# THE ISOLATION AND CHARACTERIZATION OF THREE LACTOSE BINDING PROTEINS IN THE HAEMOLYMPH OF THE PROTOCHORDATE *Botrylloides leachii*

Samuel F. Schluter, B.Sc. (Hons.) (Adelaide)
Department of Microbiology and Immunology
The University of Adelaide

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To Mum

## ABSTRACT

This thesis is concerned with the isolation and characterization of three lactose-binding proteins from haemolymph of the protochordate *Botrylloides leachii*. Initially, the haemagglutinating activity of the haemolymph for various erythrocytes was extensivley investigated and it was concluded from the results of cross-adsorption and sugar inhibition experiments together with gel filtration data that two haemagglutinins were present. Although both molecules were inhibited by lactose, their binding specificities were shown to be different both by their affinity for different types of erythrocytes and their susceptibility to inhibition by different sugars. The haemagglutinins were purified by a combination of affinity chromatography using an adsorbent consisting of lactose-substituted acid-treated Sepharose, and gel chromatography. During the purification a third protein was specifically eluted from the affinity column. This molecule was shown to bind specifically to guinea pig erythrocytes without causing haemagglutination and could be inhibited by lactose. The three proteins were designated Haemagglutinin One (HA-1), Haemagglutinin Two (HA-2) and Lactose Binding Protein Three (LBP-3). The purity of each preparation was confirmed by SDS-PAGE and immunochemical analyses.

The major findings concerning the nature of these proteins were as follows:

1) HA-2 molecules, which agglutinated every type of erythrocyte tested, have only one type of binding site and this recognises lactose (i.e., D-Gal-β1→4-D-Glu) moieties. The agglutinin is a globular ($f/f_o$ = 1.34) protein and occurs as a non-covalently linked dimer (mol. wt. = 65,000) composed of identical subunits (apparent mol. wt. = 33,400).

2) HA-1 molecules (mol. wt. = 152,000) are non-globular ($f/f_o$ = 1.8), covalently linked polymers which appear to be composed of two types of sub-units, HA-1/1a (apparent mol. wt. = 29,600) and HA-1/1b (apparent mol. wt. = 28,200). This molecule agglutinated only guinea pig erythrocytes and its activity required $Ca^{++}$ ions. It appeared to have only a single type of binding site which bound lactose, melibiose, D-galactose and D-fucose with similar affinity.

3) LBP-3 molecules (mol. wt. = 140,000 - 160,000) exhibited a binding specificity similar to that of HA-1 molecules and their activity also required $Ca^{++}$ ions. They are covalently linked polymers composed of three types of subunits, LBP-3/1a (apparent mol. wt. = 29,600, LBP-3/1b (apparent mol. wt. = 28,200) and LBP-3/2 (apparent mol. wt. = 23,600). It is proposed that the LBP-3/1a and 1b subunits are identical to the HA-1/1a and 1b subunits and that these subunits probably contain the carbohydrate binding site.

4) The HA-1 molecules appear to exist as pentamers

comprising one or the other of the HA-1/1a and 1b subunits. LBP-3 molecules appear to be hexamers comprising no more than two LBP-3/2 subunits and approximately four of either the LBP-3/1a or 1b subunits.

Preliminary studies of the haemagglutinin in *B. schlosseri* haemolymph indicated the presence of a haemagglutinin that was very similar to *B. leachii* HA-1.

## 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 of the thesis.

Samuel F. Schluter

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## ABBREVIATIONS USED IN THIS THESIS

| | |
|---|---|
| BSA | bovine serum albumin |
| c.a. | approximately |
| EDTA | ethylenediaminetetra-acetic acid di-sodium salt |
| EGTA | ethyleneglycol-bis(β-amino-ethyl ether) N,N-tetra-acetic acid |
| GPRBC | guinea pig erythrocyte |
| HA-1 | Haemagglutinin One |
| HA-2 | Haemagglutinin Two |
| HAU | haemagglutinating units |
| IgG | Immunoglobulin G |
| lactose-Sepharose | lactose substituted Sepharose |
| Lac-acid-Sepharose | lactose-substituted acid-treated Sepharose |
| mol. wt. | molecular weight |
| OD | optical density |
| PBS | phosphate buffered saline |
| SDS | sodium dodecylsulphate |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| SRBC | sheep erythrocytes |
| TEMED | N,N,N',N' tetramethylethylenediamine |
| Tris | Tris(hydroxymethyl)amino-methane |
| TSA | Tris buffered saline |
| AcNeu | N-acetylneuraminic acid |
| Gal | galactose |
| GalNAc | N-acetyl-D-galactosamine |
| Glu | glucose |
| GluN | glucosamine |
| GluNAc | N-acetyl-D-glucosamine |

CONTENTS

# CHAPTER 1

## Introduction

## 1.1 Preamble

Cells specialised for phagocytosis are found in most multicellular animals (Ratcliffe and Rowley, 1979; van Furth, 1970). These cells engulf foreign material such as viruses and bacteria or encapsulate other parasites which may be too large to be ingested and therefore appear to play an important role in host protection against disease (Sinderman, 1971; Whitcomb, Shapiro and Granades, 1974; van Furth, 1970). Before considering how the phagocytic cells of invertebrates accomplish this recognition of self from non-self it might be pertinent to describe what is known of the mechanism involved in non-self recognition by phagocytic cells of vertebrates. Wright and Douglas (1903) showed that significant levels of phagocytosis by vertebrate phagocytic cells *in vitro* occurred only in the presence of serum or plasma. Wright termed the factors present in serum which promoted phagocytosis opsonins. More recent investigations have revealed that these opsonins are specific antibodies (Review, Jenkin, 1976). Antibodies form complexes with foreign material and these complexes then become attached to the phagocyte cell surface through interactions between antibody Fc regions and membrane Fc receptors. Continued interaction between the antibodies and the Fc receptors is necessary for subsequent phagocytosis (Silverstein and Loike, 1980).

The mechanism of non-self recognition by invertebrate phagocytes is not well understood. However, it has been proposed that a system functionally analagous to that of the vertebrates may operate, i.e., recognition by phagocytic cells of invertebrates is mediated by an opsonic factor, or

factors, present in the circulation which, like antibody, can bind to both the foreign material and the phagocytes and so facilitate phagocytosis, or encapsulation if the particle is too large to be ingested. In the first part of this introduction the evidence supporting this hypothesis is discussed. The second part concerns the nature of the molecules possessing opsonic activity and examines the possibility that this function is performed by the haem-agglutinins that are found in many invertebrates. Finally, the physicochemical properties of some of the invertebrate haemagglutinins are reviewed.

## 1.2 Phagocytosis of particles *in vivo* by invertebrate phagocytic cells

In one of the first experiments which suggested the presence of opsonins in invertebrates Cantacuzene (1923) reported that bacteria injected into the circulation of the crab *Maia squinado* adhered to cell surfaces in various tissues, particularly in the brachial lacurae, and were subsequently phagocytosed by fixed and mobile phagocytes. A significantly greater degree of adherance was observed, however, if the bacteria had been pretreated with *M. squinado* haemolymph. Similar results were obtained *in vitro* using cell fragments made by macerating hypodermal or pericardial tissues.

More recent *in vivo* experiments using the freshwater crayfish *Cherax destructor* (formerly known as *Parachaeraps bicarinatus*) have also indicated the presence of opsonins (Tyson and Jenkin, 1973). Bacteria or erythrocytes injected into the ventral haemal sinus of the crayfish were cleared

from its circulation within minutes. In crayfish which had only just completed clearing a large dose of bacteria the rate of clearance of a second dose of bacteria was greatly reduced. Pretreatment of the second dose of bacteria with haemolymph restored the rate of clearance to normal levels. However if the haemolymph had first been adsorbed with the specific strain of bacteria prior to pretreating the second dose, then the rate of clearance was not restored to normal levels. These results suggested that the first dose of bacteria had depleted factors circulating in the haemolymph which were essential for the recognition of the particles by phagocytic cells.

Human type A erythrocytes injected into the circulation of the snail *Helix pomatia* first became attached to cells lining the sinuses of the digestive gland, kidney or foot muscle and were then phagocytosed by haemocytes (Renwrantz, Schäncke, Harm, Erl, Liebsch and Gercken, 1981). In much the same manner as for *C. destructor*, prior injection of erythrocytes into *H. pomatia* reduced the rate of clearance of a second dose. The rate of clearance was restored to normal levels by pretreatment of the second dose of erythrocytes with haemolymph (Renwrantz and Mohr, 1978). This suggests that the clearance of human erythrocytes from the circulation of *H. pomatia* requires an opsonin present in the haemolymph.

### 1.3 Phagocytosis of particles *in vitro* by phagocytic cells from invertebrates

Like vertebrate macrophages, the haemocytes from the clam *Mercenaria mercenaria* can adhere to glass surfaces and

form a monolayer of cells. Significant phagocytosis of a marine gram negative bacterium by such monolayers occurred in the presence of culture medium alone. However, the addition of clam haemolymph to the medium caused an approximate two fold increase in the level of phagocytosis (Arimoto and Tripp, 1977). Similarly, phagocytosis of formalin treated yeast cells by haemocyte monolayers from the snail *Otala lactea* was much higher when haemolymph was present (Anderson and Good, 1976). A possible explanation of these results with the clam and the snail is that the haemolymph supplied essential nutriments for the phagocytic cells and this accounted for the increased uptake. This conclusion is not supported by the findings that; a) the presence of haemolymph did not increase the low levels of phagocytosis by snail haemocytes of other particles such as bacteria and b) phagocytosis by clam haemocytes was not increased by haemolymph which had been adsorbed previously with bacteria.

Possible artifacts caused by the presence of haemolymph can be avoided if the monolayers are incubated in culture medium alone, and test particles added which have been pre-treated with haemolymph. In this way pretreatment with haemolymph has been shown to increase the level of phagocytosis of sheep erythrocytes by haemocytes from the lobster *Homarus americanus* (Patterson and Stewart, 1974), of rabbit erythrocytes by haemocytes from the oyster *Crassostrea virginica* (Tripp, 1966) and of sheep erythrocytes and yeast cells by amoebocytes from the snail *Lymnaea stagnalis* (Sminia, van der Knaap and Edelenbosch, 1979). However, one should note that significant phagocytosis still occurred

in these cases when the particles were not pretreated with haemolymph. In contrast, the pretreatment of the particles with haemolymph was essential for the phagocytosis of human erythrocytes, yeast cells and *Escherichia coli* by haemocytes from the octopus *Eledone cirrosa* (Stuart, 1968) and of various erythrocytes by haemocytes from the freshwater crayfish *C. destructor* (McKay and Jenkin, 1970). Ingestion of formalin treated yeast cells and formalin treated sheep erythrocytes by amoebocyte monolayers prepared from the snail *Helix aspersa* was high in the presence of *H. aspersa* serum but was completely abolished by preadsorption of the haemolymph with the respective test particle. Phagocytosis in adsorbed serum was restored by pretreating the yeast cells or sheep erythrocytes with haemolymph (Prowse and Tait, 1969). These results show clearly that there are factors present in invertebrate serum which facilitate the uptake of particles by phagocytic cells from these animals.

In some cases the presence of opsonins with different specificities is indicated. For instance, adsorption of *H. aspersa* serum with yeast cells removed the opsonic activity for yeast cells but did not affect the opsonic activity for sheep erythrocytes, and vice versa (Prowse and Tait, 1969). Similarly, adsorption of *C. destructor* haemolymph with sheep erythrocytes removed most of the opsonic activity for sheep erythrocytes but only a small portion of the activity against human erythrocytes, and vice versa (McKay and Jenkin, 1970).

Quite high levels of rossette formation and/or phagocytosis of erythrocytes from various vertebrate species by washed monolayers of haemocytes from the cockroach

*Periplaneta americana* (Scott, 1971; Rowley and Ratcliffe, 1980), the cockroach *Blaberus cranifer* (Anderson, Holmes and Good, 1973), the wax moth *Galleria mellonella* (Rabinovitch and De Stefano, 1970), the silk worm *Bombyx mori* (Wago and Ichikawa, 1979) and the stick insect *Clitumnus extradentatus* (Rowley and Ratcliffe, 1980) occurred without pretreatment of the erythrocytes with haemolymph and pretreatment had no enhancing effect.

A possible explanation for the apparent lack of requirement for haemolymph factor(s) in these cases is suggested by the work of Tyson and Jenkin, (1974). They found that phagocytosis of bacteria by crayfish (*C. destructor*) haemocytes *in vitro* did not require the bacteria being pretreated with haemolymph. However, phagocytosis of untreated bacteria was abolished by pretreating the haemocyte monolayers with trypsin. Normal levels of phagocytosis were restored to the trypsin treated monolayers by pretreating either the bacteria or the haemocytes with normal haemolymph. Thus, it seems that the crayfish opsonins may be cell bound as well as free in the haemolymph. The amount of cell bound opsonins present was apparently insufficient to facilitate the phagocytosis of the much larger erythrocyte particles, which required pretreatment with haemolymph (McKay and Jenkin, 1970). This interpretation is supported by the finding that more antibody is required to promote the phagocytosis of erythrocytes by vertebrate macrophages than is required for bacteria (Miescher, Spielgelbert and Banacerraf, 1963). It is possible, therefore, that in those cases where haemolymph had no effect in enhancing phagocytosis there was enough cell bound opsonin present to facilitate phagocytosis.

If an object is too large to be phagocytosed, say greater than 10 μm in diameter, it is usually surrounded by cells which adhere to it and to each other thus forming a capsule about the object (Lackie, 1980). This is what usually happens when the miracidia of the trematode parasite *Schistosoma mansoni* penetrate the tissues of most species of snails. However, in some strains of the snail *Biophalaria glabrata* there is very little cellular reaction against *S. mansoni* and the parasite develops normally. In other *B. glabrata* strains *S. mansoni* is destroyed. For instance, the Puerto Rican 1 strain of *S. mansoni* grows normally in the Puerto Rican albino (PR alb) strain of *B. glabrata* but is encapsulated in *B. glabrata* strain 10R2 (Bayne, Buckley and DeWan, 1980a). The basis for this host-parasite specificity has been investigated using an *in vitro* model of encapsulation which involves adding together in agarose snail haemocytes and *S. mansoni* sporocysts (Bayne *et al.*, 1980a; Bayne *et al.*, 1980b). When 10R2 haemolymph (cells plus plasma) was used the haemocytes became closely associated with the sporocysts. By 24 hours major degenerative changes had occurred to the sporocysts and the haemocytes were seen to be actively phagocytic. This was in marked contrast to the results obtained when PR alb haemolymph was used. Even though haemocytes initially became associated with the sporocysts, by 24 hours they had withdrawn from the teguement surface and the sporocysts appeared morphologically to be unharmed. Thus the *in vitro* model showed the same specificity as seen *in vivo*.

The 10R2 haemocytes retained their cytotoxic activity even in the presence of PR alb serum or culture medium alone.

When PR alb haemocytes were mixed with 10R2 plasma they became as equally cytotoxic to the sporocysts as 10R2 haemocytes. 10R2 plasma alone had no effect on the sporocysts. These results indicated that *B. glabrata* haemocyte reactions against *S. mansoni* sporocysts are mediated by a factor (i.e. an opsonin) which is present in 10R2 plasma but is absent in PR alb plasma. This factor is probably cytophilic since 10R2 haemocytes retained their activity in the absence of 10R2 plasma.

## 1.4 Clearance of proteins from the circulation of invertebrates

Radiolabelled proteins such as bovine serum albumin and bovine gamma globulin are cleared from the circulation of the crayfish *Procambarus clarkii* or the crab *Callinectes sapidus* within a few hours (McCumber and Clem, 1977; Sloan, Yocum and Clem, 1975). However, proteins that are self components such as the animal's own haemocyanin are eliminated very slowly. Injection of a large excess of unlabelled protein identical to the radiolabelled protein slowed its clearance considerably, but unlabelled unrelated protein had no effect. For instance, clearance of radiolabelled bovine serum albumin from the circulation of the crayfish was inhibited by unlabelled bovine serum albumin but not by bovine gamma globulin, and vice versa. Similar types of results were obtained for the chiton *Liolophura gaimardi* (Crichton and Lafferty, 1975; Crichton, Killby and Lafferty, 1973). It appears, therefore, that recognition and clearance of non-self components in these animals is mediated by naturally occurring receptors or recognition

molecules (opsonins) which possess some degree of specificity.

## 1.5 Nature of the opsonins promoting phagocytosis in the invertebrates

Found extensively among the invertebrates are factors which agglutinate erythrocytes (review, Yeaton, 1981). Some of these haemagglutinins are active against erythrocytes from a number of different species whilst others are specific for only one or two (review, Gold and Balding, 1975). In some instances the haemagglutinins have been shown to bind specifically to carbohydrate moities. The function of these molecules is unknown. As outlined in the last section, many groups of investigators have shown the presence of opsonins in the invertebrates, but unfortunately few have attempted to investigate fully the nature of these molecules. However, from the data accumulated to date (discussed below) it appears likely that the opsonins which promote the phagocytosis of erythrocytes may be identical to some of these haemagglutinins.

The hypothesis that the opsonins for foreign erythrocytes are the haemagglutinins is based largely on evidence derived from the observation that the presence of opsonic and haemagglutinating activities appear to be always correlated. For instance, adsorption of the haemolymph from the crayfish *C. destructor* with sheep erythrocytes removed all the haemagglutinating activity against these cells as well as the opsonic activity, but only some of the haemagglutinating activity and opsonic activity for human erythrocytes, and vice versa (McKay and Jenkin, 1970). In addition, both the opsonic and haemagglutinating activities were inhibited

by EDTA and restored by the addition of $Ca^{++}$ (Tyson, 1974). When the haemolymph was chromatographed on Biogel P-150 the opsonic and haemagglutinating activities eluted in the same fractions indicating that the opsonins and the haemagglutinins were of similar sizes (Tyson, 1974). This data provides good evidence that the opsonin(s) for sheep erythrocytes and the haemagglutinin(s) against these cells are identical.

The haemolymph of the snail *Helix pomatia* contains a haemagglutinin that can only be detected by using human type A erythrocytes that have been treated with pronase or neuraminidase. This haemagglutinating activity is inhibited best by the sugars N-acetyl-glucosamine and N-acetyl-galactosamine (Renwrantz, 1979). The opsonin present in *H. pomatia* haemolymph (section 1.3; Renwrantz and Mohr, 1978) is also inhibited by N-acetyl-glucosamine as shown by the fact that the rate of clearance of human type A erythrocytes injected as a suspension in 0.2 M N-acetyl-glucosamine is considerably reduced from the normal rate (Harm and Renwrantz, 1980). L-fucose, a sugar which had no effect on haemagglutinating activity, also had no effect on the rate of clearance of human erythrocytes. This finding that the opsonic and haemagglutinating activities in *H. pomatia* haemolymph have the same sugar specificities strongly supports the suggestion that the opsonin(s) for human blood group A cells are identical to the haemagglutinin(s) for these same cells.

A human type A erythrocyte specific haemagglutinin is present in the albumin gland of *H. pomatia*. This haemagglutinin has been purified to homogeneity (Hammarström and

Kabat, 1969; Harm and Renwrantz, 1980). The low rate of clearance of human type A erythrocytes from the circulation of *H. pomatia* snails which had previously cleared a dose of the same erythrocytes (section 1.3) was restored to normal following pretreatment of the erythrocytes with a sub-agglutinating dose of the purified *H. pomatia* haemagglutinin from the albumin gland, in exactly the same manner as achieved by pretreatment of the erythrocytes with haemolymph (Harm and Renwrantz, 1980). This result shows conclusively that a haemagglutinin can behave as an opsonin. It is possible, since *H. pomatia* has an open circulatory system, that the albumin gland releases haemagglutinin into the haemolymph, but whether haemolymph and albumin gland haemagglutinins are the same is unknown. The need to use enzyme treated erythrocytes to detect the haemagglutinins in the haemolymph may indicate that this haemagglutinin recognises different antigenic sites to the albumin gland haemagglutinin and that, therefore, these haemagglutinins are different. However, the fact that both haemagglutinins are specific for type A erythrocytes and are both preferentially inhibited by the same sugars, N-acetyl-glucosamine and N-acetyl-galactosamine, (Hammarström and Kabat, 1969; Renwrantz, 1979) suggests that they may be related. It is possible that enzyme treatment is merely necessary to increase the agglutinability (e.g., by reducing the charge of the cells) of the erythrocytes (Lis and Sharon, 1977) and so allow the low levels of albumin gland haemagglutinin in the haemolymph to be detected.

Hall and Rowlands (1974b) have purified a haemagglutinin present in the haemolymph of the crayfish *Homarus americanus*

active against human erythrocytes and have investigated the opsonic activity of this haemagglutinin *in vitro* using haemocyte monolayers. Although no data are given, the authors comment that the phagocytosis of human erythrocytes was observed only if the purified haemagglutinin was included in the culture medium. Paterson and Stewart (1974) demonstrated the presence of an opsonin for sheep erythrocytes in the haemolymph of *H. americanus* but did not investigate its relationship to the sheep erythrocyte haemagglutinin. The haemagglutinin purified by Hall and Rowlands appears to be responsible for the haemagglutinating activity for sheep erythrocytes in haemolymph and so it seems probable that this haemagglutinin is also opsonic for sheep erythrocytes.

A summary of the occurence and nature of invertebrate opsonins is shown in Table 1.1. It is clear that many invertebrates do possess opsonins. However, while the evidence supporting the hypothesis that the opsonins for erythrocytes are haemagglutinins is quite good it should be noted that most of this evidence is circumstantial. In two cases purified haemagglutinins have been shown to be opsonic but these were not shown conclusively to be responsible for the opsonic activity found in the haemolymph. The most direct evidence linking opsonins and haemagglutinins is that of Renwrantz and workers who have shown that these molecules appear to have the same sugar binding specificities.

## 1.6 Physicochemical properties of invertebrate haemagglutinins

In view of the possibility that haemagglutinins may

TABLE 1.1

Summary of the occurence and nature of invertebrate opsinic factors

| Species | Type of expt. indicating presence of opsonins* | Test particles | Number of specificities | Nature of evidence linking opsonins and agglutinins |
|---|---|---|---|---|
| ARTHROPODS | | | | |
| Maia squinado[1] | in vivo; in vitro | bacteria | - | adsorption expts |
| Homarus americanus[2] | in vitro | erythrocyes | - | purified agglutinin |
| Cherax destructor[3] | in vivo; in vitro | bacteria; erythrocytes | ≥2 | adsorption expts; same physicochemical properties |
| Procambarus clarkii[4] | in vivo | soluble protein | ≥3 | - |
| MOLLUSCS | | | | |
| Callinectes sapidus[5] | in vivo | soluble protein | ≥2 | - |
| Aplysia californica[6] | in vivo; in vitro | bacteria | - | correlation between presence of agglutinin and in vivo clearance |
| Crassostrea virginica[7] | in vitro | erythrocyte | - | - |
| Otala lactea[8] | in vitro | yeast | - | - |
| Mercenaria mercenaria[9] | in vitro | bacteria | - | adsorption expts; correlation between presence of agglutinin and opsonic activity |
| Eledone cirrosa[10] | in vitro | bacteria; yeast; erythrocytes | - | - |
| Helix aspersa[11] | in vitro | erythrocytes; yeast | ≥2 | - |
| Helix pomatia[12] | in vivo | erythrocytes | - | inhibited by the same sugars |
| Lymnaea stagnalis[13] | in vitro | erythrocytes yeast | - | - |
| Biophalaria glabrata[14] | in vitro | trematode parasite | - | - |
| Liolophura gaimardi[15] | in vivo | soluble protein | ≥2 | - |
| ECHINODERMS | | | | |
| Strongylocentrotus purpuratus[16] | in vivo; in vitro | soluble protein | ≥2 | - |

* In vitro experiments usually employed cell monolayers to assess the affect of haemolymph on phagocytosis. In vivo experiments involved measuring the clearance of introduced foriegn material from the circulation.

references: 1, Cantacuzene, 1923; 2, Patterson and Stewart, 1974; 3,Tyson and Jenkin, 1973; McKay and Jenkin, 1970; Tyson,1974; 4, Sloan et al., 1975; 5, McCumber and Clem, 1977; 6, Pauley et al., 1971; 7, Tripp, 1966; 8, Anderson and Good, 1976; 9, Arimoto and Tripp, 1977; 10, Stuart, 1968; 11, Prowse and Tait, 1969; 12, Renwrantz et al., 1981; 13, Sminia et al., 1979; 14, Bayne et al., 1980a, 1980b; 15, Crichton et al., 1973; Crichton and Lafferty, 1975; 16, Hilgard and Phillips, 1968; Hilgard et al., 1967; Hilgard et al., 1974

function as opsonins it seems pertinent to consider the physicochemical properties of these molecules. A comprehensive review of the available data on this subject has been presented by Ey and Jenkin (1982). A summary of this data is presented in Tables 1.2, 1.3, and 1.4. Generally, the haemagglutinins are multisubunit proteins many of which require divalent cations, usually $Ca^{++}$, for activity and have binding specificities for carbohydrate. This review is restricted mainly to discussing the physicochemical properties of haemagglutinins from invertebrates which appear to function as opsonins for erythrocytes.

