the HA-2 molecule, fit Cunningham's criteria is open to question, particularly as invertebrates appear to lack a vast array of opsonins with the fine specificities exhibited by antibodies. Because the production of many opsonins would be energetically expensive it may have been selectively advantageous for the invertebrates to possess a few opsonins with broad specificities for common components of cell surfaces. If self reactivity is controlled by a recognition of self determinants (H and anti-H), then opsonins would not need to have a low

affinity for self cells. Furthermore, the diversity of determinants on self cells would not have any bearing on the repertoire of the opsonins.

One could postulate that opsonins with a low affinity for self when uncomplexed were advantageous for reasons other than a prevention of self-reactivity. For example HA-2 molecules, circulating in the haemolymph and free to bind or to opsonise particles bearing the appropriate carbohydrate moiety on their surface, may induce a more rapid immobilisation of potential pathogens than is achieved by similar numbers of cell-bound HA-2 molecules. In fact some opsonins may exist both as molecules circulating in the haemolymph and bound to the surface of cells, e.g. the Helix pomatia opsonin/agglutinin (Harm & Renwrantz, 1980; Renwrantz et al., 1981). Although the functions of the haemolymph molecules HA-1 and LBP-3 are not known, it is of interest to note that at least one of them also exists on the surface of a large number of B. leachii haemocytes.

A separate aspect of this study - the possibility that the B. leachii haemagglutinins may bear some evolutionary relationship with immunoglobulin, was addressed by attempting to opsonise erythrocytes with B. leachii haemolymph for adhesion and ingestion by mouse macrophages. A factor in B. leachii haemolymph was found to promote the adhesion of sheep erythrocytes to macrophages and the phagocytosis of some of the adherent cells. Lactose was found to inhibit the adhesion of these erythrocytes to macrophages and also the agglutination of both macrophages and sheep erythrocytes. It therefore appeared that the sheep erythrocyte agglutinin was also the opsonin. This was confirmed when a purified preparation of the sheep agglutinin (HA-2) became available. It is noteworthy that binding of the HA-1 agglutinin to macrophages could not be detected. Because both

erythrocytes and macrophages could be sensitised by haemolymph for adhesion to the other and the sensitisation of both could be reversed by lactose but not by galactose or other sugars, it was concluded that the adhesion of sheep erythrocytes to macrophages was a hetero-agglutination reaction, the agglutinin cross-linking similar lactose-like sites on both cells. Thus, although the HA-2 agglutinin was acting, like antibody, as an opsonin with vertebrate cells, there was no evidence that this agglutinin was structurally related to immunoglobulin. Schluter (1982) arrived at a similar conclusion after investigating the structure of the HA-2 molecule. He found the HA-2 molecule to be dimeric, consisting of two polypeptide chains with molecular weights slightly larger than an immunoglobulin light chain but with only one disulphide loop per subunit. It was presumed that since an invariant feature of all immunoglobulin molecules is that the core of each domain is enclosed within a disulphide loop then a primordial "immunoglobulin" molecule was also likely to have possessed such a feature. The HA-2 molecule does not have this characteristic.

Recent ideas as to what may constitute a primordal immunoglobluin molecule have concentrated on cell surface components and in particular the Thy-1 glycoproteins (Williams, 1982; Williams & Gagnon, 1982). The Thy-1 glycoprotein is the size of an immunoglobulin domain, the arrangement of its disulphide bonds is similar to that of all immunoglobulin domains and furthermore there is marked homology between the amino acid sequences of Thy-1 and the variable-region immunoglobulin domains. The role of Thy-1 is not known, although it has been proposed both that Thy-1 may function as a receptor for another molecule (e.g. a hormone) or that it may be a ligand recognised by receptors on other cells and may function in cell recognition during morphogensis (Williams, 1982; Williams & Gagnon, 1982). The latter

proposal is of particular interest in view of the homology Thy-1 was shown to exhibit with β_2-microglobulin and immunoglobulin constant regions and hence with class 1 histocompatibility antigens (Williams & Gagnon, 1982).

A glycoprotein bearing distinct homologies with rodent Thy-1 has been purified from the brain of squid (Williams & Gagnon, 1982). The squid molecule had a similar molecular weight and amino acid compostion to mouse Thy-1. Furthermore, regions of amino acid sequence were found to be homologous with both mouse Thy-1 and the immunoglobulin variable domain around the COOH terminal cysteine of the conserved disulphide bond of the variable domains. If this molecule from squid does prove to be structurally related to Thy-1 then there is a distinct possibility that other "Thy-1 like" molecules will be identified from the cells of other invertebrates. It is of interest in this regard that the use of homologous and heterologous radioimmuno-assays has produced evidence for a β_2 microglobulin-like molecule in tissue extracts from the earthworm, Lumbricus terrestris, and the crayfish, Cambarus diogenes (Shalev et al., 1981). It has been suggested that immunoglobulins may have emerged as a result of the branching of the histocompatibility antigens into two groups: those that retained an importance in cell-cell contact and recognition; and those antigens that became modified and served as recognition factors for non-self antigens (Klein, 1975; Chorney & Cheng, 1980). In addition to the results noted above, other recent data are in accordance with this suggestion; the amino acid sequence of an 88 residue fragment of the heavy chain of HLA antigens was shown to have significant homology with the immunoglobulin constant domains (Orr et al., 1979) and up to 50% homology has been detected between the DNA sequences of the third external domain of H-2 antigens and the immunoglobulin C gene

(Steinmentz et al., 1981). Thus, the identification of the structure and function of any "Thy-1 like" molecules from the deuterostome invertebrates may shed light not only on the evolution of the vertebrate histocompatibility antigens but also of immunoglobulin.

It is clear both from the evidence presented in this thesis and from the work of others that in order to understand the mechanisms of recognition of foreignness in invertebrates, the problem must be approached from both a chemical and a functional viewpoint. Very few attempts have been made to investigate the function of haemolymph agglutinins or to identify the "recognition factors". This has been largely due to a profound lack of information on the properties of the molecules. The purification and characterisation of invertebrate agglutinins and recognition factors would not only provide information as to the structure and hence possible relationships between these molecules, but it could also lead to information relating to their biological function. Important questions relating to the function of agglutinins, such as:- Do agglutinins bind to self cells as well as foreign particles? If so, what is the specificity of binding to each type of cell? Do phagocytic cells secrete and/or bind agglutinin(s) or "recognition factors"? can be most rigorously addressed after the agglutinin has been purified. The agglutinin can then be tested for functional activities; labelled agglutinin can be used to study its binding properties and specific antisera, which may be used as probes for the molecules in situ, can be prepared.

It is also crucial to carry out investigations within a theoretical frame-work which relates to the requirements of the invertebrate under study rather than those of vertebrates. For an

invertebrate with a short life span and many offspring, for example, the possession of immunological memory may not have been of selective advantage, and it may have been energetically too expensive to produce a vast array of opsonins with different specificities. Nevertheless, an animal lacking these attributes does not necessarily lack the ability to respond to foreign particles. The invertebrate defence response may not display the sophistication of that of vertebrates, but the number of invertebrates surviving today indicates that it is no less effective.

APPENDIX

Material contained in this thesis has been published in the following papers.

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 42:661.

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