### 1.6.1 Arthropods

The haemagglutinins of the American lobster, *Homarus americanus*, have been purified by a combination of such techniques as gel permeation chromatography, pevikon block electrophoresis, preparative polyacrylamide electrophoresis, sucrose density gradient ultracentrifugation (Hall and Rowlands, 1974a) and affinity chromatography (Hartman, Campbell and Abel, 1978). By these methods two molecules, designated as LAg-1 and LAg-2, have been identified. LAg-2 has a sedimentation coefficient of approximately 11S while LAg-1's is greater than 19S. The haemagglutinating activity of both molecules was $Ca^{++}$ dependent and could be reversibly inactivated by EDTA. Trypsin treatment and reduction and alkylation abolished activity. Periodic acid-Schiff staining in polyacrylamide gels indicated that both molecules were glycoproteins. Both haemagglutinins were composed of non-covalently linked subunits possessing a mol. wt. of 55,000. Ouchterlony and immunoelectrophoresis tests using

TABLE 1.2

Properties of agglutinins found in molluscs

| Species | Source | Probable* number | Sensitivity to§ | | | Molecular weight ($10^3$) | | Carbohydrate content | Divalent cation requirement | Binding specificity |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | heat | reduction | proteases | native | subunit | | | |
| Helix pomatia | albumin gland | 1 | L | L | L | 73-79 | 13 | ≃7% | none ? | α-D-GalNAc |
| | haemolymph | - | - | - | - | - | - | - | - | GalNAc, GluNAc |
| Helix aspersa | albumin gland | - | - | (L) | - | ≃90 | - | ≃5% | - | GalNAc GluNAc |
| Helix hortensis | albumin gland | 2 | - | - | - | - | - | - | - | GalNAc<br>AcNeu |
| Otala lactea | albumin gland | 2 | - | L# | - | 42# | - | - | - | GalNAc<br>L-Fucose |
| Cepaea nemoralis | albumin gland | 2 | - | L# | - | 73# | - | - | - | α-D-GalNAc<br>AcNeu |
| Caucasotachea atrolabiata | albumin gland | 2 | - | L# | - | 77# | - | - | | α-D-GalNAc<br>α or β-D-GalNAc |
| Arianta arbustorum | albumin gland | - | L | L | - | - | - | - | - | α-(1→6)-Gal-Gal-Gal |
| Bradybaena fruticum | albumin gland | - | - | L | - | ≃100 | - | - | - | GalNAc |
| Euhadra callizona amaliae | albumin gland | - | - | - | - | 89 | - | 5.1% | - | GalNAc=GluNAc |
| Euhadra peliomphala | albumin gland | - | - | - | R | - | - | - | - | GalNAc=GluNAc |
| Achatina granulata | body extract | - | - | - | - | ≃200 | - | + | - | AcNeu |
| Arion empiricorum | mucous | - | - | - | - | ≃43 | - | - | - | Complex |
| Biomphalaria glabrata | albumin gland | - | - | - | - | ≃55 | - | - | $Ca^{++}$ | $GalNAc^{+}$ |
| Aplysia californica | haemolymph | 1 | L | L | R | >400 | - | - | none | - |
| Crassostrea virginica | haemolymph | >1 | L | - | - | - | 20 | 9-13% | $Ca^{++}$ | ¶ |
| Mercenaria mercenaria | haemolymph | 1 | L | - | L | - | 21 | - | $Ca^{++}$ | GalNAc, GluNAc, GluN,D-Fucose |
| Saxidomus giganteus | extract | - | L | - | - | - | - | - | - | GalNAc, GluNAc |
| Tridacna maxima | haemolymph | - | - | - | - | 470 | 10, 20, 40 | ≥7% | $Ca^{++}$ | β-D-GalNAc |
| Velesunio ambiguus | haemolymph | >1 | L | R | L | - | - | - | - | - |
| Octopus vulgaris | haemolymph | - | L | L | R | - | - | - | - | Lactose |

* Estimated mainly from adsorption experiments
§ L = labile; R = resistant; - = unknown
# Refers to human erythrocyte type A specific haemagglutinin
¶ Variable since there are a number of agglutinins
+ Agglutinin derived from PR albino strain

TABLE 1.3

Properties of agglutinins found in arthropods*

| Species | Probable§ number | Sensitivity to¶ | | | Molecular weight | | Divalent cation requirement | Binding specificity |
|---|---|---|---|---|---|---|---|---|
| | | heat | reduction | protease(s) | native | subunit | | |
| *Homarus americanus* Ag-1 | 1 | L | L | L | - | 55,000 | $Ca^{++}$ | AcNeu |
| *Homarus americanus* Ag-2 | 1 | L | L | L | - | 55,000 | $Ca^{++}$ | GalNAc |
| *Panularis argus* | - | L | - | - | 400,000 | 68,000 | $Ca^{++}$ | - |
| *Procambarus clarkii* | ≥2 | L | - | R | - | - | - | - |
| *Cherax destructor* | ≥2 | L | L | L | 81,000 | 13,500 | $Ca^{++}$ | - |
| *Callinectes sapidus* | - | L | - | R | - | - | None | - |
| *Andioctonus australis* | - | L | - | - | - | - | - | N-acetylamino-sugars |
| *Limulus polyphemus* | - | L | L | L | 400,000 | 22,500 | $Ca^{++}$ | AcNeu |
| *Tachypleus tridentatus* | ≥4 | L | - | - | - | - | $Ca^{++}$ | N-acetylamino-sugars |

* Adapted from Ey and Jenkins (1982)
§ Estimated mainly from adsorption experiments
¶ L = labile; R = resistant; - = not known

TABLE 1.4

Properties of agglutinins found in echinoderms*

| Species | Probable§ number | Sensitivity to¶ | | | | | | Molecular weight | | Divalent cation requirement | Binding specificity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | heat | reduction | periodate | pepsin | trypsin | bromelain | native | subunit | | |
| Asterias forbesi | >1 | L | - | - | - | - | - | 120,000-150,000 | 30,000 13,000 | $Ca^{++}$ | - |
| Anthocidaris crassispina | 1 | L | L | L | - | R | L | 200,000 | - | $Ca^{++}$ | $NI^{+}$ |
| Pseudocentrotus depressus | ≥2 | L | L | L | - | R | L | 200,000 | - | $Ca^{++}$ | Gal, etc£ |
| Hemicentrotus pulcherrimus | ≥3 | R | L | R | - | R | R | 200,000 | - | $Ca^{++}$ | $NI^{+}$ |
| Halothuria polli | >1 | L | L | - | L | - | - | - | - | None | NI# |
| Halothuria tubulosa | >1 | - | - | - | - | - | - | - | - | None | NI# |

* Adapted from Ey and Jenkin, (1982)
§ Estimated from adsorption experiments
¶ L = labile; R = resistant; - = not known
\+ Not inhibited by 50 mM galactose lactose, melibiose, raffinose, glucose. Inhibited by human siliva
\# Not inhibited by 0.2 M glucose, galactose, mannose, L-fucose, L-arabinose, D-xylose, N-acetylglucose, N-acetylgalactose, fructose , 2-sorbose, N-acetylneuraminic acid
£ lactose, L-glucose, melibiose, raffinose

antiserum raised against the purified agglutinins indicated that LAg-1 and LAg-2 were not antigenically related.

The LAg-1 and LAg-2 molecules also have different binding specificities as shown by the following studies (Hall and Rowlands, 1974b). LAg-1 agglutinated human, horse, hamster, chicken, sheep, and mouse erythrocytes while LAg-2 agglutinated only mouse, horse, and hamster erythrocytes. Adsorption of haemolymph with human erythrocytes did not affect activity to mouse erythrocytes. Using mouse erythrocytes, agglutination by LAg-2 was inhibited best by N-acetyl-galactosamine while LAg-1 was not inhibited by any of the sugars tested. Using human erythrocytes, agglutination by LAg-1 was inhibited best by N-acetyl-neuraminic acid while N-acetyl-mannosamine was nearly as active. All haemolymph haemagglutinating activity could be adsorbed to submaxillary mucine coupled to Sepharose (Hartman *et al.*, 1978). LAg-2 was eluted from the mucin with N-acetyl-galactosamine while LAg-1 was eluted with N-acetyl-mannosamine.

While it is clear that LAg-1 and LAg-2 are different molecules with different binding specificities, there is some confusion concerning the number of different binding specificities of LAg-1 (Hall and Rowlands, 1974b). While LAg-1 will agglutinate both mouse and human erythrocytes, only the agglutination of human erythrocytes was inhibited by N-acetyl-neuraminic acid. There are two possibilities which are compatible with this result. Either the LAg-1 molecules have two different binding sites, or there is only one binding site which has, 1) a higher affinity for mouse cells than for human cells and/ or 2) there are more receptor sites on mouse

cells than on human cells (see appendix, Chapter 4).

As already mentioned, purified LAg-1 is active as an opsonin for human erythrocytes (Hall and Rowlands, 1974b; section 1.5).

In a similar study Tyson (1974) showed that the haemagglutinins and opsonins of the crayfish *C. destructor* were both $Ca^{++}$ dependent molecules. Preliminary data indicated that the native haemagglutinin molecule, with a molecular weight of 81,000, was a hexamer composed of sub-units of mol. wt. 13,500 (Tyson, 1974). Adsorption experiments with erythrocytes indicated that this population of molecules possessed several different binding specificities (McKay, Jenkin and Rowley, 1969; McKay and Jenkin, 1970).

### 1.6.2 Molluscs

The only purified haemagglutinin so far shown to possess opsonic activity in its homologous host is the blood group A specific haemagglutinin isolated from the albumin gland of the snail *H. pomatia*. This haemagglutinin has been purified by several independent groups using techniques such as ion exchange chromatography (Knobloch, Knobloch, Vogt, Schnitzler and Böttger, 1970) and affinity chromatography (Hammarström and Kabat, 1969; Kühnemund and Köhler, 1975). The purity of these preparations was indicated by their homogeneous behaviour during electrophoresis under alkaline and acidic conditions, ultracentrifugation, and immunoelectrophoresis although isoelectric focussing has revealed up to nine components (Kühnemund and Köhler, 1975). The mol. wt. of the haemagglutinin estimated by sedimentation equilibrium varied between 79,000 ± 4,000 (Hammarström, Westöö and

Björk, 1972) and 72,400 ± 2,000 (Kühnemund, Stassburger and Triebel, 1974). By sedimentation velocity the mol. wt. appeared to be 73,000 ± 4,400 (Kühnemund *et al.*, 1974). A molar frictional ratio of 1.23 (Kühnemund *et al.*, 1974) indicated that the molecule was essentially globular and this was confirmed by electronmicrographs in which the molecule appeared slightly eliptical (Schnitzler, Uerlings and David, 1971). The haemagglutinin contains a significant amount of β structure (Hammarström, 1974).

When subjected simultaneously to denaturing and reducing conditions, the haemagglutinin dissociated into a single component of mol. wt. 13,000 (Hammarström *et al.*, 1972). Equilibrium dialysis using a tritium labelled, blood group A active pentasaccharide showed that the haemagglutinin possessed six homogeneous, non-interacting carbohydrate binding sites (Hammarström and Kabat, 1971). These results suggested that the haemagglutinin is composed of six identical subunits. If the subunits were identical as suggested, the lysine and arginine contents indicated that 12 peptides should be generated by digestion with trypsin. Tryptic digests revealed the presence of thirteen to fourteen peptides, thereby confirming that the subunits were identical.

Using $^{14}C$ iodoacetate to assess the number of free sulphydryl groups before and after reduction, Hammarström *et al.*, (1972) showed that the haemagglutinin contained 18 half-cystines, all present as disulphide bonds. As they are identical, each subunit must therefore have three half-cystines involved in disulphide bonds. When the haemagglutinin was reduced under denaturing conditions in the presence of

0.1 M N-acetyl-galactose a single component of approximate mol. wt. 13,000 resulted, but only 3-4 disulphide bonds were cleaved indicating that only one of a subunit's half cystines is involved in interchain bonding. Under denaturing conditions alone the haemagglutinin dissociated into a single component of mol. wt. 26,000 - 30,000, i.e., equal to a dimer of the subunit.

In summary, these results showed that the *H. pomatia* haemagglutinin is composed of six identical subunits, each containing one interchain disulphide bond and a single carbohydrate binding site. Thus, the native haemagglutinin is composed of 3 dimers non-covalently held together, the dimers consisting of two subunits held together by one interchain disulphide bond.

The agglutinating activity of *H. pomatia* haemagglutinin was reversibly inhibited by treatment with reducing agents in the absence of denaturing conditions. However, the agglutinin still bound to erythrocytes (Uhlenbruck, Kim and Prokop, 1967). The above treatment caused the haemagglutinin to dissociate into components of mol. wt. 30,000 which still retained their sugar-binding sites (Uhlenbruck, Pardoe, Prokop and Ishiyama, 1972). It appears, therefore, that reduction in non-denaturing conditions only affects the interchain disulphide bonds and allows the haemagglutinin to dissociate into non-covalently linked dimers. This means that there must be very little non-covalent bonding between the disulphide-linked subunits.

The specificity of *H. pomatia* haemagglutinin has been investigated by testing its ability to agglutinate various erythrocytes, to precipitate various polysaccharides and

glycoproteins and by testing the capacity of various sugars to inhibit these reactions. The results of these studies indicated that the binding activity was mainly directed against terminal α-D-linked N-acetyl-galactose groups. β linked N-acetyl-galactose was not bound. Experiments utilising human blood group type A substance oligosaccharides as inhibitors showed that the combining site accommodates a single α-D-linked glycosyl unit (Hammarström and Kabat, 1969).

The *H. pomatia* haemagglutinin is a glycoprotein containing 7-13% carbohydrate (Vogt, Oehme, Knobloch and Schnitzler, 1969; Hammarström and Kabat, 1969; Salfner, Ishiyama and Uhlenbruck, 1971). Agglutination did not seem to require divalent cations since dialysis of the haemagglutinin or addition of $Ca^{++}$ had no effect on activity, but this has not been tested by exposing the haemagglutinin to chelating agents.

The haemagglutinin of another mollusc, *Helix aspersa*, appears to be confined to the albumin gland and egg mass of the snail. It is mainly specific for human A erythrocytes although there is some activity towards human blood group O and B cells and sheep and guinea pig erythrocytes (Gold and Thompson, 1969; Grace, 1969; Khalap, Thompson and Gold, 1971). This haemagglutinin, like that isolated from *Helix pomatia*, was inhibited by N-acetyl-galactose and N-acetyl-glucose (Uhlenbruck and Weis, 1973; Ishiyama, Dietz and Uhlenbruck, 1973). In addition, a strong antigenic cross-reaction was observed between *H. pomatia* and *H. aspersa* haemagglutinins. However, there is a difference in the amino acid composition of the two agglutinins (Hammarström

and Kabat, 1969; Salfner, Ishiyama and Uhlenbruck, 1972) and the mol. wt. of the *H. aspersa* haemagglutinins is reported to be approximately 90,000 (Uhlenbruck, Pardoe, Prokop and Ishiyama, 1972) (cf. 74,000 for *H. pomatia*).

In the snail, *Otala lactea*, two haemagglutinins have been detected in whole body and albumin gland extracts (Boyd and Brown, 1965; Boyd, Brown and Boyd, 1966; Bhatia, Boyd and Brown, 1967). One was a strong human blood group A specific haemagglutinin and was inhibited by N-acetyl-galactose while the other was active against papain or ficin treated human O erythrocytes and was inhibited by L-fucose. The A-specific agglutinin was purified and shown from chromatography and ultracentrifugation data to have a mol. wt. of approximately 42,000 (Bhatia *et al.*, 1967; Bhatia, Kim and Boyd, 1968). Treatment with 2-mercaptoethanol (a reducing agent) almost totally abolished the activity of the haemagglutinin.

The agglutinin from the sea-hare *Aplysia californica* has not been well characterized (Pauley, Granger and Krassner, 1971; Pauley, 1974). It showed a broad spectrum of haemagglutinating activity but adsorption experiments with bacteria and erythrocytes indicated that a single molecule was probably responsible. The agglutinin did not require divalent cations as it was fully active in the presence of sodium citrate. It was resistant to trypsin, pronase and several other hydrolytic enzymes but was inactivated by heat ($>70^{o}$) and partially inactivated by 2-mercaptoethanol. It appeared to be a large molecule since it was excluded by Sephadex G-200 (mol. wt. > 400,000) and had a sedimentation coefficient of approximately 18S as estimated from sucrose

density gradient ultracentrifugation data.

Haemolymph from the oyster *Crassostrea virginica* contained a number of different agglutinins as shown by adsorption experiments, sugar inhibition studies and by the differing requirements for the presence of $Ca^{++}$ for the agglutination of different erythrocyte types. Low haem-agglutinating titres (1/4 - 1/16) were observed using mouse, chicken, guinea pig and bovine erythrocytes whereas high titres (1/64 - 1/512) were observed using human, sheep, rabbit and horse erythrocytes (Tripp, 1966, 1974). While adsorption with any cell type removed all the low titre activity only differential adsorption of the high titre activity occurred. For instance, adsorption with human erythrocytes removed all the activity for chicken, guinea pig, bovine (low titre group) and human erythrocytes but only partially removed the activity for sheep, rabbit and horse erythrocytes (high titre group). Furthermore, the agglutination of erythrocytes from different species of animals was inhibited by different sugars (McDade and Tripp, 1967). For example, the agglutination of horse erythrocytes, but not monkey erythrocytes, was inhibited by D-ribose while the agglutination of monkey erythrocytes, but not horse erythrocytes, was inhibited by N-acetyl-D-glucose, showing that the haemagglutinins involved must have different binding specificities. Addition of sodium citrate to oyster haemolymph abolished the haemagglutinating activity for human cells. This activity was fully restored by reconstituting the haemolymph with $Ca^{++}$ ions (McDade and Tripp, 1967). In contrast, the haemagglutinating activity for sheep and rabbit erythrocytes was unaffected by the addition of citrate.

Acton, Bennett, Evans and Schrohenloher (1969) have purified a haemagglutinin for sheep erythrocytes from *C. virginica* haemolymph by chromatography on Sepharose 4B. Analysis by SDS-PAGE revealed a single component with a mol. wt. of 20,000. This appeared to be homogeneous since N terminal amino acid analysis revealed only Thr. This haemagglutinin was a glycoprotein. An apparent contradiction to the data of McDade and Tripp (1967) was found since the haemagglutinating activity of this sheep haemagglutinin was inhibited by citrate. Ultracentrifugation analysis showed that the purified native haemagglutinin consisted of a heterogeneous group of polymeric molecules, the predominant species having a sedimentation coefficient of approximately 30S.

## 1.7 Studies on the protochordates

### 1.7.1 Recognition of foreignness by phagocytes

The protochordates are considered to be survivors of the stem line of evolution giving rise to the chordates (Berrill, 1955). In view of this it might be expected that a study of the mechanisms involved in the recognition of foreign material by phagocytic cells from these animals would lead to a better understanding of the phylogeny of the immune response of vertebrates. However, very little is known about such mechanisms in the protochordates.

The following examples illustrate that protochordate phagocytes do recognise and respond to a variety of foreign matter. Erythrocytes injected into the tunic tissues or the perivisceral cavity of *Ciona intestinalis* became agglutinated

and were then actively phagocytosed by amoebocytes (Wright, 1974). Concomitant with this response was a drop in the serum haemagglutinin titres. While this result is compatible with the hypothesis that the haemagglutinins in *C. intestinalis* are opsonic, much more work needs to be done before this can be said with confidence. Carbon and thorium dioxide particles injected into *C. intestinalis* were also phagocytosed (Perres, 1943; Brown and Davies, 1971).

Monolayers of coelomic cells from *Styela plicata* actively phagocytosed glutaraldehyde fixed rabbit erythrocytes (Fuke and Sugai, 1972), but the level of phagocytosis was the same whether saline or coelomic fluid was used as the culture medium, suggesting that serum opsonins were not involved. It is possible, however, that the phagocytes were saturated with membrane bound opsonins.

Particulate dyes injected into another protochordate *Molgula manhattensis* were quickly cleared by phagocytosis and implanted glass fragments soon encapsulated (Anderson, 1971).

### 1.7.2 Properties of the haemagglutinins from various species of protochordates within the subphylum Urochordata (class Ascidiacea)

The protochordates are made up of three subphyla (Hemichordata, Urochordata and Cephalochordata). However, apart from the work with the lancelot *Amphioxus (Branchiostoma) lanceolatus* which has been reported to contain haemagglutinins for human erythrocytes (Bretting and Renwrantz, 1973; Renwrantz and Uhlenbruck, 1974), all other work has been concerned with the Urochordata.

FIGURE 1.1

Proposed phylogenetic relationships between selected members of the Urochordata (after Berrill, 1950). Genera mentioned in this thesis are shown in brackets

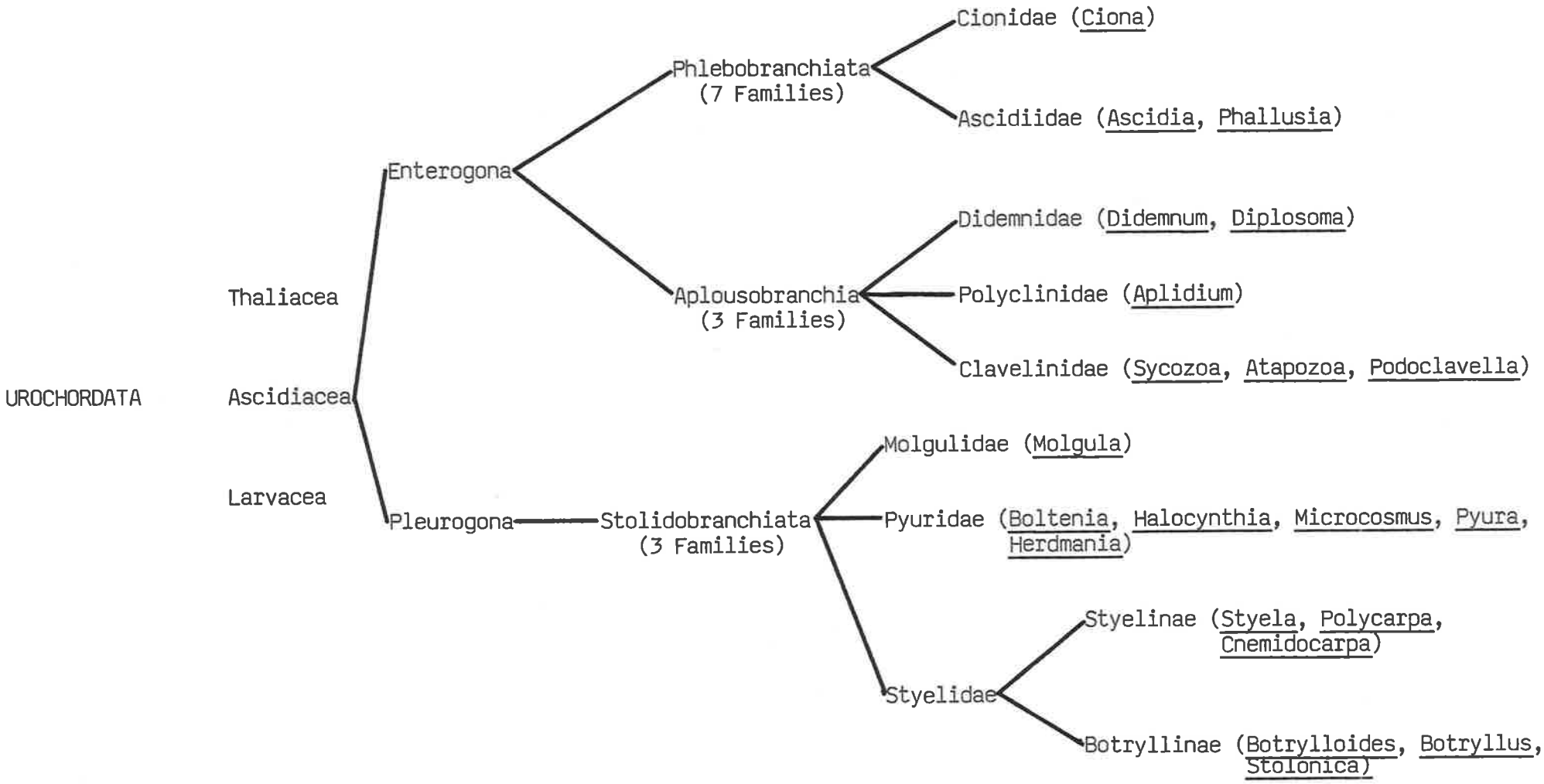

TABLE 1.5

Survey of haemagglutinins found in the haemolymph of various species of ascidians

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¶ The titre in each case is shown in parentheses and was determined using TSA containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ as the diluent. The requirement for divalent cations was determined by using trisodium citrate (0.13 M) as the diluent.

* To assess binding specificity, titrations were performed in diluents containing the following sugars (67 mM final concentration); D-arabinose (A), D-galactose (G), lactose (L), melibiose (M) and sucrose (S). NI indicates that none of these sugars were inhibitory. -- = not done.

+ The titre using the $Ca^{++}$, $Mg^{++}$ diluent was zero whereas it was 1/64 using the citrate diluent.

§ The titre was reduced by half in citrate buffer.

| Classification | Species | Type of Erythrocyte¶ | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sheep | | Mouse | | Guinea pig | |
| | | Divalent cations required? | Binding* specificity | Divalent cations required? | Binding* specificity | Divalent cations required? | Binding* specificity |
| APLOUSOBRANCHIA | | | | | | | |
| Didemnidae | Didemnum patulum | yes(1/16) | NI | no(1/16) | NI | | -- |
| | Diplosoma sp. | no(1/32) | NI | ?(1/64)+ | -- | yes(1/2) | -- |
| Polyclinidae | Aplidium australiensis | no(1/32) | NI | ?(1/128)§ | NI | no(1/128) | L |
| Clavelinidae | Sycozoa tenuicaulis | (0) | -- | (0) | -- | (0) | -- |
| | Atapozoa fantasiana | ?(1/4)§ | -- | ?(1/4)§ | -- | ?(1/4)§ | -- |
| | Podoclavella cylindrica | (0) | -- | (0) | -- | (0) | -- |
| PHLEBOBRANCHIATA | | | | | | | |
| Ascidiidae | Phallusia despressiuscula | no(1/2) | -- | no(1/4) | -- | no(1/2) | -- |
| | Ascidia thompsoni | no(1/2) | -- | (0) | -- | (0) | -- |
| Cionidae | Ciona intestinalis | no(1/2) | -- | (0) | -- | (0) | -- |
| STOLIDOBRANCHIATA | | | | | | | |
| Pyuridae | Microcosmus nichollsi | no(1/8) | L,M | yes(1/4) | -- | (0) | -- |
| | Pyura praeputiculis | no(1/4) | -- | no(1/2) | -- | (0) | -- |
| | Pyura irregularis | (0) | -- | yes(1/32) | D,L,M,S,A | yes(1/2) | -- |
| | Halocynthia hispida | no(1/16) | NI | no(1/8) | -- | yes(1/512) | L,G |
| | Herdmania momus | no(1/2) | -- | no(1/2) | -- | (0) | -- |
| Styelidae | Botrylloides magnicoecus | (0) | -- | (0) | -- | no(1/4) | -- |
| | Botryllus schlosseri | no(1/8) | G,L,M | no(1/8) | G,L,M | no(1/8) | G,L,M |
| | Cnemidocarpa etheridgii | no(1/4) | -- | yes(1/64) | NI | yes(1/128) | NI |
| | Polycarpa pedunculata | (0) | -- | (0) | -- | (0) | -- |
| | Polycarpa obtecta | no(1/2) | -- | no(1/2) | -- | -- | -- |
| | Polycarpa papillata | no(1/4) | -- | ?(1/256)§ | NI | no(1/32) | NI |
| | Stolonica australis | no(1/2) | -- | no(1/2) | -- | no(1/2) | -- |

Furthermore, among the Urochordata only a few species of the class Ascidiacea (or ascidians) have been investigated. The phylogenetic relationship of these species is shown in Figure 1.1.

The haemolymph of a variety of species within the Ascidiacea have recently been tested for the presence of haemagglutinins (Ey and Jenkin, 1982). A summary of the data is shown in Table 1.5 where it can be seen that haemagglutinins occur widely throughout the various species. However, there is considerable variation between species in the titres and properties of the haemagglutinins. Some require divalent cations for haemagglutinating activity while others do not, and sugar inhibition studies indicate that their binding specificities vary considerably. In addition, similar variation in properties is shown between haemagglutinins within the same species. For example, the haemagglutinin active against guinea pig erythrocytes in *Halocynthia hispidia* required divalent cations for activity and was inhibited by lactose and galactose, while the sheep erythrocyte haemagglutinin did not require cations and was not inhibited by these two sugars. This type of data suggests that there are several different types of haemagglutinins present in some species.

### 1.7.2.1 Family Cionidea

Low levels of haemagglutinating activity for a variety of avian and mammalian erythrocytes exist in the haemolymph of the solitary ascidian *Ciona intestinalis* (Uhlenbruck, Reifenberg and Heggen, 1970; Wright, 1974; Wright and Cooper, 1975; Anderson and Good, 1975; Parrinello and Patricolo,

1975). The haemagglutinins have been reported to be non-dialysable and to be destroyed by such treatments as heating to $70^{o}$-$100^{o}$ and by reduction (with 2-mercaptoethanol) and alkylation (Wright and Cooper, 1975; Parrinello and Patricolo, 1975). These results indicate that these haemagglutinins are at least partially composed of protein. Haemagglutinating activity was not affected by periodate treatment nor by incubation for 12 hours at room temperature in the pH range 2-10. There is some disagreement between various investigators regarding the importance of divalent cations for haemagglutinating activity. Wright and Cooper (1975), although they did not present their methodology or data, maintain that $Ca^{++}$ and $Mg^{++}$ ions are required but Parrinello and Patricolo (1975) reported that dialysis of haemolymph against EDTA had no effect on the haemagglutinating activity. No further characterization of these haemagglutinins has been carried out.

1.7.2.2 Family Ascidiidae

The haemolymph of *Phallusia mammillata* contains haemagglutinating activity to erythrocytes from amphibians, fish, mammals, and birds (Parrinello and Patricolo, 1975). Adsorption experiments indicated that this broad spectrum of activity was probably due to a single agglutinin, or at least to a limited number of agglutinins, since adsorption with either rabbit, sheep, or rat erythrocytes removed the activity for all three of these cell types (Parrinello and Patricolo, 1975). Unfortunately, the haemagglutinating activity remaining for erythrocytes from the other species was not ascertained.

The haemagglutinins were inactivated by various treatments such as heating ($75^{o}$-$100^{o}$), reduction (2-mercaptoethanol) and alkylation and by incubation with trypsin at pH 2 but were resistant to pH extremes (2-10) and periodate treatment (Parrinello and Patricolo, 1975). It appears, therefore, that these haemagglutinins are also at least partially composed of protein. Divalent cations were not required for activity since EDTA did not abolish activity.

Very similar results to those obtained for *P. mammillata* were also obtained for *Ascidia malaca* (Parrinello and Patricolo, 1975).

1.7.2.3 Family Pyuridae

High levels of haemagglutinating activity for rabbit and rat erythrocytes were detected in the coelomic fluid of *Halocynthia hilgendorfi* but none were found for sheep, guinea pig, mouse, fish, frog, and snake erythrocytes (Fuke and Sugai, 1972). Adsorption experiments indicated that a single agglutinin was responsible for the observed activities.

Haemagglutinating activities for human, rabbit, sheep, pig, goat, calf, and horse erythrocytes were detected in haemolymph from *Halocynthia pyriformis* (Anderson and Good, 1975). However, adsorption experiments indicated that a single agglutinin was probably responsible. Haemagglutinating activity for pigeon, duck, chicken, goose, turkey and guinea pig erythrocytes was also present but the specificity of this activity was not investigated by adsorption experiments.

A haemagglutinin for sheep erythrocytes has been isolated

from *H. pyriformis* by affinity chromatography on insolubilised bovine submaxillary mucin (abstract, Form, Warr and Marchalonis, 1979). The purified protein was shown, by sucrose density gradient ultracentrifugation to have a mol. wt. of approximately 800,000 and, by SDS PAGE analysis, to be composed of subunits of mol. wt. 20,000. Form *et al.*, (1979) reported that the binding specificity of *H. pyriformis* haemagglutinin was complex since although the agglutinin bound to bovine submaxillary mucin, the monosaccharide components of the mucin did not inhibit this reaction. In contrast, Anderson and Good (1975) found that sialic acid, a component of bovine submaxillary mucin, could inhibit the agglutination of human and horse erythrocytes by *H. pyriformis* haemolymph.

*H. pyriformis* haemagglutinins require $Ca^{++}$ for agglutination activity since treatment of haemolymph with EDTA abolished activity for human and horse erythrocytes (the only types tested) while the addition of $Ca^{++}$ increased the titres against erythrocytes from the other species.

Haemagglutinins have been detected in *H. papillosa*, *Microcosmus sulcatus* (Bretting and Renwrantz, 1973), and *Boltenia ovifera* (Anderson and Good, 1975) but none of these have been characterized.

### 1.7.2.4 Family Styelidae

Rabbit and mouse erythrocytes were agglutinated by coelomic fluid from *Styela plicata* but erythrocytes from fish, frogs, snakes, rats, sheep, and guinea pigs were not (Fuke and Sugai, 1972). The activity for rabbit and mouse erythrocytes was removed by adsorption with either of these

erythrocytes, but not by rat erythrocytes, suggesting that only a single agglutinin was involved. Treatment with EDTA indicated that divalent cations were not required for haemagglutinating activity. The haemagglutinins appeared to be large molecules since they were excluded by Sephadex G-100 during chromatography. The haemagglutinins were very heat stable; heating at 140$^{o}$ for 30 minutes had no effect on the ability of these molecules to agglutinate erythrocytes from various species of animals. Incubating coelmic fluid with trypsin for several hours also had no effect on haemagglutinating activity. However, treatment with periodate, which oxidises 1,2 dihydroxy sugars, did abolish the activity. Based on these results, Fuke and Sugai (1972) suggested that the haemagglutinin was a polysaccharide or a mucosaccharide rather than a protein. Although this could be possible, with the observed agglutinating activity being due to carbohydrate-carbohydrate interactions or to the presence of a lectin on the surface of the erythrocytes, experience with other invertebrate and plant lectins suggest that this is unlikely. The results are also consistent with the haemagglutinin being a glycoprotein. However, until more data is presented the character of this haemagglutinin remains in doubt.

A summary of the properties of agglutinins found in ascidians is shown in Table 1.6.

## 1.8 Aims of this thesis

The work reported in this thesis concerns; a) the determination of the number and specificity of the haemagglutinins present in the haemolymph of the protochordate

TABLE 1.6

Properties of agglutinins found in ascidians*

| Species | Probable§ number | Sensitivity to# | | | | | Molecular weight | | Divalent cation requirement | Binding specificity |
|---|---|---|---|---|---|---|---|---|---|---|
| | | heat | reduction | periodate | trypsin | pepsin | native | subunit | | |
| Ciona intestinalis | - | L | L | R | - | - | - | - | $Ca^{++}$, $Mg^{++}$ None¶ | - |
| Phallusia mammillata | 1 | L | L | R | - | L | - | - | None | - |
| Ascidia malaca | 1 | L | L | R | - | L | - | - | None | - |
| Halocynthia hilgendorfi | 1 | - | - | - | - | - | - | - | - | - |
| Halocynthia pyriformis | 1 | L | - | - | - | - | 800,000 | 20,000 | $Ca^{++}$ | AcNeu |
| Styela plicata | 1 | R | - | L | R | - | excluded by G-200 | - | None | - |

* Adapted from Ey and Jenkin, (1982)
§ Estimated from adsorption experiments.
# L = labile; R = resistant; - = not known
¶ There two contradictory reports in the literature regarding this point. See text.

*Botrylloides leachii*, b) the purification of these haemagglutinins and c) their physicochemical characterization.

In view of the lack of data in this field and in view of the evidence suggesting that invertebrate haemagglutinins may act as opsonins, this work was undertaken as a preliminary step in a study aimed at determining the role, if any, the haemagglutinins of *B. leachii* play in the recognition of foreigness by *B. leachii* phagocytes.

# CHAPTER 2

## Materials and Methods

## 2.1 Protochordates used

Specimens of *Botryllus schlosseri* were collected from sea grass beds off the coast of St. Kilda, South Australia using Scuba. Colonies of *Botrylloides leachii* were collected from jetty piles at Rapid Bay, South Australia also using Scuba. Colonies could only be collected during the winter since colony regression occurred during summer. The animals were bled immediately after collection.

## 2.2 Collection of haemolymph

After removing surface water with a paper towel, the colonies were cut into pieces and the exuding fluid collected in a large glass Petri dish. Gentle squeezing by hand ensured that most of the haemolymph was collected. Usually 500-1000 ml of pooled haemolymph could be collected from 10-20 colonies of *B. leachii*. The haemolymph, which does not clot, was centrifuged (900 g, 5 min.) to remove cells and stored at $-20^{o}$. When required, it was thawed and clarified by centrifuging at 30,000 g for 30 minutes at $4^{o}$ in a Sorvall RC-5 centrifuge using a SS34 rotor (Dupont Instruments, U.S.A.).

## 2.3 Media and Reagents

Only deionised, filtered water (Milli-RO6O; Millipore Corporation, U.S.A.) was used to make the solutions and buffers used in this thesis. The following solutions were prepared and stored at $4^{o}$ before use. *Saline:* 0.9% w/v (0.154 M) dispensed in 500 ml aliquots and autoclaved at $120^{o}$ for 20 minutes. *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$^{o}$ for 20 minutes. *Phosphate buffered saline pH 7.4* (PBS): 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* (TSA): TSA was made by combining two solutions, each prepared separately. Solution one 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. Solution two 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.

Reagents were obtained as follows: Sephadex G-200, Sepharose 6B and Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden); Papain (2X crystallized) (Sigma Chemical Company, pronase (Calbiochem-Behring Corporation); Mouse IgG (Gift of Dr. P.L. Ey, this department); Standard proteins (Sigma Chemical Company and Calbiochem-Behring Corporation); Sodium iodide-125 (IMS:30, The Radiochemical Centre, Amersham, England); 1,4-butanediol diglycidyl ether (ICN Pharmaceuticals Inc., New York); Sodium dodecylsulphate (specially purified for biochemical work, BDH Chemicals Ltd, Product No = 44215); Acrylamide and bis-acrylamide (Eastman Kodak Company); N,N,N$^{1}$,N$^{1}$ tetramethylethylene-diamine (TEMED) and 2-Mercaptoethanol (Sigma Chemical

Company); Bromophenol blue (May and Barker Ltd., England); Coomassie Blue R-250 and G-250 (ICI Australia Ltd). All other reagents were of laboratory or analytical grade.

Except where indicated, sugars were purchased from BDH Chemicals Ltd. Laboratory Grade: D-arabinose, L-arabinose, L-fructose, N-acetyl-D-galactosamine (B grade, Calbiochem.), maltose, D-xylose. Analytical grade: L-fucose (A grade, Calbiochem.), D-galactose, D-glucose, lactose, mannose, melibiose (A grade, Calbiochem.), stachyose and sucrose. The D-fucose and stachyose (Sigma Chem. Co.), were kindly donated by Dr. J. Redmond, Chemistry Department, Macquarie University, New South Wales.

## 2.4 Erythrocytes used in haemagglutination assays

Sheep and human blood were drawn aseptically into Alsever's solution and stored at $4^{o}$ for no longer than 3 weeks. Blood collected by cardiac puncture from guinea pigs, rats and pigeons was also mixed with Alsever's solution but discarded after three days. When required, the erythrocytes were centrifuged at 900 *g* for 5 minutes, washed 3 times in 0.15 M NaCl and stored at $4^{o}$ as a 10% v/v stock suspension for no longer than 3 days. Blood collected from (BALB/c x C57Bl) $F_1$ mice was mixed with 0.013 M trisodium citrate/ 0.168 M NaCl. The erythrocytes were washed in 0.168 M NaCl and used immediately.

## 2.5 Haemagglutination assays

Haemagglutinating activity was usually titrated by mixing two-fold serial dilutions of the sample with an equal

volume (50 µl) of an 0.5% v/v erythrocyte suspension in the wells of a microtitre tray (Linbro Scientific Co., U.S.A., Model lS-FB-96). The diluent was phosphate-buffered saline, pH 7.4 (PBS) or Tris-buffered saline pH 7.5 (TSA). Agglutination was evaluated after the cells had settled at room temperature. Titres were expressed as the greatest dilution of the sample which caused complete agglutination.

For titrating agglutinating activity in adsorption experiments, greater endpoint accuracy was required. Thus, after determining the approximate agglutination titre as above, each sample was retitrated using fractional dilutions around the endpoint dilution. For example, if the titre was found to be 1/a then the actual titre was between 1/a and 1/2a. Therefore, fractional dilutions were obtained by making sample dilutions of 1/a and 1/2a and combining various aliquots (total volume 21 µl) of these solutions together in the wells of a microtitre tray using a 25 µl glass syringe (see Figure 4.5). After adding the erythrocytes (21 µl: 0.5% v/v) and allowing them to settle, the trays were then photographed and the cell diameters measured from enlarged prints. A plot of diameter versus dilution allowed the dilution corresponding to 50% agglutination to be accurately determined. This was chosen as the endpoint.

## 2.6 Inhibition of haemagglutination by sugars

To assess the capacity of a given sugar to inhibit haemagglutination, the agglutinin sample was titrated in the presence of various concentrations of sugar. The sugar solutions were made by dissolving the sugar in water to give

a solution iso-osmotic with saline (see Table 2.1) and then diluting in PBS. Replicate titrations (serial two-fold dilutions; 25 µl) of the sample were made across the rows of a microtitre tray. An equal volume of PBS was then added to each well of the first two rows (duplicate controls), whilst the next two rows received PBS containing a fixed amount of the test sugar. This procedure was repeated using a range of sugar concentrations. Erythrocytes (50 µl, 0.5% v/v) were then added and allowed to settle. The concentration of sugar which reduced the agglutinating titre by 50% was thus determined.

## 2.7 Preparation of erythrocyte membrane vesicles for adsorption experiments and for use in the purification of the haemagglutinins

Sheep, human and mouse erythrocytes were centrifuged, the buffy layer was removed and the cells were re-suspended to 10% v/v in TSA, plus 0.1% sodium azide. Vesicles were prepared by sonicating these suspensions (MSE Ultrasonic Power Unit) for 2 minutes, cooling for 1 minute and finally sonicating for a further 1 minute. Undisrupted cells were removed by centrifuging at 900 g for 5 minutes. The vesicle suspensions were stored at $4^{o}$.

## 2.8 Adsorption of haemagglutinins with vesicles

To 100 µl aliquots of vesicle suspensions in 400 µl conical polyallomer tubes (3-4 x 20-30 mm, Camelec Medical Products, No. MCP 225C) were added 300 µl of purified haemagglutinin(s) with rapid mixing on a vortex mixer. The suspensions were incubated at $37^{o}$ for 1 hour and then

TABLE 2.1

Composition of the isotonic sugar solutions

| Sugar | Molecular weight | Isotonic solution | |
|---|---|---|---|
| | | percent w/v | molarity |
| melibiose | 342.3 | 9.75 | 0.285 |
| D(+)-xylose | 150.4 | 4.1 | 0.273 |
| D-fructose* | 180.16 | 5.05 | 0.280 |
| stachyose | 666.6 | 18 | 0.270 |
| D(+)-mannose | 180.16 | 4.92 | 0.273 |
| mannitol* | 182.18 | 5.07 | 0.278 |
| L(+)-rhamnose | 182.18 | 5.07 | 0.278 |
| D(-)-arabinose | 150.14 | 4.1 | 0.273 |
| L(+)-arabinose | 150.14 | 4.1 | 0.273 |
| L(-)-fucose | 164.2 | 4.5 | 0.274 |
| N-acetyl-D-glucosamine | 222.2 | 6.0 | 0.270 |
| N-acetyl-D-galactosamine | 239.2 | 6.5 | 0.272 |
| methyl-α-D-mannopyranoside | 194.2 | 5.24 | 0.270 |
| glucose | 180.16 | 5.05 | 0.280 |
| lactose* | 360.32 | 9.75 | 0.270 |
| galactose* | 180.16 | 4.92 | 0.273 |
| maltose | 360.32 | 9.75 | 0.270 |
| D-fucose | 164.2 | 4.5 | 0.274 |
| sucrose* | 342.3 | 9.25 | 0.270 |

* The composition (percent w/v) of isotonic solutions of these sugars were obtained from "The Merck Index" (Merck and Co., Inc. U.S.A. Eighth edition). For the other sugars the composition of the isotonic solutions were estimated by assuming that they would have similar molar concentrations as the known solutions.

overnight at 4°. The tubes were centrifuged at 27,000 g for 30 minutes at 4° using a Sorval SS34 rotor (Dupont Instruments, U.S.A.). The supernatants were removed and assayed for haemagglutinating activity.

## 2.9 Sephadex G-200 chromatography of haemolymph: large column

Sephadex G-200 was rehydrated according to the manufacturer's instructions (Pharmacia Gel Filtration Handbook, 1979). The slurry was poured into a glass column measuring 108 cm by 5 cm and allowed to flow under gravity. Extra slurry was continually added until the column was full of packed Sephadex. The column was initially equilibrated at 4° with PBS containing 0.5 mM EDTA and 0.1% azide and later with the same buffer containing 50 mM lactose. Approximately 25 ml of haemolymph was applied to the column. The flow rate was set using an LKB (LKB-Productor AB) peristaltic pump and fractions were collected using an LKB Ultrorac fraction collector. Bovine serum albumin and mouse immunoglobulin $IgG_1$ radio-labelled with iodine-125 were used as markers.

## 2.10 Sephadex G-200 chromatography of haemolymph: small column

This column (glass; 2.5 x 98.5 cm) was to be used for the accurate measurement of Stoke's radii, it was therefore necessary to pack the Sephadex as uniformly as possible (Pharmacia Gel Filtration Handbook, 1979). To do this, an extension was temporarily added to the column so that enough slurry could be added to enable the column to be poured in a

single operation. During packing the flow rate was set at 20 ml/hr. The column was equilibrated at 4° with PBS, containing 50 mM lactose, 100 mM galactose and 8 mM $NaN_3$ (eluant buffer). A zone of Blue Dextran 2000 moved through the bed with a sharply defined, horizontal front indicating that the Sephadex was correctly packed (Pharmacia Handbook: Blue Dextran 2000, 1972). The column was calibrated with Blue Dextran 2000, iodine-125 labelled mouse $IgG_1$, bovine liver catalase, horse spleen ferritin, bovine serum albumin, and horse skeletal muscle myoglobin. Various combinations of these markers were applied to the column in 5 ml of buffer and the column washed at a flow rate of 16 ml/hr, 4.9 ml fractions being collected. The elution positions of the various markers were measured by absorbance at 280 nm or by assaying the radioactivity in the various fractions in a Packard Autogamma Counter.

## 2.11 Sephacryl S-300 chromatography

The procedure used to pack this column (103 cm x 3.7 cm) was similar to that used for the large G-200 column. The eluate buffer was TSA, ph 7.5 containing 0.5 mM EDTA and 0.5 mM EGTA. Five to ten millilitres of material was loaded on to the column and the eluate collected in 6 ml fractions at a flow rate of 18 ml/hr.

## 2.12 Sedimentation velocity ultracentrifugation of haemagglutinin preparations

Gradients were ultracentrifuged at 40,000 r.p.m. at 5° in a Beckman Model L2-65B ultracentrifuge (Beckman Instruments Inc., Palo Alto, California) using an SW41 six place

swinging bucket titanium rotor. Both linear and isokinetic sucrose density gradients were used.

### 2.12.1 Preparation of linear sucrose gradients

Sucrose solutions containing the appropriate amount of sugar were made up in PBS containing 0.1% sodium azide. The gradients were established by utilising a Technicon Model 1 multi-channel pump as shown in Figure 2.1A. A 7.5% w/v sucrose solution was pumped into the mixing vessel which initially contained 23 ml of a 25% w/v sucrose solution. Mixing was achieved by the use of a magnetic stirrer. The sucrose solution was pumped from the mixing chamber into Beckman cellulose nitrate tubes (13.2 ml capacity) and allowed to flow down the sides. Pumping was continued until the mixing vessel was empty and each tube had received 11 ml. The gradients were cooled on ice prior to sample application. Samples (250 µl) were carefully applied to the top of each gradient using a Pasteur pipette.

### 2.12.2 Preparation of isokinetic sucrose gradients

The sedimentation velocity of a particle in an isokinetic gradient is considered to be constant throughout the length of the gradient. To achieve this situation, the increased centrifugal force experienced by the particle as it moves away from the centre of the rotor must be exactly compensated for by an equivalent increase in the opposing forces of viscous drag and buoyancy. It has been calculated (Noll, 1967) that a convex exponential gradient is required to satisfy these conditions. These gradients can be

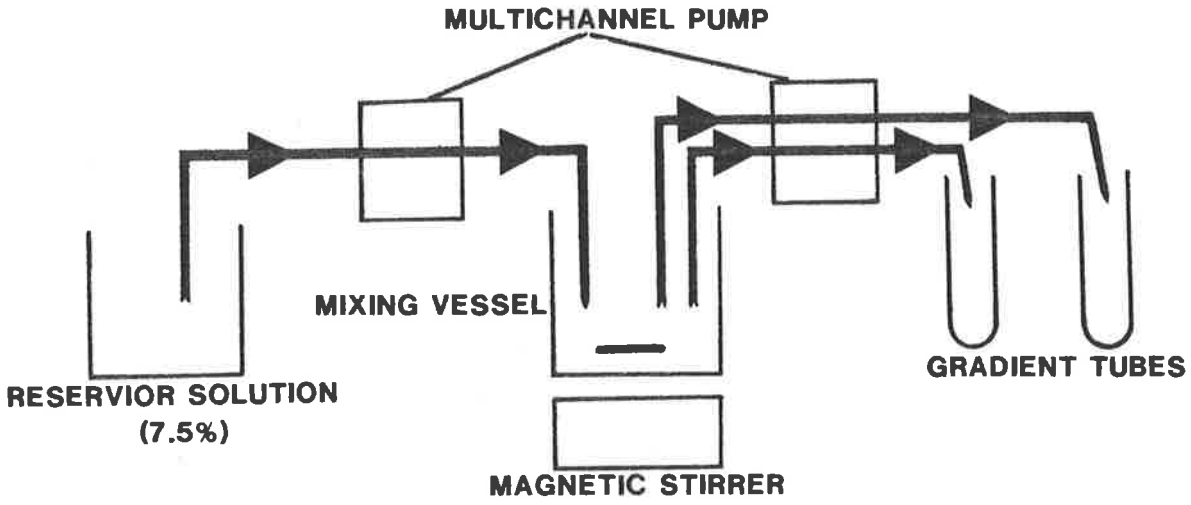
FIGURE 2.1

Diagram showing the arrangement of equipment used to make sucrose density gradients.

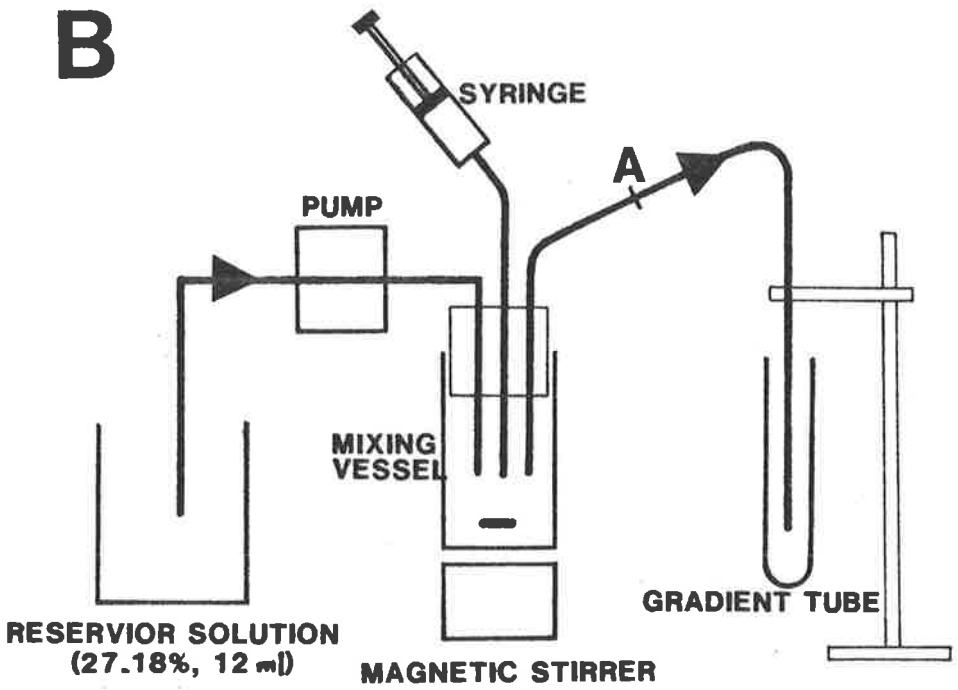
A. Linear sucrose gradients.

B. Isokinetic sucrose gradients.

A


MULTICHANNEL PUMP
MIXING VESSEL
RESERVIOR SOLUTION
(7.5%)
MAGNETIC STIRRER
GRADIENT TUBES

B


SYRINGE
A
PUMP
MIXING
VESSEL
RESERVIOR SOLUTION
(27.18%, 12 ml)
MAGNETIC STIRRER
GRADIENT TUBE

prepared using a constant volume gradient maker.

The particular device used was an adaptation of the gradient maker described by Noll (1967) and is illustrated in Figure 2.1B. The mixing vessel was a 20 ml McCartney bottle made airtight by a rubber stopper. The sucrose solution was delivered to the cellulose nitrate gradient by a piece of thin metal tubing extending completely to the bottom of the tube. This allowed the less dense solution introduced first to float to the top as solutions of increasing density were pumped in. The interconnecting tubing was made of silicon rubber and the pump used was an LKB Varioperpex peristaltic pump. Weighed amounts of sucrose were dissolved in half the final volume of 20 mM phosphate buffered 0.3 M NaCl (pH 7.2) containing 0.1% sodium azide and diluted with water to the final volume. This ensured the same salt concentration in all parts of the gradient (Johns and Stanworth, 1976).

To make the gradient, a volume (Vm, see next section) of 5% w/v sucrose was added to the mixing vessel. The air pressure in the mixing chamber was increased with the syringe until the solution had risen to an arbitarily chosen point A (Fig. 2.1B). Twelve millilitres of sucrose solution of concentration CR (see next section) was then pumped into the mixing chamber forcing an equal volume of sucrose solution out of the mixing chamber and into the gradient tube. The constants Vm and CR, which depend on the type of rotor and centrifugation conditions, were calculated for these experiments to be 9.9 ml and 27.18% w/v respectively.

2.12.2.1 Derivation of Vm and CR

The calculation of these values is described in detail by Johns and Stanworth (1976). Briefly, a particle in a gravitational field moves according to the equation

$$\frac{dr}{dt} = \omega^2 r S_{20,w} \frac{\eta_{20,w}}{Dp - D_{20,w}} \frac{Dp - Dm}{\eta_m}$$

where

r = radial distance of particle (cm); $\omega$ = angular velocity of rotor ($sec^{-1}$); Dp = density of particle (g $cm^{-3}$); Dm = density of medium at r cm from axis (g $cm^{-3}$); $\eta_m$ = viscosity of medium at r cm from axis (centipoise); $D_{20,w}$ = density of water at $20^o$ (g $cm^{-3}$); $\eta_{20,w}$ = viscosity of water at $20^o$ (centipoise); $S_{20,w}$ = sedimentation coefficient in water at $20^o$.

In order to produce an isokinetic gradient (where dr/dt is constant), the factor F must be kept constant throughout the gradient, where

$$F = \frac{r(Dp - Dm)}{\eta_m}$$

Since the initial sucrose concentration (Ct = 5%) was known, this factor was calculated for the top of the gradient, assuming Dp = 1.41 g $cm^{-3}$. The density of the *B. leachii* agglutinins was not known, but because bovine serum albumin was to be used as an internal marker, a Dp value (= 1.41 g $cm^{-3}$) similar to vertebrate plasma proteins was chosen (Johns and Stanworth, 1976). The values of Dm and $\eta_m$ for aqueous sucrose solutions at $5^o$ were obtained from the tables of Anderson (1970). Thus, the concentrations of sucrose required at the mid-point (C½) and bottom (C1)

of the gradient to keep F constant were calculated to the nearest 0.1% by iteration utilising the predetermined value of F and the tables of Anderson (1970). The gradient making parameters Vm and CR were then calculated using the equations

$$CR = \frac{CtCl - (C\frac{1}{2})^2}{Ct + Cl - 2C\frac{1}{2}}$$

and

$$Vm = \frac{Vl}{\ln (CR-Cl)/(CR-Ct)}$$

where Vl = the total volume of the gradient (12 ml).

## 2.13 Calculation of Stoke's radii of the marker proteins

With the exception of ferritin (Siegel and Monty, 1966) the Stoke's radii of the marker proteins were calculated from their diffusion constants (Sober, 1970) using the equation $a = kT/6\Pi MD$ where a = the Stoke's radius; k = the Boltzman constant; T = the absolute temperature ($^{O}K$); M = the viscosity of the medium (centipoise); D = the diffusion coefficient ($cm^2\ sec^{-1} \times 10^{-7}$). The values obtained are shown in Table 2.2.

## 2.14 Preparation of material for affinity chromatography of haemolymph

### 2.14.1 Coupling of lactose to Sepharose 4B

The method used was that described by Uy and Wold (1977). Sepharose 4B was washed with deionised water and dried by suction on a sintered glass funnel. Eight grams of this material was added to 8 ml of 1,4-butanediol

TABLE 2.2

Stoke's radii of the standard proteins*

| Standard protein | Diffusion constant§ ($cm^2\ sec^{-1} \times 10^{-7}$) | Stoke's radius (nm) |
|---|---|---|
| Bovine spleen ferritin | - | 79# |
| Bovine liver catalase | $4.1 \times 10^7$ | 52 |
| Bovine serum albumin | $5.9 \times 10^7$ | 36 |
| Horse heart myoglobin | $11.3 \times 10^7$ | 19 |

* Stoke's radii were determined from diffusion constants as explained in the text.
§ Determined in aqueous medium, corrected for water at $20^o$.
# Siegel and Monty, 1966.

diglycidyl ether and 8 ml of 0.6 M NaOH containing 16 mg of sodium borohydride in a 100 ml flask. The mixture was incubated at room temperature for 10 hours while being rotated on an orbital shaking water bath (Patton Industries, South Australia). This epoxy-activated Sepharose 4B was then washed with 1 litre of water on a sintered glass funnel and added to 16 ml of 0.1 M NaOH containing 0.8 g of lactose in a 100 ml flask. The mixture was rotated mechanically at 37$^{o}$ for 24 hours and then washed on a sintered glass funnel with 2 litres of 0.1 M borate buffer, pH 8.0 followed by 1 litre of water. The conjugated gel was packed into a 20 ml glass syringe and equilibrated with TSA. The above procedure resulted in approximately 8 ml of packed Sepharose.

### 2.14.2 Acid treatment of Sepharose 4B

Eight grams of suction-dried Sepharose 4B (approximately 8 ml) was added to 8 ml of 0.2 M HCl in a 100 ml flask and incubated in a 56$^{o}$ water bath for 100 minutes. It was then washed with 1 litre of water on a sintered glass funnel, packed into a 20 ml glass syringe and equilibrated with TSA.

## 2.15 Protein determinations

### 2.15.1 Lowry (Folin-Phenol) method

#### (i) Assay of proteins in the absence of interfering materials (e.g. tris, glycine, sugars)

The method used was a modification of the procedure of Schacterle, G.R. and Pollack, R.L., (1973), which was based

on the original method of Lowry, Rosebrough, Farr and Randall (1951). The following reagents were prepared:-

Alkaline Copper Reagent

| Ingredients: | Amount: |
|---|---|
| Sodium hydroxide | 2 g |
| Anhydrous sodium carbonate | 10 g |
| 5% disodium tartrate | 2 ml |
| 5% copper sulphate | 1 ml |
| Sodium dodecylsulphate | 0.8 g |
| Water to | 100 ml |

The disodium tartrate and copper sulphate were added as solutions to avoid precipitation problems. The reagent was stored at $4^{o}$ for no longer than 2 months. Prior to use it was warmed in order to re-dissolve the dodecylsulphate.

Folin's Reagent

The Folin-Ciocalteu phenol reagent was diluted 1:1 by volume with water and stored at $4^{o}$ in the dark. Immediately before use 1 volume of this solution was mixed with 8 volumes of water. A sample (0.1 ml) of the material to be assayed was mixed with 0.1 ml of the alkaline copper reagent in a 5 ml disposable plastic test tube (Medical Plastics Pty. Ltd., South Australia). This mixture was incubated for 10 minutes at room temperature and then 0.4 ml of Folin's reagent was added with rapid mixing. After incubating for 5 minutes in a $56^{o}$ water bath, the tubes were cooled in tap water (1-2 min.) and the absorbance at 650 nm of the solutions was measured in a Perkin-Elmer spectrophotometer. The protein concentration was estimated from a standard curve constructed using 25, 50, 100, 150,

200 and 250 µg per ml solutions of BSA in water. Standards and buffer blanks were always included.

(ii) Assay of proteins in the presence of interfering materials

To remove materials, such as lactose and galactose, which interfered with the protein assay it was necessary to precipitate the protein using a modification of the method of Bensadoun and Weinstein (1976). To 1 volume of protein solution in a 5 ml disposable plastic test tube was added 0.5 volumes of sodium deoxycholate solution (0.495 mg/ml in water) with vigorous mixing. After allowing the solution to incubate for 15 minutes at room temperature, 0.5 volumes of 24% trichloroacetic acid was added with vigorous mixing. The tubes were then centrifuged at 3000 g for 30 minutes in a MSE Coolspin centrifuge (MSE Scientific Instruments, England) using the 4 x 380 ml swing out rotor (MSE#63301). The supernatants were decanted and the inverted tubes were drained on tissue paper for 5 minutes. After draining, 0.1 ml of water was added to the precipitate and the normal protein assay was continued.

2.15.2 Ninhydrin method

The procedure used was that of Kabat and Mayer (1961).

2.16 Discontinuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein samples

A vertical slab gel electrophoresis apparatus employing a discontinuous buffer system originally devised by

Laemmli (Polyacrylamide Gel Electrophoresis, laboratory techniques. Handbook, Pharmacia Fine Chemicals, 1981) was used. The method involved forming a separating acrylamide gel over which was layered a more porous stacking gel. The stacking gel, the separating gel and the electrode buffer were each of different buffer composition. In this system, the components of the sample loaded into slots in the stacking gel are concentrated into a sharp band while migrating through the stacking gel, thus ensuring maximum resolution in the separating gel.

### 2.16.1 Stock solutions

44.8% w/v Acrylamide stock solution (1)

| | |
|---|---|
| Acrylamide | 44 g |
| Bis-acrylamide | 0.8 g |
| Water to | 100 ml |

30.8% w/v Acrylamide stock solution (2)

| | |
|---|---|
| Acrylamide | 30 g |
| Bis-acrylamide | 0.8 g |
| Water to | 100 ml |

The acrylamide solutions were stored at $4^{o}$ in the dark.

0.75 M Tris/HCl pH 8.8

| | |
|---|---|
| Tris | 45.51 g |
| dissolve in 400 ml water | |
| 10% azide | 5 ml |
| pH with 32% w/w HCl to pH 8.8 | |
| add water to 500 ml | |

0.25M Tris/HCl pH 6.8

| | |
|---|---|
| Tris | 7.57 g |
| dissolve in 200 ml water | |
| 10% azide | 2.5 ml |
| pH with 1 M HCl to pH 6.8 | |
| add water to 250 ml | |

Reservoir buffer

| | |
|---|---|
| Tris | 12.11 g |
| Glycine | 57.06 g |
| Sodium dodecylsulphate | 2 g |
| water | to 2 litres |

## 2.16.2 Preparation of the acrylamide gel[1]

### A) Separating gel

The acrylamide gel was formed between two glass plates which were separated by 1.5 mm thick by 5 mm wide perspex spacers. One of the glass plates had a notch cut from its top edge as shown in Figure 2.2A. The glass plates and spacers were assembled as shown in Figure 2.2A and clamped to a perspex backing plate using spring clips. This assembly was then clamped to a retort stand through a metal rod screwed into the perspex plate. To seal the assembly 1.5% agar was poured around the outside edges. The separating acrylamide gel solution was prepared in a 250 ml Buchner flask as shown below:-

| Stock Solution | Final acrylamide concentration | | |
|---|---|---|---|
| | 7% | 11% | 13% |
| | mls | | |
| Acrylamide Solution 1 | 3.9 | 6.25 | 8.4 |
| 1% Ammonium persulphate* | 0.63 | 0.63 | 0.63 |
| 0.75 M Tris/HCl | 12.5 | 12.5 | 12.5 |
| Water | 7.47 | 5.12 | 3 |

*The Ammonium persulphate solution was prepared in water immediately before use.

[1] As recommended by Wyckoff, Rodbard and Chrambrach (1977), SDS was not included in the acrylamide gels. During electrophoresis the SDS was supplied by the reservoir buffer.

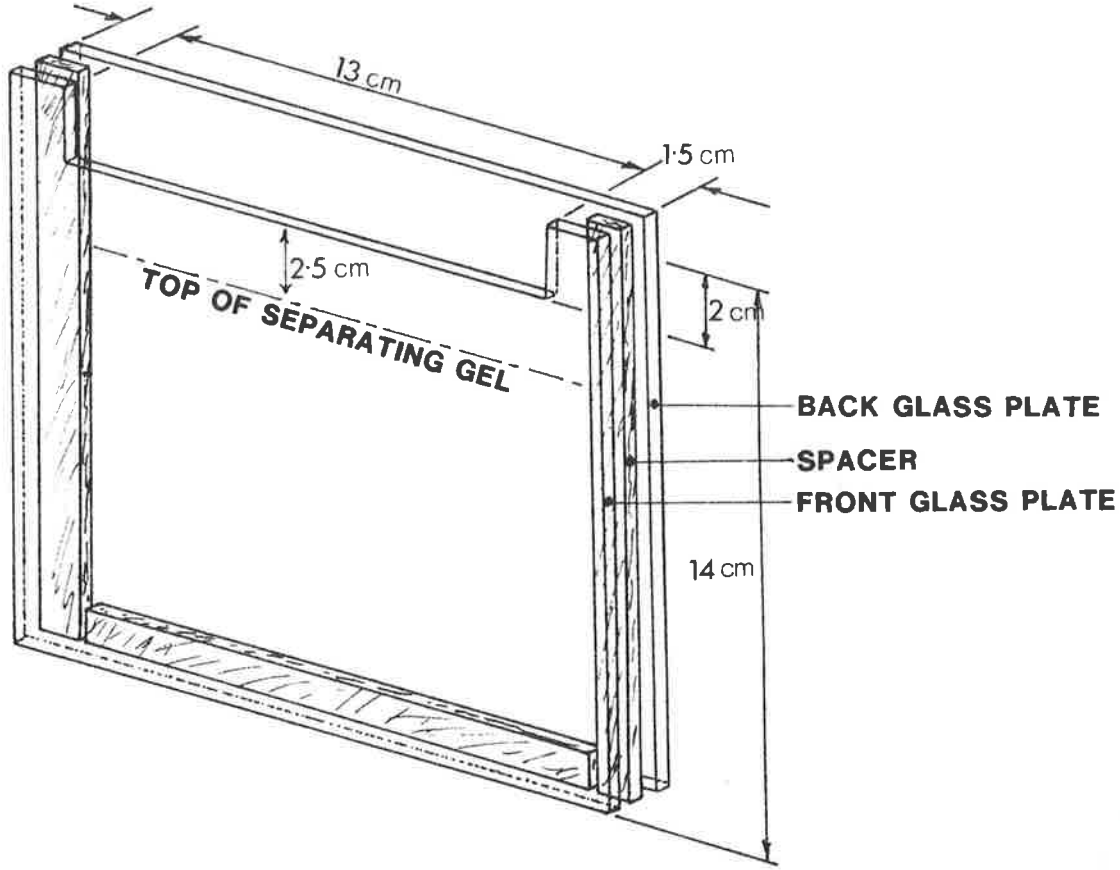
FIGURE 2.2

Diagram showing the equipment used in casting gel slabs for discontinuous SDS-PAGE.

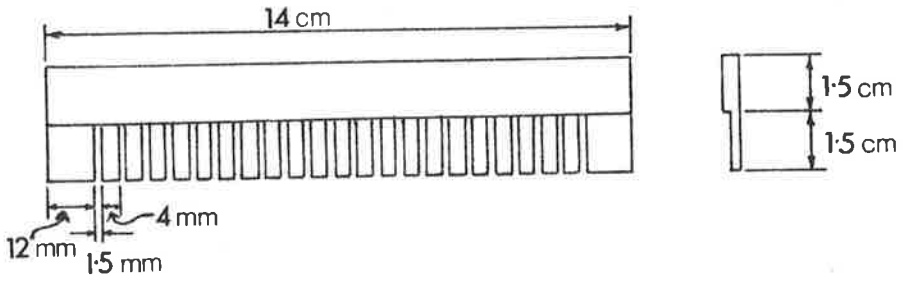
A. Gel cassette.

B. Comb used for forming sample wells.

# A


13 cm
1·5 cm
2·5 cm
TOP OF SEPARATING GEL
2 cm
BACK GLASS PLATE
SPACER
FRONT GLASS PLATE
14 cm

# B


14 cm
1·5 cm
1·5 cm
4 mm
12 mm
1·5 mm

The acrylamide solution was deaerated under vacuum using a water vacuum pump for 10 minutes while the solution was stirred with a magnetic mixer. Twenty five microlitres of N,N,N$^{1}$,N$^{1}$ tetramethylethylenediamine (TEMED) were added and the solution was immediately poured between the glass plates to a level 2.5 cm below the notch, as shown in Figure 2.2A. The acrylamide solution was carefully overlayed with water using a Pasteur pipette and left to set at room temperature (at least 1 hour). If the gels were to be kept overnight before use, the water was replaced with separating gel buffer and the gels were placed in a humidified chamber.

B) Stacking gel

The stacking gel acrylamide solution was made up in a 50 ml Buchner flask as shown below and deaerated as above:-

| Stock Solution | Volume (ml) |
|---|---|
| Acrylamide Solution 2 | 0.5 |
| 1% Ammonium persulphate | 0.12 |
| 0.25 M Tris/HCl | 2.5 |
| Water | 1.83 |

TEMED (5 µl) was added and the solution was immediately poured over the separating gel. A perspex comb (see Fig. 2.2B) was then inserted between the glass plates and into the acrylamide solution, care being taken that no air bubbles were trapped. After the acrylamide had set (1 hour at room temperature) the comb was removed. The sample wells were filled with stacking gel buffer containing 0.004%

bromophenol blue and left for 10-30 minutes to allow the dye to diffuse into the gel.

2.16.3 Preparing the protein samples for SDS-PAGE

A 2X concentrated solution of sample buffer was made up as follows:-

| Reagent | Amount |
|---|---|
| SDS | 0.8 g |
| Glycerol | 4 ml |
| 2-Mercaptoethanol | 2.0 ml (optional) |
| 1 M Tris | 0.4 ml |
| 0.25 M EDTA | 0.16 ml |
| Water to | 20 ml |
| pH to pH 8.0 with 1 M HCl | |

Samples to be analysed were mixed 1:1 by volume with 2X sample buffer and a maximum of 25-50 µl was applied to the gel. Standard protein solutions were made up in distilled water (50-100 µg/ml), 10 µl being used. This gave optimal results with bands containing 0.5-1 µg of protein. If the protein concentration of the sample was too low or if the sample buffer concentration was too high to give good results, the proteins were precipitated using the trichloroacetic acid-deoxycholate method as described in Section 2.15.1 except that 400 µl conical polyallomer tubes (Eppendorf Gerätebau, Netheler + Hinz GmbH, West Germany) and a Beckman micro-fuge were used. The pellet was washed with a 1:1 ethanol:ether solution to remove trichloroacetic acid and deoxycholate and then dissolved in 10 µl water plus 10 µl 2X sample buffer. Samples were boiled for 5 minutes before loading onto the gel.

### 2.16.4 SDS-PAGE electrophoresis conditions

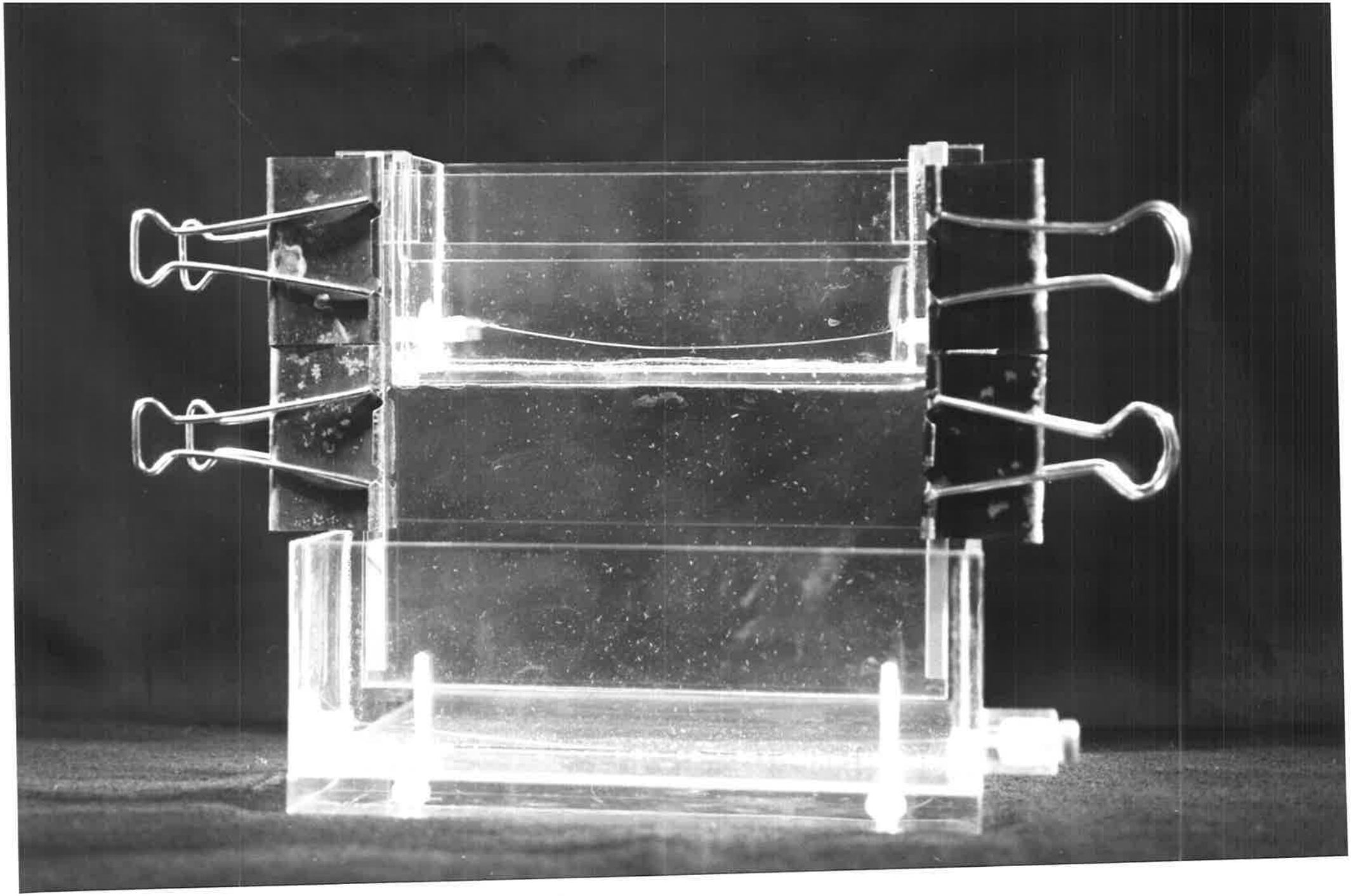
The gel assembly was removed from the perspex backing plate and after removing the bottom spacer, clamped to the electrophoresis apparatus as shown in Figure 2.3. The notch in the front glass plate was aligned with the notch in the upper buffer vessel so as to allow access of the electrode buffer to the acrylamide gel. The interface between the front glass plate and perspex wall of the electrophoresis apparatus was sealed with 1.5% agar to prevent leakage of buffer between the upper and lower buffer vessels. Sufficient electrode buffer was added to the buffer vessels to cover the exposed acrylamide gel. The samples were applied to the sample wells by layering the solutions under the electrode buffer. The upper buffer vessel was connected as the cathode. Electrophoresis proceeded at constant voltage (50 V) until the dye front had moved into the separating gel (approximately 90 minutes) and the voltage was then increased to 80 V. When the dye front had moved to the bottom of the gel (4-6 hours) electrophoresis was stopped. The gel assembly was removed and the gel separated from the glass plates by forcing water between the gel and glass surfaces with a 10 ml syringe and 22 gauge needle.

### 2.16.5 Staining the acrylamide gels

To fix the proteins, the gels were incubated in 50% trichloroacetic acid (approximately 100 ml) with gentle shaking for 30-60 minutes. The trichloroacetic acid was drained off and the gel rinsed with distilled water. Approximately 100 ml of staining solution was then added.

FIGURE 2.3

Gel electrophoresis apparatus used for discontinuous SDS-PAGE, shown with gel slab in place.



This consisted of 0.06% Coomassie Blue G-250 in 3.5% perchloric acid and was made by dissolving 1.2 g of G-250 in 2 litres of water and then adding slowly with stirring 100 ml of 70% perchloric acid. The gel was left in the staining solution overnight with gentle shaking and de-stained with several changes of 5% acetic acid.

## 2.17 Two dimensional discontinuous SDS-PAGE

In this procedure electrophoresis in non-reducing conditions was followed by electrophoresis in reducing conditions. The techniques used were essentially the same as described above.

### 2.17.1 The first dimension

This was performed exactly as for normal discontinuous SDS-PAGE using a 7% separating gel. After electrophoresis, gel strips (1 cm wide) containing the individual sample tracks were sliced from the slab, wrapped in adhesive plastic wrapping ("Gladwrap", Union Carbide) and stored at $-18^{o}$.

### 2.17.2 The second dimension

The thawed gel strip was immersed in 20 volumes of sample buffer containing 5% v/v 2-mercaptoethanol and incubated for 1 hour at $37^{o}$. The strip was then placed in the assembled gel former such that its top long edge was about 1 mm below the top edge of the front glass plate. Both glass plates were 2 cm longer than normal in order to obtain a longer stacking gel. An 11% separating gel was formed in the normal manner (top edge 4-5 cm below the

top of the front plate) above which the stacking gel solution was poured to about 2 mm below the first dimension gel strip. After the acrylamide had set, the plates were filled with hot (56$^{o}$) 1% agarose (Pharmacia Fine Chemicals; low electroendosmosis, type C) dissolved in stacking gel buffer. This completely embeded the first dimension gel within the agarose stacking gel, avoiding the use of ammonium persulphate which has been reported to significantly degrade proteins (Tuszynski, Buck and Warren, 1979). Electrophoresis was performed in the normal manner. The gels were fixed and stained as in section 2.16.5.

## 2.18 Drying polyacrylamide gel slabs for autoradiography

Polyacrylamide gel slabs were dried on a piece of Whatman number one filter paper as follows: 1) a piece of Gladwrap was placed upon a 26 cm x 26 cm x 0.5 cm rubber mat; 2) the gel was placed upon the Gladwrap and then a piece of Whatman number one filter paper was layered on the gel. The Gladwrap and filter paper were cut so as to extend 1 cm beyond the edges of the gel; 3) a piece of porous plastic, extending 0.5 cm beyond the edges of the filter paper, was placed over the filter paper; 4) a second rubber mat (identical to the first) was then placed on top. A tube through the top rubber mat allowed a vacuum to be applied to the gel using a vacuum water pump. The whole assembly was placed in a 70$^{o}$ water bath for 4 hours.

## 2.19 Autoradiography using polyacrylamide gel slabs

The dried gel slab was placed on Fugi medical X-ray

film which rested on an Ilford fast tungstate intensifying screen and encased in an Ilford R.R. cassette. After exposure the film was developed for 5 minutes in Kodak D19 developer and fixed for 2 minutes in Kodak liquid X-ray fixer.

## 2.20 Immunochemical techniques

### 2.20.1 Production of antisera

Antisera were raised in adult rabbits obtained from the University of Adelaide Central Animal House and housed in this department. Antigen solution was emulsified with an equal volume of Freund's complete adjuvant by adding 4 drop aliquots to the adjuvant and forcing the mixture several times through a 19 gauge needle using a 5 ml disposable syringe before the next addition. The animals were injected subcutaneously with 0.2 ml aliquots of the emulsion in several locations. The second immunisation was given 2-3 weeks later, using an emulsion made from Freund's incomplete adjuvant. Animals were bled from the ear vein 4 weeks after the second injection.

### 2.20.2 Purification of rabbit IgG

The IgG immunoglobulin was isolated from the rabbit antisera by the method of Steinbuch and Audran (1969). This yielded essentially pure IgG which was used in the immunoelectrophoretic procedures.

### 2.20.3 Rocket immunoelectrophoresis

The procedures used are described in detail in the

Scand. J. Suppl. 1 (1973) and are summarised here.

Five times concentrated immunoelectrophoresis (IEP) buffer was prepared 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 | |

Add solutions 1 and 2 and adjust volume to 5000 ml with water. Working IEP buffer was prepared by diluting the stock solution five fold and checking the pH to be exactly pH 8.6. One percent agarose was prepared by dissolving 1 g of agarose (Seakem HGT, Marine Colloids) in 100 ml of IEP buffer containing 1 mM EDTA.

Fifty five microlitres of anti-(HA-1) rabbit IgG (12.26 mg/ml) was mixed with 7 ml of 1% agarose in a 56$^{o}$ water bath and poured onto a 7.6 cm x 5 cm glass plate. After the agarose had set, 3 mm diameter holes were punched (Bio-Rad gel puncher) at 6 mm spacing in a line 1 cm from the long edge. The plates were connected to each buffer reservoir using 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 minutes and pressed again before being dried and stained.

2.20.4 Staining procedure

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

2.20.5 Crossed immunoelectrophoresis

A) 1st dimension: 6.8 ml of agarose (containing no antibodies) was poured onto a 7.6 x 5 cm glass plate and sample wells punched as shown in Figure 2.4A. Bromophenol blue was added to the samples (final conc. = 0.005%) and electrophoresis was carried out at 10 volts/cm until the dye had migrated approximately 5 cm. The agarose was cut and trimmed as indicated by the dashed lines in Figure 2.4A.

In some cases two sample wells were included in the same 1st dimension gel. These were placed at 5 mm spacing, either adjacent to each other or staggered, as shown in Figure 2.4C.

B) 2nd dimension: The agarose slab from the 1st dimension was placed on a 7.6 x 5 cm glass plate as shown in Figure 2.4B and 4.7 ml of agarose containing the appropriate anti-sera was poured over the rest of the plate. Electrophoresis was continued overnight at 2 volts/cm. The gel was pressed and stained as for rocket immunoelectrophoresis.

2.20.6 Crossed line immunoelectrophoresis

Crossed line immunoelectrophoresis was performed as

FIGURE 2.4

Diagram of gel plates used in crossed immuno-electrophoresis. Arrows indicate the direction of electrophoresis.

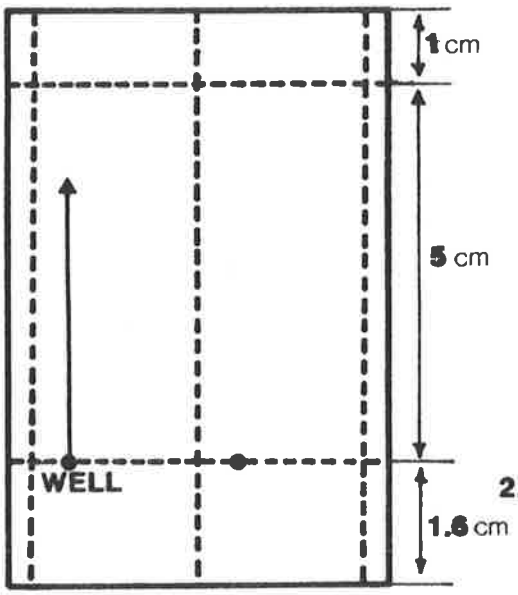
A. A 1st dimension gel showing position of sample wells. Dashed lines show where the agarose gel was trimmed in preparation for the 2nd dimension.

B. A 2nd dimension gel plate showing the arrangement of the 1st dimension gel and the antibody containing gel.

C. Position of the sample wells for the first dimension of tandem crossed immunoelectrophoresis.

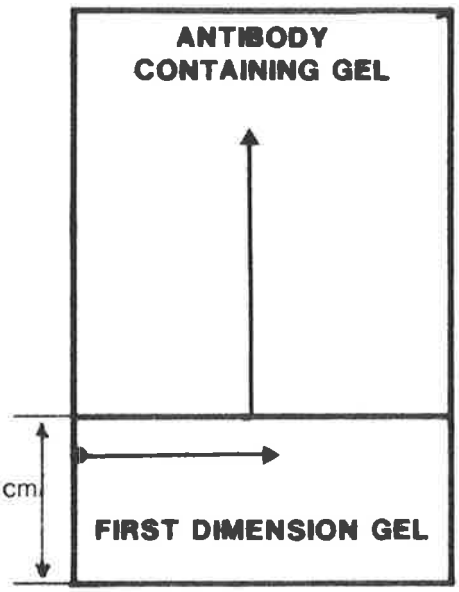
A

1st Dimension

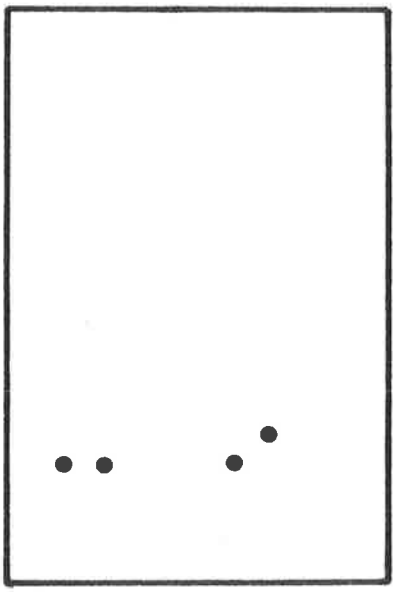



B

2nd Dimension




C



for crossed immunoelectrophoresis with the following modification. In the second dimension, a trough 4 mm wide was cut into the antibody containing gel immediately adjacent to the first dimension gel. This was filled with 0.36 ml of agarose containing the appropriate antigen.

## 2.21 Continuous SDS-PAGE analysis of haemagglutinin preparations

### 2.21.1 Stock solutions

50% Acrylamide

| | |
|---|---|
| Acrylamide | 48.75 g |
| Bis-acrylamide | 1.25 g |
| distilled water to | 100 ml |

10X Buffer, pH 7.4

| | |
|---|---|
| Tris | 121.1 g |
| Sodium acetate | 42 g |
| $Na_2$ EDTA | 18.6 g |
| distilled water | 2 litres |
| n-Butanol | 12.5 ml |

pH to 7.4 with glacial acetic acid

distilled water to 2.5 litres

### 2.21.2 Preparation of 10% w/v acrylamide gels

The following acrylamide solution was prepared in a 50 ml Buchner flask and deaerated by applying a vacuum with a water pump for 5 minutes:-

| Stock Solution | Amount |
|---|---|
| 10X buffer | 3 ml |
| 50% acrylamide | 6 ml |
| 1.5% Ammonium persulphate | 3 ml |
| Water | 15 ml |

After the addition of 1.5 ml of 20% SDS and 1.5 ml of 1%

v/v TEMED, the acrylamide solution was immediately added to glass tubes (0.50 x 12 cm) whose bottom ends had been sealed with parafilm (American Can Company). Each tube was filled to approximately 1 cm below the top. Using a Pasteur pipette, the acrylamide was carefully overlayed with water and allowed to set (approximately 3 hours).

### 2.21.3 Preparing the protein samples

A 2X concentrated solution of disintegration buffer was prepared as follows:-

| Reagent | Amount |
|---|---|
| 0.25 M EDTA, pH 7.4 | 0.1 ml |
| 20% SDS | 0.5 ml |
| Urea | 1.35 g |
| Water to | 2.5 ml |

The disintegration solution was mixed 1:1 by volume with the sample and boiled for 3 minutes. To 100 µl of this solution was added 10 µl of 0.02% bromophenol blue solution.

### 2.21.4 Electrophoresis conditions

The electrophoresis apparatus consisted of two sets of buffer compartments which were fixed to retort stands and could be moved independently. The glass tubes were passed through holes lined with rubber grommets in the bottom of the top compartment and the buffer compartments were then positioned so that the tubes (parafilm removed) protruded into the bottom buffer compartment. Enough electrode buffer (approximately 400 ml) consisting of 100 ml of 10X buffer, 10 g of SDS and 900 ml of water, to cover

the ends of the glass tubes were then added to each buffer compartment. An aliquot (25-100 µl) of sample was layered beneath the electrode buffer. The upper compartment was connected as the cathode and electrophoresis continued in constant current mode at 5 mA/gel until the bromophenol blue migrated to the bottom of the gels. The gels were removed from the tubes by forcing water between the gel and glass surfaces with a 10 ml syringe and 25 gauge needle. They were cut at the position of the bromphenol blue marker.

### 2.21.5 Staining the gels

The staining procedure was the same as that outlined for discontinuous SDS-PAGE. After staining, the gels were scanned in a Quick Scan Jr. scanner (Helena Laboratories Corp., U.S.A.).

### 2.21.6 Sectioning of gels for measuring radioactivity

The stained gels were wetted with a 1% w/v SDS solution to provide lubrication and sucked under slight vacuum into a 1 ml disposable tuberculin syringe (Pharmoceal Laboratories, U.S.A.), from which the plunger had been removed. Using a Hamilton repeating syringe apparatus, equal fractions of the gel were extruded from the syringe into counting tubes. About 60 fractions were usually collected. The fractions were counted in a Packard Autogamma Counter.

## 2.22 Radiolabelling of haemolymph components

The procedure used was a modification of the method

of Hunter and Greenwood (1962). Twenty microlitres of haemolymph (containing approximately 10 µg of protein), 2 µl of iodine-125 (200 µCi) and 10 µl of chloramine T (5 mg in 25 ml of 0.066 M phosphate pH 7.4) were mixed in the order shown in a small glass tube and incubated for 5 minutes at room temperature. After this procedure, 10 µl of sodium metabisulphate (10 mg in 25 ml of 0.066M phosphate pH 7.4), 10 µl of 0.1 M potassium iodide and 460 µl of BSA diluent (2.5% w/v in PBS) were added. The preparation was then dialysed extensively against TSA. The iodination efficiency was usually 20-50%.

## 2.23 Method of radiolabelling pure proteins

The procedure used was a modification of the method of Sonada and Schlamowitz (1970). Fourty microlitres of protein solution (10 µg), 25 µl of iodine-125 diluted 1/20 in 0.066 M phosphate pH 7.4 (125 µCi) and 50 µl of chloroamine T (0.009 mg/ml in phosphate buffer) were sequentially mixed in the above order in a 4 ml low adsorption plastic test tube (Minisorp tube; A/S Nunc, Denmark) and incubated in ice for 60 minutes. Fifty microlitres of sodium metabisulphite (0.0065 mg/ml in phosphate buffer) followed by 50 µl of potassium iodide (0.1 M) were then added. After a further 5 minutes incubation, 0.8 ml of BSA diluent (2.5% w/v in PBS) was added and the solution was exhaustively dialysed against TSA. Chloroamine T and sodium metabisulphite were prepared immediately before use and all solutions were precooled in ice. Labelling efficiency was usually 10-40%.

## 2.24 Photography

An Olympus OM2 camera set on automatic mode, Recordak AHV microfilm 5460 (Eastman Kodak Co., U.S.A.) and Ilfospeed Grade 1, Glossy, medium weight paper (Ilford Pty. Ltd., Australia) were used and processed according to the manufacturer's instructions.

## 2.25 Concentration of samples by ultrafiltration

Samples were concentrated using Amicon (Amicon Corporation, U.S.A.) stirred cells and Diaflo ultrafiltration membranes as per the manufacturer's instructions. Model 202 stirred cell (200 ml) was used with PM10 and PM30 membranes and Model 52 (50 ml) was used with YM10 and YM30 membranes.

## 2.26 Amino acid analysis

Amino acid analyses were performed by Mike Calder of the Biochemistry Department, University of Adelaide. A Beckman 120C amino acid analyser, modified for use with Durrum DC1A resin and the Pico buffer II system, was used.

The protein (1 mg) was dissolved in 3.0 ml of 6 M HCl plus 3 Pasteur pipette drops of 5% aqueous phenol. One millilitre of this was then transferred to each of 3 hydrolysis tubes, which were then flushed with nitrogen, sealed and incubated at $105^{o}$ for 24, 48 and 72 hours, respectively, before being evaporated to dryness. Each was then dissolved in 1 ml of loading solution and 0.5 ml was analysed.

# CHAPTER 3

## Some properties of the haemagglutinins present in the haemolymph of *B. leachii*

## 3.1 Preamble

As is the case with other ascidians (Chapter 1), the haemolymph from *B. leachii* was found to contain haemagglutinating activity for erythrocytes from various vertebrate species (Table 3.1). In this chapter the susceptibility of these activities to various chemical and physical treatments is investigated. This was done to gain information on the general physical properties and stabilities of the haemagglutinins and to establish whether any special precautions were necessary to ensure maintenance of activity during purification procedures.

## Experimental

## 3.2 Haemagglutinating activity of *B. leachii* haemolymph

The haemagglutinating activity of *B. leachii* haemolymph was assayed using erythrocytes from a variety of vertebrate species as indicator cells (see Table 3.1). The titre was similar (1/16 - 1/64) for most of the erythrocytes tested, but especially high for those from the guinea pig and pigeon. No difference in titre was observed when human type A, B or O erythrocytes were used. Little variation was observed between different batches of haemolymph, except for guinea pig erythrocytes where the variation was quite large (1/1,600 - 1/32,000). The titres shown in Table 3.1 are for undialysed haemolymph. Dialysis against TSA or PBS had no effect on the titres obtained.

In the experiments to be described in this chapter the haemagglutinating activity to sheep and guinea pig erythrocytes was measured. As the titres obtained with these cells

TABLE 3.1

Haemagglutinating activity of *B leachii* haemolymph

| Source of erythrocytes | Agglutination titre$^{-1}$ |
|---|---|
| Guinea pig | 2,000 - 32,000 |
| Chicken | 8 - 16 |
| Pigeon | 16,000 |
| Mouse | 64 |
| Rat | 64 |
| Rabbit | 32 |
| Sheep | 64 - 128 |
| Human | 64 |

were indicative of the extremes of activity in *B. leachii* haemolymph, it was thought that their use would yield results most representative of the haemagglutinins in *B. leachii* haemolymph.

## 3.3 Divalent cation requirements of the haemagglutinins

To determine whether divalent cations were required for haemagglutinating activity, samples of haemolymph were exhaustively dialysed against either TSA containing 1 mM $CaCl_2$ plus 1 mM $MgCl_2$ or TSA containing 1 mM EDTA. Each sample was then assayed using the following diluents; isotonic trisodium citrate (0.13 M), TSA containing 1 mM $CaCl_2$ plus 1 mM $MgCl_2$, TSA containing 0.05 mM EDTA plus either 0.1 mM $CaCl_2$ or 0.1 mM $MgCl_2$. Citrate and EDTA were used since they are excellent chelators of divalent metal ions. The results are shown in Table 3.2.

The haemagglutinating activity of haemolymph for sheep erythrocytes was unaffected by any of these treatments. In contrast, the haemagglutinating activity for guinea pig erythrocytes was completely inhibited by titration in citrate, indicating that divalent metal ions were required for this activity. This inactivation was reversible since addition of $Ca^{++}$ plus $Mg^{++}$ to haemolymph dialysed against EDTA restored activity. Calcium ions alone could also fully restore activity, but $Mg^{++}$ alone was completely ineffective. Thus, while the sheep erythrocyte haemagglutinin in *B. leachii* haemolymph has no divalent metal ion dependency, the guinea pig erythrocyte haemagglutinin specifically requires $Ca^{++}$ for activity.

TABLE 3.2

Divalent cation requirements of the haemagglutinins

| Erythrocyte type | Haemolymph dialysed against | Diluent used for titration | | | |
|---|---|---|---|---|---|
| | | Citrate | $Ca^{++}$ + $Mg^{++}$ | $Ca^{++}$ | $Mg^{++}$ |
| | | Agglutination $titre^{-1}$ | | | |
| Guinea pig | $Ca^{++}/Mg^{++}$ | 200 | 16,000 | - | - |
| | EDTA | 200 | 16,000 | 16,000 | 200 |
| Sheep | $Ca^{++}/Mg^{++}$ | 160 | 160 | - | - |
| | EDTA | 160 | 160 | - | - |

## 3.4 Effect of acid pH on the activity of the *B. leachii* haemagglutinins

Half millilitre aliquots of *B. leachii* haemolymph (exhaustively dialysed against saline) were added to 0.5 ml aliquots of 0.1 M trisodium citrate solutions whose pH varied from pH 2.0 to pH 7.0. The solutions were incubated at room temperature for 5 hours and then dialysed against a 20 fold excess of TSA containing 10 mM $CaCl_2$. The haemagglutinating activity of each dialysed sample is shown in Table 3.3.

After incubation at pH 2.0, 20% of the guinea pig erythrocyte haemagglutinating activity remained. At least 50% of the activity remained after incubation at pH 4.0 and higher. Activity was unaffected at pH's near neutrality.

The sheep erythrocyte haemagglutinating activity was very labile at pH 2.0 (only 3% remained) but was apparently stable at pH's higher than pH 3.0.

## 3.5 Sensitivity of the haemagglutinins to digestion with pepsin

The pH optimum for the proteolytic activity of pepsin is pH 2.0 (Fruton, 1971); at neutral pH the enzyme is virtually inactive. This experiment was therefore conducted in conjunction with the pH sensitivity experiment using the same stock solutions. Pepsin was added to aliquots of haemolymph (dialysed against saline), which were then treated in exactly the same manner as described in the last section. The final concentration of pepsin was 1 mg/ml. The results are shown in Table 3.3.

TABLE 3.3

Effect of acid pH and pepsin on the haemagglutinating activities of *B. leachii* haemolymph*

| Buffer pH | Haemagglutination titre$^{-1}$ (% of control) | | | |
|---|---|---|---|---|
| | Guinea pig erythrocytes | | Sheep erythrocytes | |
| | No pepsin | + pepsin | No pepsin | + pepsin |
| 2.0 | 3200 (20) | 100 ( <1) | 8 (3) | <2 |
| 2.5 | 6400 (40) | 100 ( <1) | 32 (12) | <2 |
| 3.0 | 6400 (40) | 100 ( <1) | 128 (50) | <2 |
| 3.5 | 6400 (40) | 100 ( <1) | 256 (100) | <2 |
| 4.0 | 8000 (50) | 100 ( <1) | 256 (100) | 4 |
| 4.5 | 8000 (50) | 100 ( <1) | 256 (100) | 8 |
| 5.0 | 8000 (50) | 100 ( <1) | 256 (100) | 8 |
| 6.0 | 16000 (100) | 8000 (50) | 256 (100) | 8 |
| 7.0 | 16000 (100) | 16000 (100) | 256 (100) | 8 |
| TSA | 16000 (100) | 16000 (100) | 256 (100) | 8 |
| inactivated§ pepsin | - | 16000 (100) | - | 8 |

* Samples were incubated for 5 hours under the indicated conditions.

§ Boiled for 5 minutes in TSA.

While 20% - 50% of the guinea pig erythrocyte haemagglutinating activity remained after incubation at pH values between pH 2.0 - pH 5.0 in the absence of pepsin, samples incubated with pepsin in this pH range had a residual activity of less than 1%. At pH 7.0 the activity was unaffected by pepsin. These results show that the guinea pig erythrocyte haemagglutinin is sensitive to pepsin digestion.

The results for the sheep erythrocyte haemagglutinating activity were complex. Samples incubated with pepsin at pH 7.0 or with heat inactivated pepsin lost greater than 95% of their haemagglutinating activity for sheep erythrocytes. This effect was clearly not due to the enzymatic activity of the pepsin. While no definite reason can be given for these results, it is possible that the pepsin contained carbohydrates which inhibited the sheep erythrocyte but not the guinea pig erythrocyte haemagglutinins.

## 3.6 Sensitivity of the haemagglutinins to the proteases trypsin, pronase and papain

Half millilitre aliquots of *B. leachii* haemolymph (dialysed against TSA) were added to 0.5 ml aliquots of TSA containing 100 µg/ml of enzyme and appropriate activators or inhibitors of enzyme activity (see below). The solutions were incubated at $37^{o}$ for 5 hours and then assayed in TSA containing 1 mM $CaCl_2$ for residual haemagglutinating activity. The inhibitors were used in controls to check whether any observed reduction in titre was due to enzymatic activity. The activators and inhibitors on their own had no effect on

TABLE 3.4

Susceptibility of the haemagglutinins to digestion with trypsin

| Erythrocyte type | $Titre^{-1}$ of haemolymph after incubation* with trypsin plus | | | |
|---|---|---|---|---|
| | $Ca^{++}$ | EDTA | PMSF# | No enzyme |
| Guinea pig | 6,400 | 200 | 12,800 | 12,800 |
| Sheep§ | 256 | 256 | 256 | 256 |

* 5 hours at 37°.

# Phenylmethylsulphonyl fluoride. Incubation of haemolymph with PMSF and EDTA without enzyme had no effect on titres.

§ These results were the same for 5 hour and overnight incubation.

TABLE 3.5

Susceptibility of B. leachii haemagglutinins to digestion with pronase

| Erythrocyte type | Titre$^{-1}$ of haemolymph after incubation* with pronase plus | | |
|---|---|---|---|
| | $Ca^{++}$ | EDTA | No enzyme |
| Guinea pig | 3,200 | 200 | 12,800 |
| Sheep§ | 256 | 256 | 256 |

* 5 hours at 37°.

§ These results were the same for 5 hour and overnight incubation.

the haemagglutinating activities of haemolymph. None of the enzymes exhibited any haemagglutinating activity of their own, nor did they affect the haemagglutinating activity when added to haemolymph immediately prior to titration. The results are shown in Tables 3.4, 3.5 and 3.6.

a) Trypsin: Calcium chloride (100 µl of 0.1 M solution) was used to activate trypsin and either EDTA (50 µl of 0.25 M solution) or phenylmethylsulphonyl fluoride (25 µl of 6 mg/ml solution in ethanol) were used to inhibit activity (Keil, 1971; Fahrney and Gold, 1963). The results (Table 3.4) show that 50% of the guinea pig erythrocyte haemagglutinating activity remained after incubation of the haemolymph with trypsin plus $Ca^{++}$, but only 2% remained after incubation with trypsin plus EDTA. This reduction in activity was attributed to the proteolytic activity of trypsin since (1) the haemagglutination titre was unaffected if the trypsin inhibitor phenylmethylsulphonyl fluoride was present and (2) the EDTA by itself had no effect on the haemagglutination titre. The sheep erythrocyte haemagglutinating activity was resistant to trypsin treatment, even after overnight incubation.

b) Pronase: This enzyme was activated with $CaCl_2$ (100 µl of 0.1 M solution) and inhibited with EDTA (50 µl of 0.25 M solution; Calbiochem pamphlet, document number 4941). Pronase destroyed guinea pig erythrocyte haemagglutinating activity (98% reduction after 5 hours) in the presence of EDTA but had much less effect when $Ca^{++}$ was present (75%

TABLE 3.6

Susceptibility of B. leachii haemagglutinins to digestion with papain

| Erythrocyte type | Titre$^{-1}$ of haemolymph after incubation* with papain plus | | |
|---|---|---|---|
| | EDTA# | Iodoacetamide§ | No enzyme |
| Guinea pig | 100 | 12,800 | 12,800 |
| Sheep¶ | 256 | 256 | 256 |

* 5 hours at 37°.

# Cysteine was also present. Incubation of haemolyph with EDTA plus cysteine without enzyme had no effect on titres.

§ Incubation of haemolymph with iodoacetamide without enzyme had no effect on titres.

¶ These results were the same for 5 hour and overnight incubation.

reduction). The sheep erythrocyte haemagglutinating activity was resistant to pronase activity (overnight incubation).

c) Papain: EDTA (50 µl of 0.25 M solution) plus cysteine-HCl (50 µl of 0.5 M solution) was used to inhibit activity (Biochemica Information, pg. 146, Boehringer Mannheim GmbH, Biochemica 1973). As with trypsin and pronase, guinea pig erythrocyte haemagglutinating activity was labile to the action of papain (98% reduction after 5 hours) in the presence of EDTA but sheep erythrocyte haemagglutinating activity was totally resistant (overnight incubation). The reduction in titre could be attributed to enzymatic activity since the titre was unaffected when iodoacetamide was present.

### 3.7 Sensitivity of the haemagglutinins to reduction, alkylation and denaturation

The effects of reduction, alkylation and/or denaturation on haemagglutinating activity were investigated by adding dithiothreitol, iodoacetic acid and/or urea to dialysed *B. leachii* haemolymph. To 1 ml aliquots of haemolymph was added 0.75 ml of 0.15 M NaCl and 0.25 ml of 0.75 M Tris-HCl (pH 8.0) ± 20 mM EDTA or 0.31 M dithiothreitol. The solutions (2 ml) were incubated for 5 hours at $28^{o}$ or $58^{o}$ and then mixed with 2 ml of 0.75 M Tris buffer ± 0.1 M iodoacetic acid. This amount of iodoacetic acid represented 2.5 molar equivalents of the dithiothreitol. After a further 30 minutes incubation at room temperature, the solutions were dialysed against a 1000 fold excess of saline and assayed for

TABLE 3.7

Sensitivity of the B. leachii haemagglutinins to reduction, alkylation and denaturation.

| Treatment | Haemagglutination titre$^{-1}$ | | | |
|---|---|---|---|---|
| | Guinea pig erythrocytes | | Sheep erythrocytes | |
| | No urea | Plus urea | No urea | Plus urea |
| | % activity* | | | |
| Control | 100 | 15 (<2) | 100 | 46 (<30) |
| Alkylation | 100 | 9 (<2) | 80 | 35 (<30) |
| Reductn + Alkn | 2 | 2 (<2) | 100 | 46 (<30) |

*)At room temperature (28°) or at 58° (brackets).

haemagglutinating activity. As a precaution, solutions containing dithiothreitol were dialysed separately and those containing iodoacetic acid were kept in the dark. A duplicate set of samples were treated identically except that the saline was replaced with 0.97 gm of urea. This gave solutions 8 M in urea with a final volume of 2 ml.

The results are shown in Table 3.7. Both haemagglutinating activities appeared sensitive to denaturation with urea, especially at 58°, although the sheep erythrocyte haemagglutinating activity was reasonably stable at 28°. In the absence of urea, alkylation alone had no effect. However, reduction (followed by alkylation) completely destroyed the guinea pig erythrocyte haemagglutinating activity whereas the sheep erythrocyte activity remained unaffected, even in the presence of urea.

## 3.8 Sensitivity of the haemagglutinins to heat

The sensitivity of the haemagglutinins to heat was investigated by incubating haemolymph (dialysed against TSA) containing either 10 mM EDTA or 10 mM $Ca^{++}$ in glass test tubes at various temperatures in a water bath. At various times samples were removed, immediately cooled on ice and assayed for haemagglutinating activity. The results for incubation at 60° are shown in Table 3.8.

In the presence of $Ca^{++}$, approximately 95% of the guinea pig erythrocyte haemagglutinin was inactivated within 30 minutes at this temperature. In EDTA, however, the activity remained unchanged after 2 hours and was reduced by only 50% after 3 hours. The sheep erythrocyte haemagglutinating

TABLE 3.8

Sensitivity of the haemagglutinins to heating at 60° in the presence or absence of EDTA

| Time (hr) | Haemagglutination titre$^{-1}$ after incubation in | | | |
|---|---|---|---|---|
| | Ca++ | | EDTA | |
| | SRBC* | GPRBC§ | SRBC* | GPRBC§ |
| 0 | 256 | 3,200 | 256 | 3,200 |
| 0.5 | 256 | 200 | 256 | 3,200 |
| 1.0 | 256 | 200 | 256 | 3,200 |
| 1.5 | 256 | 200 | 256 | 3,200 |
| 2.0 | 256 | 200 | 256 | 3,200 |
| 2.5 | 256 | 200 | 256 | 1,600 |
| 3.0 | 256 | 200 | 256 | 1,600 |

* Sheep erythrocytes.

§ Guinea pig erythrocytes.

activity was unaffected in either $Ca^{++}$ or EDTA during this period. Incubation for 3 hours at $40^{o}$ and lower temperatures in $Ca^{++}$ or EDTA affected neither activity. In contrast, both activities were reduced by at least 95% after one hour incubation at $70^{o}$ in either $Ca^{++}$ or EDTA.

## 3.9 Discussion

The experiments described in this chapter were carried out with the aim of obtaining information on the basic physico-chemical properties of the haemagglutinins in *B. leachii* haemolymph, particularly with regard to their resistance to inactivation by proteolytic enzymes and by heat. In general the haemagglutinins appeared to be quite stable, no loss of activity being observed after routine manipulations such as dialysis, freeze-thawing and lyophilisation (data not shown). Additionally, it was found that full expression of the guinea pig erythrocyte haemagglutinating activity depended on the availability of $Ca^{++}$ ions and that the inhibitory effect of chelating agents such as EDTA or citrate was wholly reversible.

The results obtained in this chapter are summarised in Table 3.9. It is clear that the properties of the sheep and guinea pig erythrocyte haemagglutinating activities are quite different. The sheep erythrocyte haemagglutinating activity was resistant to proteolysis, heat and reduction and did not require $Ca^{++}$ ions. In contrast, the guinea pig erythrocyte haemagglutinating activity was labile to all the proteases tested, to heating as well as reduction and $Ca^{++}$ ions were required for activity. While both agglutinins were sensitive

TABLE 3.9
Some properties of the *B. leachii* haemagglutinins

| Haemagglutinating activity for erythrocytes from | Sensitivity to* | | | | Divalent cation requirement |
|---|---|---|---|---|---|
| | Denaturation | Reduction | Proteolysis | heat (60°) | |
| Sheep | + | R | R | R | None |
| Guinea pig | ++ | L | L | L | $Ca^{++}$ |

* R = resistant; L= labile

to denaturation in 8 M urea, the sheep erythrocyte haemagglutinating activity was significantly more stable than the guinea pig erythrocyte activity. These results provided strong evidence that different molecules were responsible for the sheep erythrocyte and guinea pig erythrocyte haemagglutinating activities.

An interesting aspect of these experiments was the finding that the haemagglutinating activity for guinea pig erythrocytes was more resistant to heat in the presence of EDTA than it was in the presence of $Ca^{++}$ ions. In most cases where ascidian agglutinins have been reported to be $Ca^{++}$-dependent (Chapter 1) the influence of $Ca^{++}$ and EDTA on heat stability has not been investigated. However, in the few reported cases where $Ca^{++}$-dependent haemagglutinins of both plant and invertebrate origin have been tested for heat sensitivity, $Ca^{++}$ has enhanced the heat stability of the molecules (e.g. McDade and Tripp, 1967; Arimotto and Tripp, 1977; Doyle, Thomasson, and Nicholson, 1976). The polypeptide chains of proteins are not arranged as random coil polymers but are maintained in a precise conformation by the non-covalent intermolecular forces of hydrogen, ionic and hydrophobic bonding (White, Handler and Smith, 1973). Heating causes increased movement of the chains, ultimately leading to the breaking of the bonds and denaturation of the protein. Thus, differences in the susceptibility of proteins to heat denaturation reflect differences in the non-covalent bond structure (i.e. the conformation) of the proteins (Tanford, 1968). It appears, therefore, that the $Ca^{++}$ dependent proteins adopt different conformations

depending on whether $Ca^{++}$ ions are present or absent. The *B. leachii* guinea pig erythrocyte haemagglutinin seems to adopt a looser, less stable conformation in the presence of $Ca^{++}$ ions whereas the opposite appears to be true for the other molecules cited.

The guinea pig erythrocyte haemagglutinating activity was much more sensitive to digestion with pronase and trypsin in the presence of EDTA than it was in the presence of $Ca^{++}$. This result was unexpected since the EDTA had been included in control tubes to inhibit enzymatic activity. The loss of haemagglutinating activity was obviously due to enzymatic activity since EDTA alone had no effect and incubation, at least in the case of trypsin, with phenylmethylsulphonyl fluoride (an inhibitor of trypsin) prevented inactivation of the haemagglutinin(s). Evidently, these enzyme(s) were still active in the presence of EDTA, although independent spectrophotometric measurements of trypsin activity using the synthetic substrate TAME (p-Tosyl-L-arginine methyl ester HCl; Hummel, 1959), showed that the enzyme was considerably more active in $Ca^{++}$ than it was in EDTA (data not shown). We are left with the conclusion that the guinea pig erythrocyte haemagglutinin(s) was more sensitive to proteolytic digestion in the presence of EDTA than it was in $Ca^{++}$. Clearly, the removal of $Ca^{++}$ ions from this agglutinin must cause conformational changes which increase the susceptibility of the molecule to proteolytic digestion, a conclusion consistent with the results of the heat sensitivity experiments described above.

A comparison of Tables 1.5 and 1.6 with Table 3.9 shows

that the *B. leachii* haemagglutinins share some common features with haemagglutinins from other ascidians. Thus, there are some like the *B. leachii* guinea pig erythrocyte haemagglutinin that require $Ca^{++}$ for activity and are particularly labile to heat and to reduction. Others like the *B. leachii* sheep erythrocyte haemagglutinin do not require $Ca^{++}$ or other divalent cations for activity and are relatively protease resistant. These features, however, do not always occur together. For instance, in common with the *B. leachii* sheep erythrocyte haemagglutinin, the haemagglutinins from *Phallusia mamillata* and *Ascidia malaca* do not require $Ca^{++}$ ions for activity but, unlike the *B. leachii* sheep erythrocyte haemagglutinin, they lose activity upon reduction. Unfortunately, this kind of information is really too limited to allow one to derive any significant conclusions about the similarity or otherwise of these molecules, except to say that they are almost certainly proteins. This situation is unlikely to change appreciably until these molecules are purified and properly characterized as has been done for the agglutinins from, for example, *Helix pomatia* (review; Chapter 1), *Homarus americana* (review; Chapter 1), *Tridacna maxima* (Baldo, Sawyer, Stick and Uhlenbruck, 1978) and *Limulus polyphemus* (Marchalonis and Edelman, 1968; Fernandex-Moran, Marchalonis and Edelman, 1968; Kaplan, Li and Kehoe, 1977).

## 3.10 Summary

1) The haemolymph from the ascidian *B. leachii* was found to contain haemagglutinating activity for erythrocytes from

a variety of mammalian and avian species.

2) The susceptibility of the agglutinating activity for sheep and guinea pig erythrocytes to such treatments as dialysis, heating, denaturation, alkylation, proteolysis and exposure to low pH was investigated. It was concluded from the results (Table 3.9) that the agglutination of these erythrocytes was due to different haemagglutinins.

# CHAPTER 4

## Resolution of the haemagglutinins and characterization of their binding specificities

## 4.1 Preamble

The haemagglutinins found in plants and invertebrates have been shown in all the cases where specificity has been investigated to bind carbohydrates. In the protochordates, however, there was at the time this work commenced only one example of a sugar-specific haemagglutinin. This was from the haemolymph of the ascidian *Halocynthia pyriformis* (Anderson and Good, 1975).

As reported in Chapter 3, *B. leachii* haemolymph can agglutinate erythrocytes from a large variety of vertebrate species. Broad spectrum haemagglutinating activity has also been reported to be displayed by haemolymph from a number of other ascidian species (Chapter 1). The question of whether such activity is due to a single, monospecific agglutinin or to a number of agglutinins with different specificities has usually been investigated by cross adsorption studies. These studies have led to the conclusion that the broad spectrum activity present in the haemolymph of many ascidians (e.g., *Phallusia mamillata, Acidia malaca*; Parrinello and Patricolo, 1975: *Halocynthia pyriformis*; Anderson and Good, 1975: *Styela plicata*; Fuke and Sugai, 1972) is due in each case to a single agglutinin. However, most of these experiments were essentially qualitative in nature. For example, haemolymph was adsorped one or more times with a particular type of erythrocyte and the effect on the haemagglutinating activity for a variety of erythrocyte types then assessed. More quantitative studies (e.g., measuring the effect of erythrocyte dose) may have revealed heterogeneities in specificity not detected in

these experiments. In addition, in many of these experiments only a small subset of the erythrocytes to which agglutinating activity had been detected were used in adsorption tests.

In contrast to the experiments described above, experiments in which the agglutinating activity for erythrocytes from sheep, mouse and guinea pigs had different requirements for divalent cations (Table 1.5) indicated the presence of several agglutinins in haemolymph from the ascidians *Didemnum patulum*, *Micocosmus nichollsi*, *Halocynthia hispidia* and *Cnemidocarpa etheridgii*. Similarly, differences in the susceptibility of the sheep and guinea pig erythrocyte haemagglutinating activity of *B. leachii* haemolymph (Chapter 3) to various treatments suggested that several agglutinins might be present in the haemolymph of this ascidian.

In this chapter the number of agglutinins, and their specificities, present in the haemolymph of *B. leachii* and the closely related ascidian *B. schlosseri* were investigated.

## Experimental

### 4.2 The inhibition by sugars of the haemagglutinins present in *B. leachii* haemolymph

Many of the agglutinins detected in the haemolymph or coelomic fluid of a variety of invertebrates, including one ascidian, have been characterized as carbohydrate binding proteins. It was therefore considered a distinct possibility that the agglutinins in *B. leachii* haemolymph may also bind carbohydrates. Accordingly, the ability of a number of simple sugars to inhibit the agglutination of

erythrocytes from several species of vertebrates by *B. leachii* haemolymph was tested.

Erythrocytes from three different vertebrates (sheep, pigeon and guinea pig) were chosen for use for the following reasons:-

a) The titres obtained with these cells spanned the range of haemagglutinating activity found in haemolymph, viz the activity was high for guinea pig erythrocytes (1/4000), intermediate for pigeon (1/1600) and low for sheep (1/64).

b) The results presented in Chapter 3 on the susceptibility of the haemagglutinin(s) to various chemical treatments had indicated that the agglutinins for sheep and guinea pig erythrocytes were probably different.

The inhibitory effect of simple sugars was investigated by titrating the haemolymph, which had been exhaustively dialysed against PBS, in different concentrations of sugar, as described in Chapter 2, section 2.6. Some of the results obtained using guinea pig erythrocytes are shown in Table 4.1. It can be seen that the activity was progressively decreased in the presence of increasing concentrations of lactose. Thus, starting at 0.5 mM, the titre was approximately halved every time the lactose concentration was doubled. The same general pattern occurred for D-galactose and D-glucose. However, the absolute concentrations of sugar required for inhibition varied quite markedly. Lactose began to inhibit at 0.5 mM, D-galactose at 0.5 - 1.0 mM, D-glucose at 8 mM and D-arabinose did not inhibit even at 16 mM. In order to compare the effect of the different sugars on a quantitative basis, the inhibitory

TABLE 4.1

The effect of several saccharides on the agglutination of guinea pig erythrocytes by *B. leachii* haemolymph

| Inhibitor | Inhibitor concentration (mM)* | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | 4.0 | 8.0 | 16.0 |
| | Agglutination titre$^{-1}$ | | | | | | |
| Lactose | 3200 | 1600 | 800 | 400 | 200 | <200 | <200 |
| D-Galactose | 3200 | 1600 | 1600 | 400 | 400 | <200 | <200 |
| D-Glucose | 3200 | 3200 | 3200 | 3200 | 3200 | 1600 | 800 |
| D-Arabinose | 3200 | 3200 | 3200 | 3200 | 3200 | 3200 | 3200 |

* Final concentration in each well of the microtitre tray.

capacity was defined as that concentration of sugar found to reduce the haemagglutination titre to exactly 50% of the uninhibited (control) value. This was considered valid since the inhibition by each sugar showed a similar concentration dependence. Further data are presented in Table 4.2 using a more extensive array of sugars.

The inhibition of agglutination was considered to occur because of competition between free sugars and the erythrocyte binding sites for the agglutinin. Artifacts arising through masking of the binding sites because of non-specific binding of sugar to the agglutinins or to the cells appeared unlikely for the following reason. In the case of guinea pig erythrocytes, D-galactose and D-fucose were highly inhibitory. However, D-glucose and L-fucose, both of which have essentially the same physicochemical properties as D-galactose and D-fucose, showed a very much lower inhibitory capacity. If the inhibition observed was non-specific, one may have reasonably expected these sugars to have exhibited similar inhibitory activity. Indeed, all the sugars used may on this basis be expected to have the same non-specific inhibitory activity.

The varying capacities of the different sugars to inhibit the haemagglutination of guinea pig, sheep and pigeon erythrocytes (Table 4.2) suggested that the agglutinins for these cells had different binding specificities. Thus agglutination of guinea pig cells was effectively inhibited at a concentration of $\leq$ 8 mM by seven sugars (melibiose $\equiv$ lactose $\geq$ D-galactose $\equiv$ D-fucose $\geq$ stachyose $\equiv$ N-acetyl-D-galactosamine $\geq$

TABLE 4.2

The capacity of sugars to inhibit the agglutination of erythrocytes by *B. leachii* haemolymph*

| Sugar | Concentration of sugar (mM) required to halve the agglutination titre for | | |
|---|---|---|---|
| | Guinea pig erythrocytes | Sheep erythrocytes | Pigeon erythrocytes |
| Melibiose+ | 0.5 | >67 | 4 |
| Lactose§ | 0.5 | 8 | 0.1 |
| D-Fucose | 1 | >32 | >32 |
| D-Galactose | 1 | >67 | 2 |
| Stachyose# | 2 | >67 | 4 |
| N-Acetyl-D-Galactosamine | 2 | >4 | ND |
| L-Arabinose | 2 | ND | >67 |
| D-Glucose | 8 | >67 | >67 |
| D-Xylose | 8 | >67 | >67 |
| L-Fucose | 16 | >67 | >67 |
| D-Arabinose | 32 | >67 | >67 |
| Maltose | 32 | >67 | >67 |
| Mannose | >67 | >67 | >67 |
| Sucrose | >67 | >67 | >67 |
| D-Fructose | >67 | >67 | >67 |

* Haemolymph was dialysed against 100 volumes of PBS (4°, overnight). ND = not done

+ D-Gal-α1→6-D-Glu

§ D-Gal-β1→4-D-Glu

# D-Gal-α1→6-D-Gal-α1→6-D-Glu-β1→2-D-Fructose

L-arabinose), that of pigeon erythrocytes by four sugars (lactose > D-galactose > stachyose ≡ melibiose) and sheep erythrocytes by only one sugar (lactose). Although lactose was in each case the most effective inhibitor, the concentration required to half the titre was different for each cell type.

## 4.3 The inhibition by sugars of the haemagglutinins present in *B. schlosseri* haemolymph

A study similar to that undertaken for *B. leachii* was performed using *B. schlosseri* haemolymph. In this case only guinea pig cells were used since the haemagglutinating titre to sheep and pigeon cells was too low. As can be seen from Table 4.3, the results were similar to those obtained using *B. leachii* haemolymph, suggesting that the guinea pig erythrocyte agglutinins from the two species have a similar binding specificity.

## 4.4 Divalent cation requirements of the haemagglutinins present in *B. schlosseri* haemolymph

The haemagglutinating activity for guinea pig erythrocytes in *B. schlosseri* haemolymph was tested for its divalent cation requirements as was done for *B. leachii* in Chapter 3, section 3.3. It was found (results not shown) that, like *B. leachii* haemolymph guinea pig erythrocyte haemagglutinating activity, the activity in *B. schlosseri* haemolymph was abolished by the removal of divalent cations and restored by the subsequent addition of $Ca^{++}$, but not $Mg^{++}$, ions.

TABLE 4.3

The capacity of sugars to inhibit the agglutination of guinea pig erythrocytes by *B. schlosseri* haemolymph*

| Sugar | Concentration of sugar (mM) required to halve the agglutination titre |
|---|---|
| Lactose | 0.125 |
| D-Galactose | 0.25 |
| L-Arabinose | 2 |
| D-Glucose | 4 |
| D-Xylose | 4 |
| L-Fucose | 16 |
| D-Arabinose | 32 |
| Mannose | 32 |
| Sucrose | 32 |

* Haemolymph was dialysed against 100 volumes of PBS (4°, overnight).

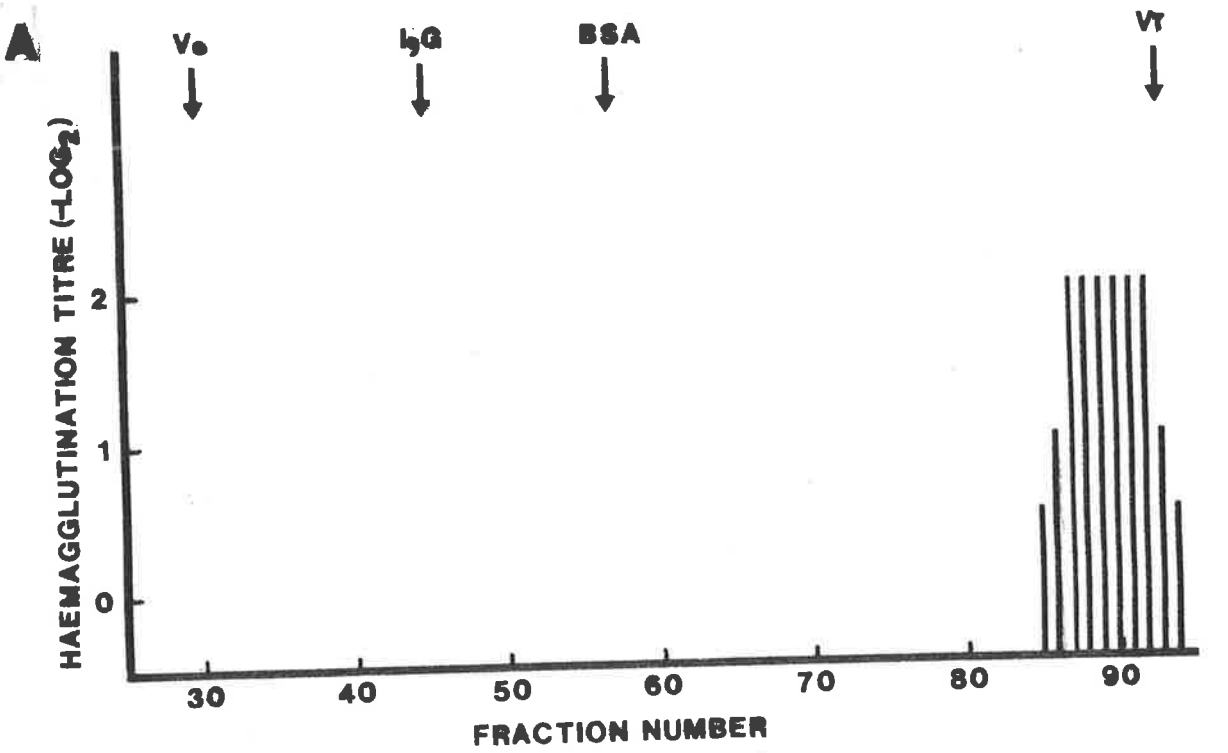
## 4.5 Fractionation of *B. leachii* haemolymph by chromatography on Sephadex G-200

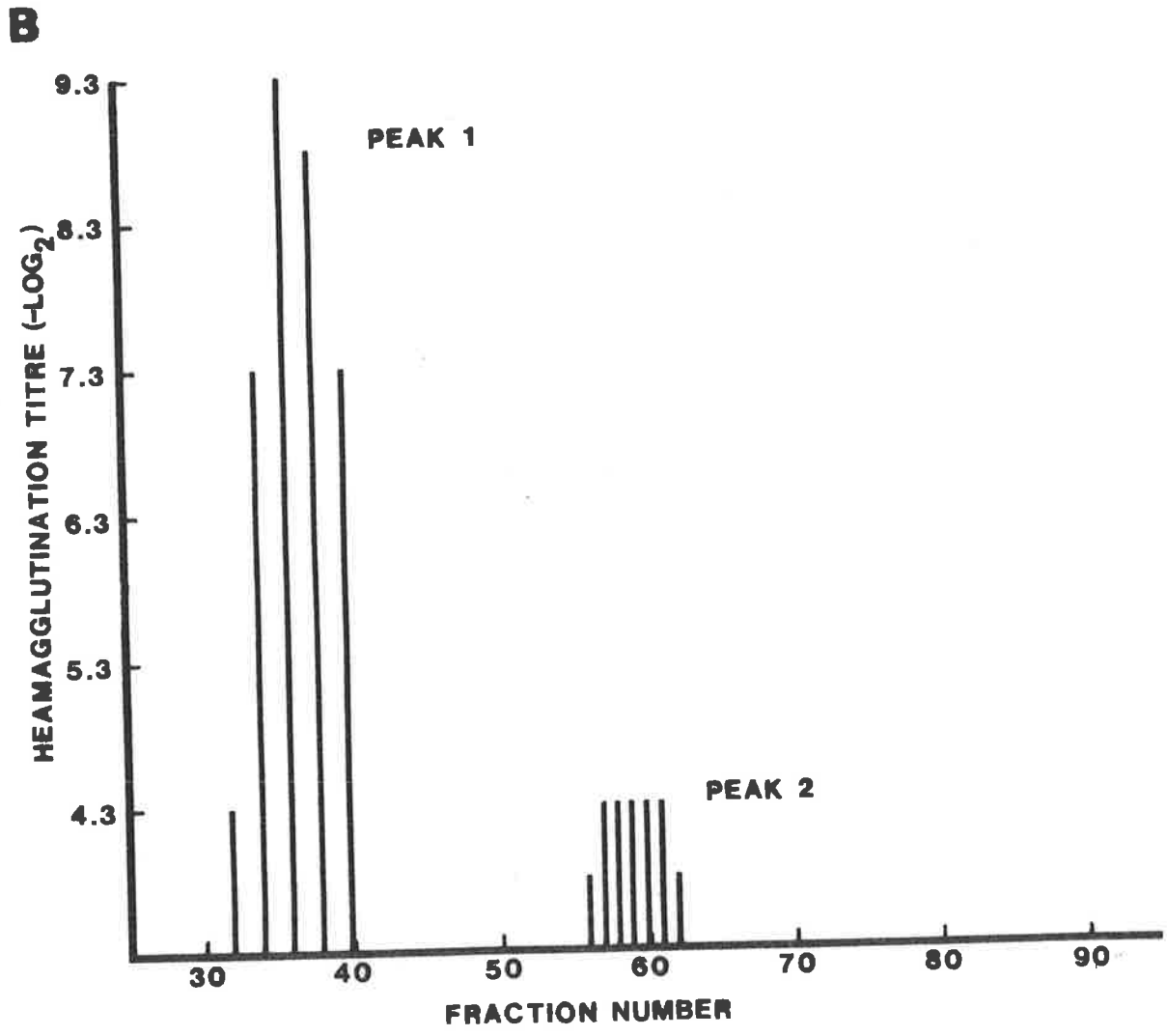
As a preliminary step in the physicochemical characterization of the haemagglutinins in the haemolymph from *B. leachii*, a sample of haemolymph which had been dialysed against PBS was chromatographed on the large Sephadex G-200 column. The fractions were tested for agglutinating activity with guinea pig erythrocytes and the results are shown in Figure 4.1A. Only a small peak of activity representing < 1% of the applied activity was detected. The apparent mol. wt. of the agglutinin, estimated from its elution position, was less than 10,000. This seemed rather unlikely if the agglutinin was a protein. However, since Sephadex is a polymer of glucose, it was considered possible that the passage of the agglutinin(s) through the column had been retarded through specific interaction with the gel matrix (c.f. Table 4.2). To test this proposition, the column was re-equilibrated with buffer containing 50 mM lactose and the haemolymph sample was made 50 mM with respect to lactose before addition to the column. The fractions were diluted 1/20 in PBS and tested for agglutinating activity using guinea pig erythrocytes. The results depicted in Figure 4.1B show that the agglutinins were resolved into two peaks which together contained more than 50% of the activity applied to the column. These results confirmed the hypothesis that the haemagglutinin(s) interacted specifically with the Sephadex in the previous run. The elution position of the agglutinin in peak 1 gave an apparent mol. wt. greater than

FIGURE 4.1

A. Chromatography of B.leachii haemolymph on Sephadex G-200 in the absence of lactose. Sample, 25 ml of haemolymph. Eluant buffer, PBS-azide containing 0.5 mM EDTA. Flow rate = 72 ml/hr. Temperature = 4°. Fraction size = 21 ml. Fractions were tested for agglutinating activity using guinea pig erythroctes. The elution positions of blue dextran (Vo), mouse immunoglobulin G (IgG), bovine serum albumin (BSA) and para-nitrophenol (Vt) are indicated.

B. Chromatography of B.leachii haemolymph on Sephadex G-200 in the presence of lactose. Sample, 25 ml of haemolymph containing 50 mM lactose. Eluant buffer, PBS-azide containing 0.5 mM EDTA and 50 mM lactose. To circumvent inhibition by lactose, all fractions were diluted 1/20 in saline before being tested for agglutining activity using guinea pig erythrocytes.


A
Vo
IgG
BSA
Vt
HAEMAGGLUTINATION TITRE (-LOG2)
0
1
2
30
40
50
60
70
80
90
FRACTION NUMBER


B
9.3
8.3
7.3
6.3
5.3
4.3
HEAMAGGLUTINATION TITRE (-LOG2)
PEAK 1
PEAK 2
30
40
50
60
70
80
90
FRACTION NUMBER

150,000 while the agglutinin in peak 2 appeared to have a mol. wt. less than 67,000. The haemagglutinating activity recovered in peak 2 was approximately 4% of the activity recovered in peak 1.

## 4.6 Analysis of the haemagglutinins by sedimentation velocity in sucrose density gradients

To confirm that the peak 1 and peak 2 agglutinins did indeed differ in mol. wt. as indicated by the gel filtration data, the material in each peak was further analysed by sedimentation velocity in sucrose density gradients. This was considered necessary because of the possibility that in the previous experiment (Fig. 4.1B) the lactose had not completely prevented the interaction of the agglutinins with the Sephadex, i.e. due to residual retardation effects, the elution positions did not reflect true molecular size. This complication does not occur in sedimentation velocity.

The fractions representing peak 1 and peak 2 (Fig. 4.1B) were separately pooled and concentrated to 20 ml in an Amicon Model 202 ultrafiltration unit using PM30 and PM10 membranes, respectively. The sucrose gradients were prepared, run and fractionated as described in Chapter 2, section 2.12. Iodine-125 labelled BSA was included as a marker in all samples so that the different gradients could be correctly aligned and compared.

The Sephadex G-200 peak 2 agglutinin(s) moved in sucrose gradients as a homogeneous band at a rate just slower than BSA (Fig. 4.2), indicating that these

FIGURE 4.2

Sedimentation velocity ultracentrifugation in linear sucrose gradients of B. leachii haemagglutinins.

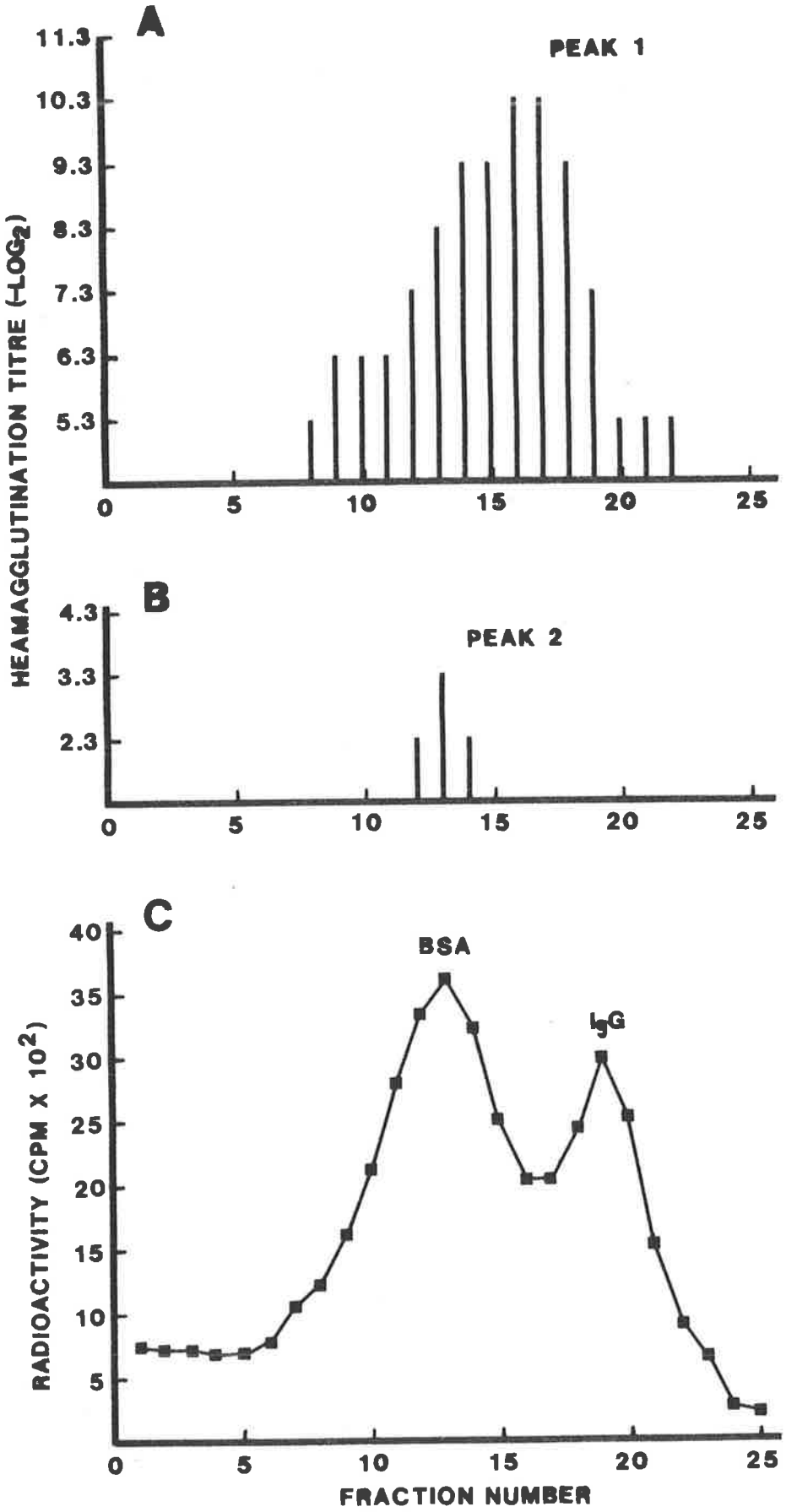
A. Sample: 250 ul of concentrated Sephadex G-200 peak 1 material (Fig. 4.1B), titre vs GPRBC = 1/1,600; 10 ul each of radiolabelled BSA and mouse IgG. Fraction size was 0.2 ml.

| haemagglutination titre for guinea pig erythrocytes

B. Sample: 250 ul of concentrated peak 2 material, (Fig. 4.1B), titre vs SRBC = 1/64; 10 ul each of radiolabelled BSA and mouse IgG. Fraction size was 0.2 ml.

| haemagglutination titre for sheep erythrocytes

C. Radioactivity profile (■——■) of gradient in (A). Profile of gradient in (B) coincided exactly with this.


A
PEAK 1
11.3
10.3
9.3
8.3
7.3
6.3
5.3
HEAMAGGLUTINATION TITRE (-LOG2)
0
5
10
15
20
25
B
PEAK 2
4.3
3.3
2.3
0
5
10
15
20
25
C
BSA
IgG
40
35
30
25
20
15
10
5
RADIOACTIVITY (CPM X 10^2)
0
5
10
15
20
25
FRACTION NUMBER

agglutinins had a mol. wt. slightly less than 67,000. This conclusion was in complete agreement with the gel filtration data. The peak 1 agglutinin(s) migrated as a homogeneous band at a rate much faster than peak 2 material but slower than IgG, indicating that they had a mol. wt. less than 150,000. This was in apparent contradiction with the gel filtration data, which indicated that the peak 1 agglutinin(s) were larger than 150,000. Thus, these molecules migrated faster on Sephadex G-200 than would have been expected from their apparent size as estimated by sedimentation velocity and it is clear, therefore, that no interaction could have occurred with the Sephadex in the presence of lactose in the experiment of Figure 4.1B.

The anomalous behaviour of the peak 1 agglutinin(s) in gel filtration and sedimentation velocity will be discussed in detail in Chapter 7. Because the sedimentation velocity results confirmed that the peak 1 and peak 2 agglutinins differed in size, they were termed Haemagglutinins type one (HA-1) and Haemagglutinins type 2 (HA-2) respectively.

The results obtained when *B. leachii* haemolymph was subjected to sedimentation velocity in the presence and absence of lactose are shown in Figure 4.3. The single guinea pig erythrocyte agglutinin peak observed probably represents HA-1 molecules. The presence of HA-2 molecules was probably obscured by overlap with this peak. It should be noticed that the position of the peak was not altered by the presence of lactose in the sucrose gradient. This rules out the remote possibility that the larger apparent size for

FIGURE 4.3

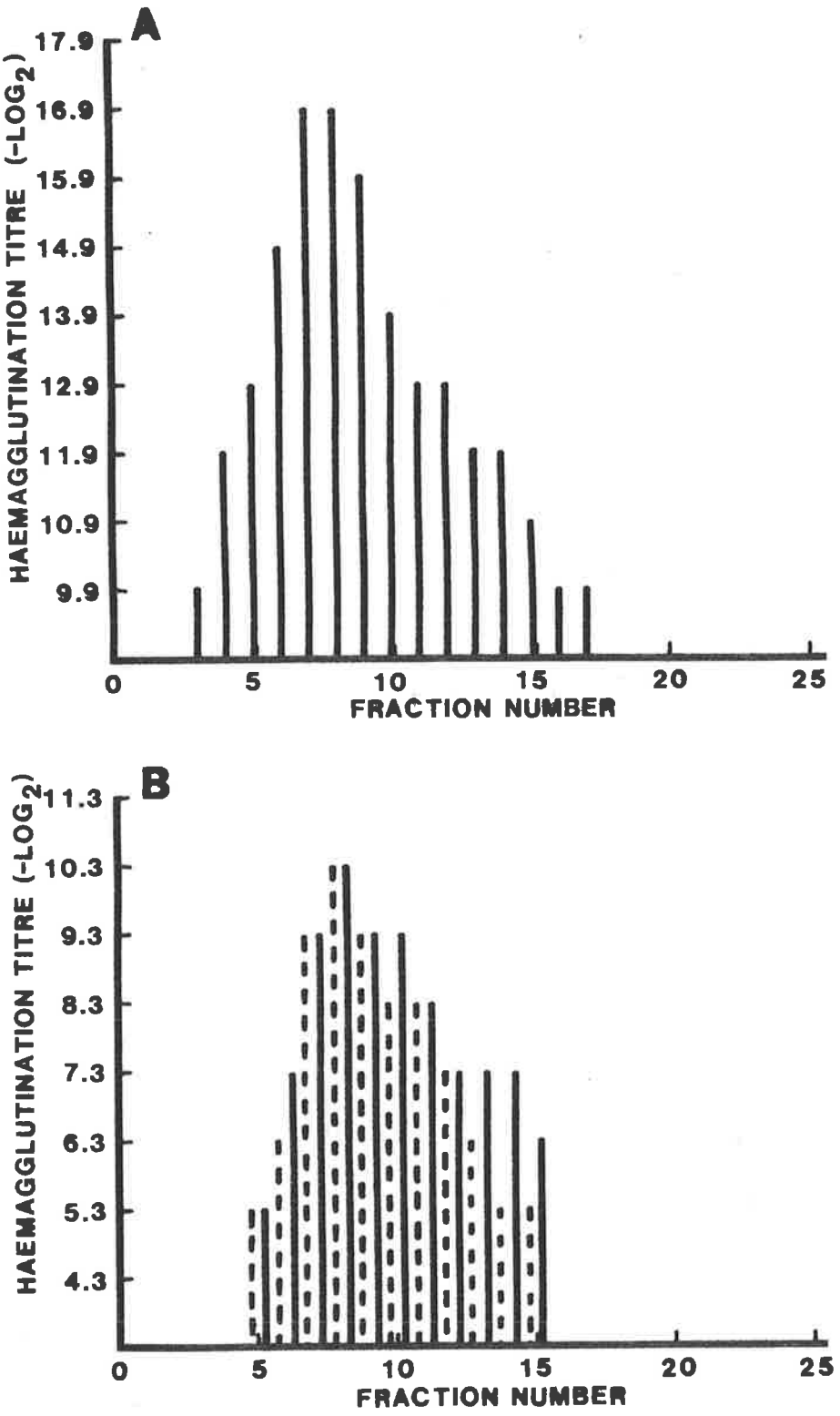
Sedimentation velocity ultracentrifugation of B. leachii haemolymph through linear sucrose gradients. Fraction size was 0.2 ml. Each sample (200 ul) contained 10 ul of radiolabelled BSA. The figures have been drawn by aligning these radioactivity peaks. Haemagglutinating activity for guinea pig erythrocytes is shown.

A. Sample; 200 ul of B. schlosseri haemolymph. Gradient contained no lactose.

B. Sedimentation of B. leachii haemolymph in the presence and absence of lactose.

gradient contained no lactose

gradient contained 0.025 M lactose


A
17.9
16.9
15.9
14.9
13.9
12.9
11.9
10.9
9.9
HAEMAGGLUTINATION TITRE (-LOG2)
0
5
10
15
20
25
FRACTION NUMBER
B
11.3
10.3
9.3
8.3
7.3
6.3
5.3
4.3
HAEMAGGLUTINATION TITRE (-LOG2)
0
5
10
15
20
25
FRACTION NUMBER

the HA-1 agglutinin observed during chromatography on Sephadex G-200 (Fig. 4.1B) was due to cross-linkage of the agglutinin molecules by lactose.

The results obtained when *B. schlosseri* haemolymph was subjected to sedimentation velocity analysis are shown in Figure 4.3. A point to note is that the agglutinin(s) for guinea pig erythrocytes present in *B. schlosseri* haemolymph sedimented in an identical manner to the *B. leachii* HA-1 agglutinins. These agglutinins from these ascidians must therefore be similar in size.

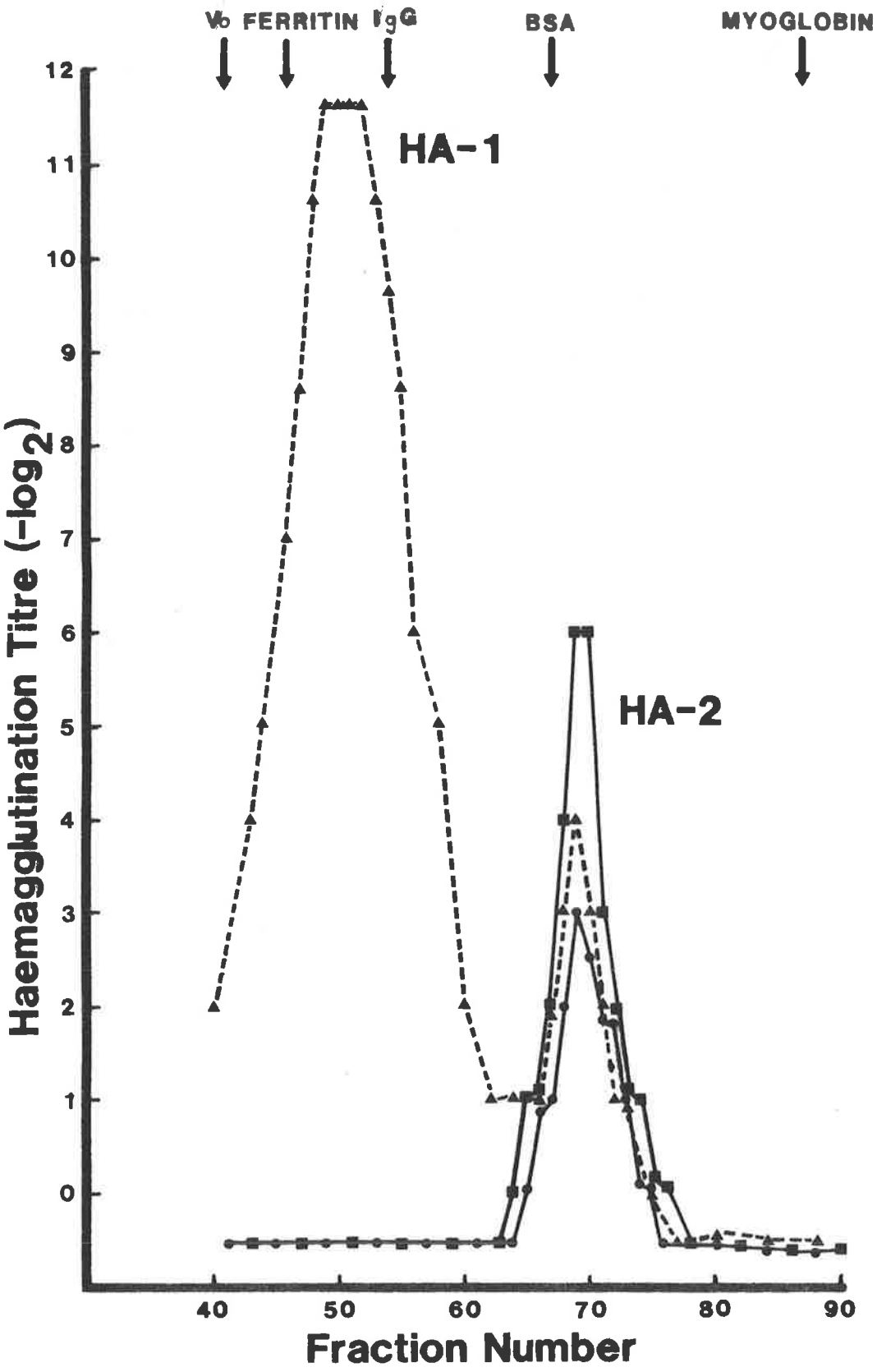
## 4.7 Further analysis of the *B. leachii* HA-1 and HA-2 agglutinins by Sephadex G-200 chromatography

An analytical column of Sephadex G-200 was prepared in order to obtain more precise data on the chromatographic behaviour of the haemagglutinins. To prevent adsorption of the haemagglutinins to the column, lactose and galactose were included in the eluant (50 mM and 100 mM respectively) since these sugars were among the most highly inhibitory of those tested (Table 4.2). The procedure is outlined in Chapter 2, section 2.10.

The analysis of 5 ml of dialysed *B. leachii* haemolymph on this column is depicted in Figure 4.4. Each fraction was assayed after dialysis against guinea pig, sheep, pigeon, human and mouse erythrocytes. As was previously found (Fig. 4.1B) the HA-1 peak contained essentially all of the guinea pig erythrocyte specific activity applied to the column. No activity to any of the other cell types was found in this peak. In contrast, the

FIGURE 4.4

Separation of B. leachii haemagglutinins by chromatography of haemolymph on Sephadex G-200. The eluant buffer contained 50 mM lactose and 100 mM galactose. The elution positions of marker proteins are shown. The fractions were dialysed against TSA before being assayed for agglutinating activity using guinea pig (▲------▲), pigeon (■——■) and sheep erythrocytes (●——●). The OD (280 nm) of fractions 51 and 70 was negligible.


Vo
FERRITIN
IgG
BSA
MYOGLOBIN
HA-1
HA-2
Haemagglutination Titre (-log2)
Fraction Number
0
1
2
3
4
5
6
7
8
9
10
11
12
40
50
60
70
80
90

material in the HA-2 peak accounted for only 0.5% of the applied activity for guinea pig erythrocytes. However, these fractions agglutinated erythrocytes from all the other species tested. The recovery of the latter activities was at least 50%. The agglutinating titre of fraction 69 (the HA-2 peak fraction) for all the erythrocyte tested was 1/8 - 1/16 except for pigeon erythrocytes for which it was 1/64.

The column was calibrated using proteins of known mol. wt. (Chapter 2, section 2.10) and using an empirically-derived relationship between Kav (a standardised partition coefficient; Laurent and Killander, 1964) and mol. wt. (Pharmacia Gel Filtration Handbook, 1979), the mol. wts. of the HA-1 and HA-2 agglutinins were estimated to be at least 200,000 and 63,000 respectively.

## 4.8 The binding specificities of the HA-1 and HA-2 agglutinins

The preceding results demonstrated the presence of at least two agglutinins in *B. leachii* haemolymph. Although the agglutinins appeared to differ both in size and in binding specificity, the possibility could not be excluded that the HA-2 molecules were sub-units or a degraded form of the HA-1 agglutinin. Apparent differences in agglutinating specificity and/or titre might in such cases result from differences in the nature and density of receptor sites on various cells and from differences in the avidity and agglutinating efficiency of the different forms of the agglutinin (Ehrlich, 1979).

The binding specificities of both agglutinins were therefore examined by testing the capacity of various sugars to inhibit haemagglutination, as was done previously for unfractionated haemolymph. The HA-1 and HA-2 preparations were obtained by pooling and concentrating the fractions representing each peak. The results, presented in Table 4.4, revealed marked differences in the binding specificity of the two agglutinins. The agglutinating titre of the HA-1 preparation for guinea pig cells was halved, for example, in the presence of 0.5 mM lactose or 1 mM D-galactose, whereas that of the HA-2 preparation was halved in the presence of 4 mM lactose but not affected by even 67 mM galactose. When these results (Table 4.4) are considered in conjunction with those obtained for whole haemolymph (Table 4.2) and with the overall recovery of agglutinating activity in the two peaks (Fig. 4.2), it is clear that these two haemagglutinins account for the whole activity of unfractionated haemolymph.

## 4.9 Adsorption of HA-1 and HA-2 agglutinins with guinea pig and sheep erythrocytes

The different binding specificities of the HA-1 and HA-2 agglutinins were confirmed by adsorption experiments with guinea pig and sheep erythrocytes. Guinea pig erythrocytes adsorbed all the activity against guinea pig erythrocytes from each preparation and also adsorbed the sheep erythrocyte agglutinin(s) from the HA-2 preparation. In contrast, sheep cells adsorbed both sheep and guinea pig erythrocyte agglutinating activity from the HA-2 preparation but caused no loss of the HA-1 activity. These

TABLE 4.4

The capacity of various sugars to inhibit the agglutination of erythrocytes by HA-1 and HA-2 agglutinins*

| Sugar | Concentration of sugar (mM) required to halve the haemagglutination titre | | | | | |
|---|---|---|---|---|---|---|
| | HA-1 | HA-2 | | | | |
| | Guinea pig erythrocytes§ | Pigeon erythrocytes | Guinea pig erythrocytes | Sheep erythrocytes | Human erythrocytes | Mouse erythrocytes |
| Lactose | 0.5 | 0.1 | 4 | 8 | 8 | 8 |
| D-Galactose | 1 | 2 | >67 | >67 | >67 | >67 |
| Melibiose | 0.5 | 4 | >67 | >67 | >67 | >67 |
| L-Arabinose | 4 | >67 | >67 | >67 | >67 | >67 |
| D-Glucose | 8 | >67 | >67 | >67 | >67 | >67 |
| D-Xylose | 16 | >67 | >67 | >67 | >67 | >67 |
| L-Fucose | 16 | >67 | >67 | >67 | >67 | >67 |
| D-Arabinose | 32 | >67 | >67 | >67 | >67 | >67 |
| Maltose | 67 | >67 | >67 | 67 | >67 | >67 |

* Each haemagglutinin was isolated by fractionation of haemolymph on Sephadex G-200 as per Figure 4.2.

§ Agglutinating activity for other types of erythrocytes was undetectable.

results further indicated that the HA-1 and HA-2 agglutinins have different binding specificities and that the receptor site(s) recognized by HA-1 molecules are unique to guinea pig erythrocytes.

## 4.10 Divalent cation requirements of the HA-1 and HA-2 agglutinins

In Chapter 3 it was shown that the agglutinating activity of *B. leachii* haemolymph for guinea pig erythrocytes required $Ca^{++}$ ions. Since both HA-1 and HA-2 were active against guinea pig erythrocytes, it was of interest to see whether their divalent cation requirements were the same. This was done as described for haemolymph in Chapter 3. It was found that HA-1 required $Ca^{++}$ for activity. The HA-2 activity exhibited no divalent cation requirement whatsoever.

## 4.11 The binding specificity of the HA-2 agglutinins

The sugar inhibition data (Table 4.4) indicated that the agglutinins in the HA-2 peak responsible for the activity towards mouse, sheep, human and guinea pig erythrocytes had a similar binding site specificity. However, one could not be certain from this result alone that the HA-2 activity was the result of a single homogeneous agglutinin. For instance, the agglutinins from the jack bean and the lentil are both specifically inhibited by mannose and glucose derivatives suggesting that they have the same binding site specificity. However, since the lentil agglutinin can bind to two glycoproteins on the

surface of human erythrocytes (protein III and glycophorin) whereas the jack bean agglutinin can only bind to one (protein III), it is clear that these agglutinins have different binding site specificities (Lis and Sharon, 1977).

To clarify this point, further adsorption experiments were performed. Although each type of erythrocyte could adsorb the activity toward the others, it was felt that a quantitative assessment of the effect of cell dose on the amount of activity adsorbed might reveal differences in binding specificity. Initially whole cell suspensions were used for adsorption but consistent results could not be obtained. The cells rapidly agglutinated and clumped upon being mixed with the agglutinin preparations and this may have prevented complete adsorption of the agglutinins. In an attempt to alleviate this problem, membrane vesicles, which are much smaller than whole cells, were prepared (Chapter 2, section 2.7) and used for adsorption. For each type of erythrocyte (mouse, sheep and human) a suspension equivalent to 8% v/v intact cells was serially diluted 2-fold in 100 µl aliquots, yielding six dilutions representing concentrations from 8% v/v to 0.25% v/v. The HA-2 solution (Fig. 4.2; peak 2 concentrate) (300 µl) was added to these suspensions and immediately mixed on a vortex mixer. The adsorption procedure is described fully in Chapter 2, section 2.8.

Accurate determinations of the agglutinating activity remaining in each supernatant for sheep, human, mouse and guinea pig erythrocytes were obtained as described in

Chapter 2, section 2.5. For example, the results obtained when supernatants recovered after adsorption with sheep erythrocyte vesicles were assayed against sheep erythrocytes are presented in Figure 4.5. When the settled cell pellet diameters (Fig. 4.5B), measured from the photograph, were plotted against haemagglutinin dilution (well number), symmetrically shaped, sigmoidal curves were obtained (Fig. 4.6). The exact dilution required to give 50% agglutination could then be easily derived from these plots (Fig. 4.6). A compilation of all the results obtained is shown in Table 4.5. There was some variation in the results, but the general trend is clear. At all vesicle concentrations, each cell type was able to adsorb to a similar degree the haemagglutinating activity against all the other cell types as well as against itself, i.e. no significant differential adsorption of activities occurred.

These results are consistent with the sugar inhibition data (presented in Table 4.4) and indicate that a single agglutinin was responsible for the haemagglutinating activity of the HA-2 fraction against the above panel of erythrocytes.

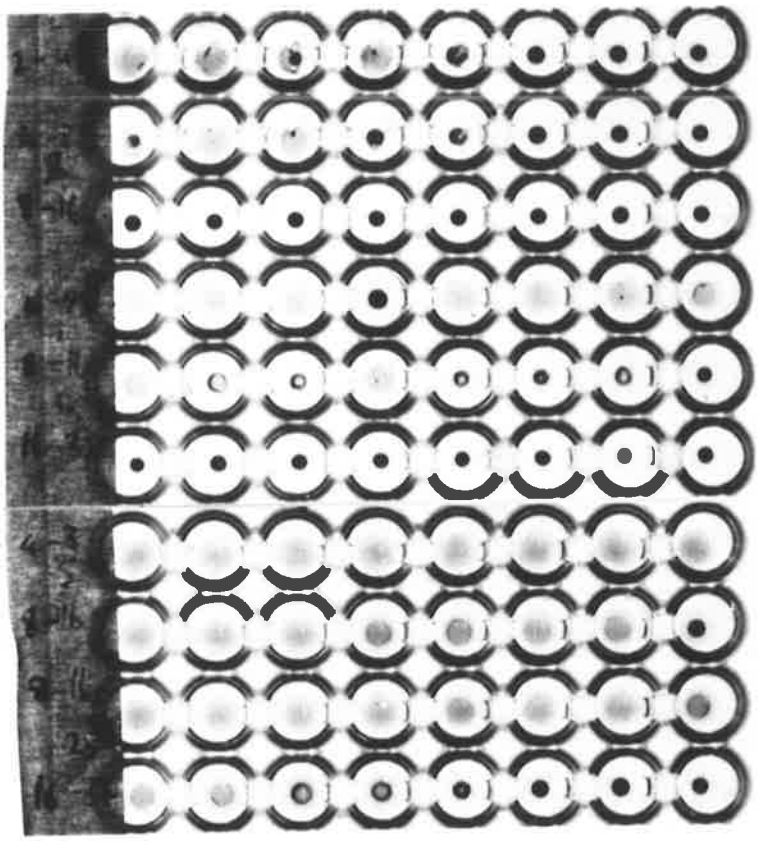
## 4.12 Further studies on the HA-2 binding site specificity: pigeon erythrocytes

The results of the sugar inhibition studies (Table 4.4) in which the agglutination of pigeon erythrocytes was more easily inhibited by lactose and galactose, suggested that the agglutinin for pigeon erythrocytes may be distinct from the agglutinins for the other types of erythrocytes.

FIGURE 4.5

Adsorption of HA-2 activity by various concentrations of sheep erythrocyte vesicles: titration against sheep cells of the supernatants recovered after adsorption. The details of the adsorption procedure are given in the text. After determining the approximate agglutination titre of each supernatant using the standard serial 2-fold dilution assay, the exact dilution required to give a 50% agglutination end point was accurately determined by retitrating each sample using fractional dilutions around the approximate end-point, as described in Chapter 2, section 2.5. For example, the supernatant obtained after adsorbing the HA-2 with 4% vesicles caused full agglutination at a 1/2 dilution but negligible agglutination at a 1/4 dilution. The 50% agglutination titre must therefore lay between 1/2 and 1/4. Accordingly, 1/2 and 1/4 dilutions of the supernatants were prepared (solutions (a) and (b) respectively) and these were mixed in the wells of the microtitre tray in varying proportions ranging from 100% of (a) to 100% of (b) (total volume = 21 ul). Thus, the ratio of solution (a) to solution (b) in the 8 wells from left to right was 7:0, 6:1, 5:2, 4:3, 3:4, 2:5, 1:6, 0:7. This ratio series was used for all supernatants, the dilution of solutions (a) and (b) in each instance depending on the approximate agglutination titre of the supernatant. The pattern obtained after fractional titration of the supernatants is shown in the photograph. Each row depicts the titration of the supernatant obtained by adsorption with the concentration of membrane vesicles indicated. The dilution range represents the reciprocal dilution of solutions (a) and (b) (eg, row 1, solution a = 1/2; solution b = 1/4). The results are summarised in the table and plotted in Figure 4.6.

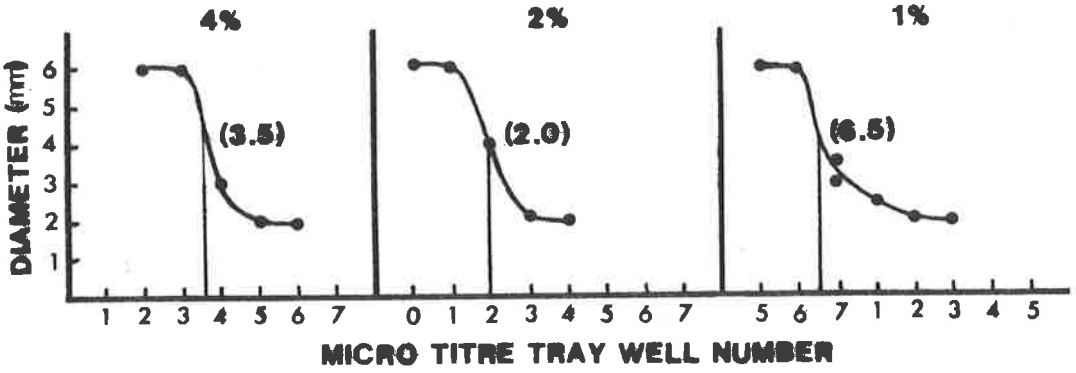
| VESICLE CONC. | DILUTION RANGE |
|---|---|
| 4 | 2-4 |
| 2 | 4-8 |
| 2 | 8-16 |
| 1 | 4-8 |
| 1 | 8-16 |
| 1 | 16-32 |
| 0.5 | 4-8 |
| 0.5 | 8-16 |
| 0.25 | 8-16 |
| 0.25 | 16-32 |



| Vesicle concentration | Dilution range | Diameter(mm) of pellet in well number | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 4 | 2-4 | 6 | 6 | 6 | 6 | 3 | 2 | 2 | 2 |
| 2 | 4-8 | 6 | 6 | 4 | 2 | 2 | 2 | 2 | 2 |
| 1 | 4-8 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 3 |
| 1 | 8-16 | 3.5 | 2.5 | 2 | 2 | 2 | 2 | 2 | 2 |
| 0.5 | 8-16 | 6 | 6 | 6 | 5 | 5 | 5 | 3 | 2 |
| 0.25 | 8-16 | 6 | 6 | 6 | 6 | 6 | 6 | 5 | 3 |
| 0.25 | 16-32 | 3 | 3 | 2.5 | 2.5 | 2 | 2 | 2 | 2 |

FIGURE 4.6

Adsorption of HA-2 activity by various concentrations of sheep erythrocyte vesicles; determination of the sheep erythrocyte agglutination titre of the supernatant recovered after adsorption using the data obtained in Figure 4.5. The diameters of the settled cell pellets obtained after titrating each supernatant (Fig. 4.5) are plotted against well number (see Table in Fig. 4.5 ). The titre of the supernatants was derived as follows. The plots were used to estimate the well number (shown in parentheses in the Figure.) corresponding to a 50% agglutination endpoint and the dilution was then determined.


4%
2%
1%
DIAMETER (mm)
(3.5)
(2.0)
(6.5)
MICRO TITRE TRAY WELL NUMBER

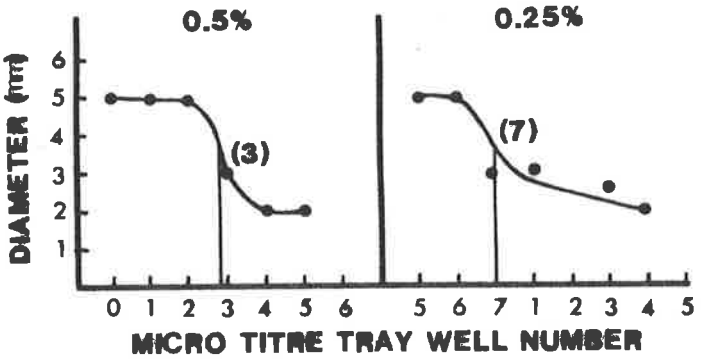

0.5%
0.25%
DIAMETER (mm)
(3)
(7)
MICRO TITRE TRAY WELL NUMBER

TABLE 4.5

Haemagglutinating activity remaining in supernatants after adsorption of HA-2 with different concentrations of various types of erythrocyte vesicles*

1) Mouse erythrocyte vesicles

| Vesicle concentration§ | % agglutinating activity remaining for | | | |
|---|---|---|---|---|
| | Sheep erythro-cytes | Human erythro-cytes | Guinea pig erythro-cytes | Mouse erythro-cytes |
| 0.25 | 80.4 | 78.9 | 108.3 | 112.7 |
| 0.5 | 74.8 | 54.0 | 54.1 | 98.6 |
| 1.0 | 52.3 | 39.4 | 34.5 | 65.5 |
| 2.0 | 37.4 | 18.3 | 13.5 | 41.5 |
| 4.0 | 26.2 | 10.3 | - | 24.6 |

2) Sheep erythrocyte vesiciles

| Vesicle concentration§ | % agglutinating activity remaining for | | | |
|---|---|---|---|---|
| | Sheep erythro-cytes | Human erythro-cytes | Guinea pig erythro-cytes | Mouse erythro-cytes |
| 0.25 | 75.2 | 68.6 | 48.7 | 75.4 |
| 0.5 | 47.7 | 48.7 | 29.1 | 75.4 |
| 1.0 | 35.0 | 25.6 | 20.3 | 56.3 |
| 2.0 | 22.0 | 11.2 | 9.5 | 37.3 |
| 4.0 | 12.6 | 5.5 | - | 16.9 |

3) Human erythrocyte vesicles

| Vesicle concentration§ | % agglutinating activity remaining for | | | |
|---|---|---|---|---|
| | Sheep erythro-cytes | Human erythro-cytes | Guinea pig erythro-cytes | Mouse erythro-cytes |
| 0.25 | 49.5 | 34.1 | 47.4 | 68.3 |
| 0.5 | 40.2 | 30.2 | 39.9 | 63.4 |
| 1.0 | 49.5 | 34.1 | 27.1 | 65.5 |
| 2.0 | 29.0 | 14.2 | 13.2 | 49.3 |
| 4.0 | 14.5 | 4.8 | - | 16.2 |

* HA-2 was isolated by fractionation of haemolymph on Sephadex G-200 as per Figure 4.2.
§ Equivalent to the same % solution of intact cells.

In initial adsorption experiments it was found that pigeon and sheep erythrocytes each adsorbed activity against the other. Since the agglutination of pigeon erythrocytes was more readily inhibited by lactose than was that of sheep erythrocytes (Table 4.4), it was anticipated that the agglutinins might be separated, if they were in fact distinct, by differential adsorption to sheep or pigeon erythrocytes in the presence of a low concentration of lactose. That is, even though each agglutinin was capable of binding to both cell types, low concentrations of lactose should preferentially prevent binding of the agglutinins responsible for pigeon cell agglutination (if as suggested these were distinct and had a greater affinity for lactose) but not of those responsible for sheep cell agglutination. The pigeon cell agglutinin should therefore be recoverable from the supernatant.

The proposition that two agglutinins were present implied that the sheep cell agglutinin bound to but did not agglutinate pigeon erythrocytes since agglutination of these cells would not otherwise have been inhibited by the lower lactose concentrations. Although this seemed unlikely, a precedence for such a phenomenon existed in the case of haemagglutinins which bind to but do not agglutinate tumor cells (Lis and Sharon, 1977).

Samples of haemolymph were incubated with sheep erythrocytes in the presence of a range of concentrations of lactose and the supernatants obtained after centrifugation were titrated against sheep and pigeon erythrocytes. The results are shown in Table 4.6. The experiment was

repeated using pigeon erythrocytes as the adsorbing cell. However, in this case vesicles were used since a large amount of membrane material (equivalent to 26% v/v suspension of erythrocytes) was needed to achieve complete adsorption. The results are shown in Table 4.7. At all the concentrations of lactose used to inhibit adsorption to either type of cell, the agglutinating activity for sheep and pigeon erythrocytes was adsorbed in parallel, i.e. in no case did differential adsorption of activity occur. However, the concentration of lactose needed to inhibit adsorption of the agglutinins by 50% was different for the two adsorbants, being 5 mM for sheep erythrocytes (Table 4.6) and 0.1 - 1.0 mM for pigeon erythrocyte vesicles (Table 4.7). Thus it appeared that a single agglutinin was responsible for the activity to pigeon and sheep cells but that a different concentration of lactose was required to inhibit agglutination in each case.

## 4.13 Discussion

It was shown in this chapter by two independent methods, namely gel filtration chromatography and sedimentation velocity ultracentrifugation, that the haemolymph of *B. leachii* contains two distinct populations of haemagglutinins which have been designated HA-1 and HA-2. HA-1 molecules are the larger (mol. wt. approximately 200,000 by gel filtration or 150,000 by sedimentation velocity) and bind specifically to guinea pig erythrocytes, whereas the smaller (mol. wt. approximately 63,000) HA-2 molecules agglutinated every type of erythrocyte tested. Both these agglutinins are carbohydrate binding molecules.

TABLE 4.6

Effect of lactose on the capacity of sheep erythrocytes to adsorp the HA-2 agglutinins for sheep and pigeon erythrocytes

| Concentration of lactose present during adsorption* (mM) | Agglutinating activity recovered in the dialysed supernatant for (%)§ | |
|---|---|---|
| | Sheep erythrocytes | Pigeon erythrocytes |
| 0.3 | <6 | 6 |
| 0.7 | 6 | 6 |
| 1.4 | 6 | 12 |
| 2.8 | 25 | 25 |
| 5.6 | 50 | 50 |
| 11.0 | 100 | 100 |
| 22.0 | 100 | 100 |

* Samples of dialysed haemolymph (0.5 ml) were mixed with 1 ml aliquots of a 5% suspension of sheep erythrocytes in PBS containing an appropriate concentration of lactose . After a 1 hour incubation (22°), each sample was centrifuged (900 g, 5 min.). The supernatants were dialysed overnight before being titrated against sheep and pigeon erythrocytes.

§ Unadsorbed (100%) titres were 1/32 (sheep erythrocytes) and 1/256 (pigeon erythrocytes).

TABLE 4.7

Effect of lactose on the capacity of pigeon erythrocyte vesicles to adsorp the HA-2 agglutinins for sheep and pigeon erythrocytes

| Concentration of lactose present during adsorption* (mM) | Agglutinating activity recovered in the dialysed supernatant for (%)§ | |
|---|---|---|
| | Sheep erythrocytes | Pigeon erythrocytes |
| 0.00057 | 12.5 | 6.25 |
| 0.0017 | 12.5 | 6.25 |
| 0.005 | 25 | 12.5 |
| 0.015 | 25 | 12.5 |
| 0.046 | 50 | 25 |
| 0.14 | 100 | 50 |
| 0.42 | 100 | 100 |
| 1.25 | 100 | 100 |
| 3.75 | 100 | 100 |
| 11.25 | 100 | 100 |

* Samples of dialysed haemolymph (0.5 ml) were mixed with 1 ml aliquots of a suspension of pigeon erythrocyte vesicles in PBS (equivalent to a 26% suspension of intact cells) containing an appropriate concentration of lactose. After a 1 hour incubation (22°), each sample was centrifuged (27,000 g, 30 min). The supernatants were dialysed overnight before being titrated against sheep and pigeon erythrocytes.

§ Unadsorbed (100%) titres were 1/32 (sheep erythrocytes) and 1/256 (pigeon erythrocytes).

It was initially considered that HA-1 and HA-2 might be different forms of a single agglutinin. This possibility was rejected for the following reasons. Firstly, the HA-1 molecules appeared monospecific whilst the HA-2 molecules were heterospecific, with respect to haemagglutinating activity. Secondly, although guinea pig erythrocytes could adsorb out both HA-1 and HA-2 activities, erythrocytes from other species were able to adsorb only HA-2 activities. Thirdly, the agglutination of guinea pig erythrocytes by HA-1 and HA-2 exhibited different sensitivities to inhibition by various sugars. Loontiens, Van Wauve and De Bruyne, (1975) have shown experimentally that the inhibitory capacity of a sugar is related to its intrinsic association constant for the binding sites of an agglutinin. Although their system was based on the precipitation of a complex carbohydrate by a plant lectin, there is no reason to suspect that this conclusion is not valid for sugar inhibition of haemagglutination reactions generally (see later discussion). Thus, it was concluded from the sugar inhibition data that the individual binding sites of the two *B. leachii* haemagglutinins were different and that the agglutinins were therefore unrelated. Fourthly, the two haemagglutinins had different divalent cation requirements.

Initially in this work it was thought that the concentration of sugar required to inhibit haemagglutination was a consequence only of the interaction of the sugar and the agglutinin and was independent of the type of cell used. Thus, the sugar inhibition results for HA-2 were interpreted

to mean that the agglutinins responsible for the activity to mouse, human, sheep and guinea pig cells had similar if not identical binding sites and that they were, therefore, identical molecules. The different inhibitory capacities shown by lactose and D-galactose, for example, for the inhibition of pigeon cells were taken to indicate that the association constants of these sugars for the pigeon cell agglutinin differed from those for the other agglutinins. Consequently it was suspected that the pigeon cell agglutinin might be a different molecule. The conclusion that there was a single agglutinin for mouse, human, sheep and guinea pig erythrocytes was supported by quantitative adsorption experiments in which all activities were equally affected by adsorption to any single type of cell (Table 4.5). Unexpectedly, however, the pigeon cell agglutinin was shown to be identical with this agglutinin since the binding activities for pigeon and sheep cells were found to be equally sensitive to inhibition by lactose (Tables 4.6 and 4.7). The concentration of lactose required for inhibition depended on the cell type used for adsorption. The association constants of the pigeon and sheep cell agglutinins for lactose must therefore be identical.

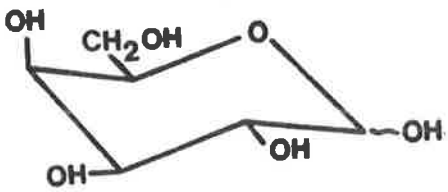
It is worthwhile at this point to consider the thermodynamics involved in the sugar inhibition reaction. Although a precise mathematical analysis would be quite complex (e.g. Reynolds, 1979), the parameters which must be considered when one evaluates sugar inhibition data can be identified by a simple examination of the reactions involved. An analysis of this kind (see Appendix), in the light of the

experimental results, reveals that the differences in sugar concentration required for inhibition of sheep and pigeon cell haemagglutination are best explained as reflecting differences in the affinity of the HA-2 molecules for the respective cell surface receptor sites (i.e. differences in the structure of these receptors) and in the number of receptor sites on each cell, rather than differences in agglutinin binding sites. The thermodynamic analysis also indicates that the assumption that the inhibitory capacity of a sugar is related to its intrinsic association constant for the binding sites of a particular agglutinin is valid providing the cell type is not changed. Sugar inhibition data derived using a single cell type should therefore reveal information about the agglutinin binding site(s).

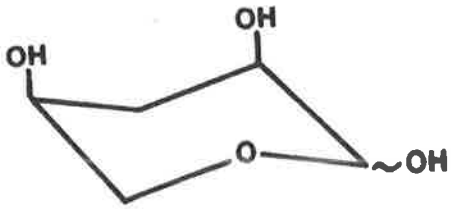
The structures of the sugars which inhibited the agglutination of guinea pig erythrocytes by the HA-1 are depicted in Figure 4.7. Simple sugars exist in equilibrium between open forms and ring structures. The ring structures also exist in equilibrium between chair and boat forms; however the chair form is the more stable since all carbons and hydrogens are staggered. Furthermore in substituted cyclohexanes (at C5), the most stable chair form has the substitution in an equatorial position since this avoids steric replusion with axial hydrogens. The sugars in Figure 4.7 have been drawn in this form. An examination of Figure 4.7 reveals that sugars which are the most galactosyl-like are the best inhibitors. Lactose (D-gal-$\beta$1$\rightarrow$4-D-glu) and melibiose (D-gal-$\alpha$1$\rightarrow$6-D-glu) are

FIGURE 4.7

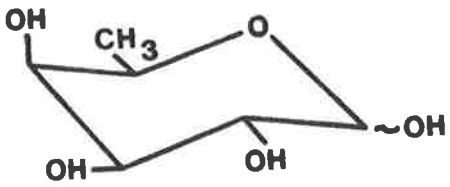
Structures of sugars used in inhibition experiments. The concentration required to inhibit HA-1 by 50% is shown in parentheses.



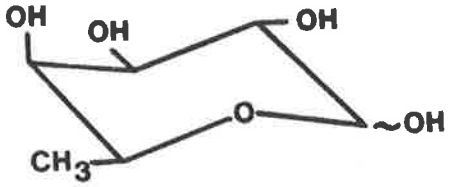

D-GALACTOSE (1 mM)



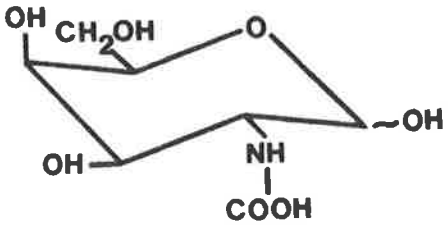

D-XYLOSE (16 mM)



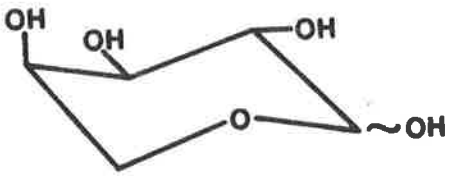

D-FUCOSE (1 mM)



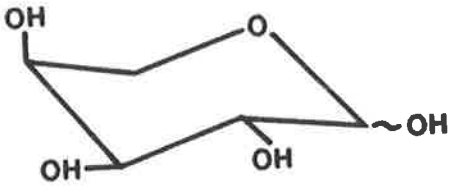

L-FUCOSE (16 mM)



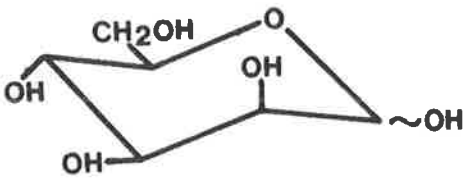

N-Ac-D-GALACTOSE (2 mM)



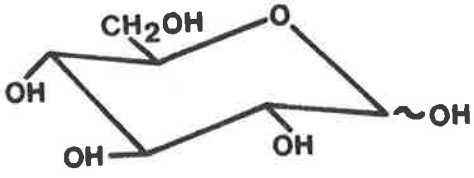

D-ARABINOSE (32 mM)




L-ARABINOSE (4 mM)




MANNOSE (>67 mM)




D-GLUCOSE (8 mM)

both more potent inhibitors than galactose. Although these disaccharides have the same inhibitory capacity, the orientation of the glucose is markedly different. This suggests that the glucose *per se* is not important, but that the increased potency is probably due to the locking of the galactose in the ring form. Thus it seems likely that the HA-1 molecule preferentially recognises the ring form.

Additionally the HA-1 binding site appears to be fairly non-discriminatory, since changing the orientation or the nature of the substituent groups at carbons 1,2,4, and 5 of the inhibiting sugar had a relatively small effect on HA-1 agglutinating activity. However, a marked reduction of inhibitory activity was apparent in sugars containing changes at more than one location. Surprisingly, differences in the relative location of the oxygen atoms at the reducing end of the sugar (e.g. D-xylose versus D-fucose) resulted in only a 16 fold difference in inhibitory capacity. This finding, and the equal activity of lactose and melibiose, suggests that the HA-1 binding site is probably quite small, recognising the non-reducing end of D-galactose.

It seems that the HA-2 binding site recognises parts of both the glucose and galactose moieties of lactose since lactose was inhibitory whereas galactose and melibiose were not. The orientation of the glucose with respect to the galactose appears to be critical.

## 4.14 Summary

1) Two haemagglutinins, designated HA-1 and HA-2, have

been identified in *B. leachii* haemolymph. The size of these haemagglutinins was investigated by gel chromatography and sedimentation velocity experiments and their binding specificities by sugar inhibition and adsorption experiments.

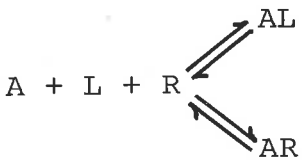
2) HA-1, which only agglutinated guinea pig erythrocytes, was found to be approximately the size of mouse IgG (mol. wt. = 150,000). Haemagglutinating activity required $Ca^{++}$ ions for expression and could be reversibly abolished by EDTA. The haemagglutinin was found to be a galactosyl binding molecule, primarily recognising the non-reducing end of galactose.

3) HA-2 (approximate mol. wt. = 63,000) agglutinated erythrocytes from all the species tested and did not require $Ca^{++}$ ions for activity. Agglutination of all erythrocytes was specifically inhibited by lactose. It was concluded from quantitative adsorption experiments that HA-2 haemagglutinating activity was due to a single agglutinin.

4) *B. schlosseri* haemolymph was found to contain a $Ca^{++}$-dependent, guinea pig erythrocyte specific haemagglutinin that was inhibited by the same sugars and had a similar sedimentation coefficient as *B. leachii* HA-1.

## 4.15 Appendix

A theoretical analysis of the inhibition of haem-agglutination reactions by small ligands is not available, but the parameters which must be considered when one evaluates such data can be identified by a simple examination of the reactions involved. For example, consider the interaction of a cell surface-associated receptor (R) and a small ligand (L) with a homogeneous, multivalent agglutinin (A). The system can be approximately described by the expression




The association constants for these reactions may be defined as

$$K_L = \frac{[AL]}{[A][B]} \quad \ldots\ldots\ldots \text{ Equation 1}$$

$$K_R = \frac{[AR]}{[A][R]} \quad \ldots\ldots\ldots \text{ Equation 2}$$

where [A], [L], [R], [AL] and [AR] represent the concentrations of the respective molecular species when the system is at equilibrium. For simplicity, the association constants are considered to include all species arising from the multivalency of the agglutinin (e.g. AL, $AL_2$, ...., $AL_n$). Also, although the concentration of R (and consequently also of AR) can only be described by complex mathematical functions (Reynolds, 1979), for the purposes of this analysis it suffices to recognize that [R] depends on both the number

of molecules of R present on each cell and the cell concentration. Dividing equation (2) by equation (1), one obtains

$$\frac{[AR]}{[AL]} = \frac{K_R \cdot [R]}{K_L \cdot [L]} \quad \ldots\ldots\ldots \text{Equation 3}$$

Providing $K_R$ is large (e.g. >100) and the initial concentrations $[R]_o > [A]_o$, then at equilibrium [A] will be negligible and $[AR] + [AL] \simeq [A]_o$. These conditions can be expected to exist when erythrocytes are agglutinated by a limiting amount of agglutinin, e.g. near a titration end-point. Since, in this system, ligand molecules compete with the cell receptors for the binding sites of the agglutinin molecules, one may consider that, at the concentration of ligand ($[L]_{\frac{1}{2}}$) which halves the titre of the agglutinin with respect to the uninhibited titre (observed in the absence of ligand), $[AL] = [AR]$. Thus from equation (3),

$$[L]_{\frac{1}{2}} = \frac{K_R \cdot [R]}{K_L} \quad \ldots\ldots\ldots \text{Equation 4}$$

It is evident from equation (4) that in any given experimental system, the concentration of ligand required to halve the titre of a haemagglutinin depends not only on the affinity of the agglutinin for the ligand ($K_L$) (Loontiens *et al.*, 1975), but also on the affinity/avidity of the agglutinin for the cell receptors ($K_R$) and on the receptor concentration ([R]). Thus, using a given assay, one agglutinin and one type of cell, the concentration of a sugar needed to halve the agglutinating titre is

inversely proportional to the affinity of the agglutinin for that sugar. When one compares the capacity of a particular sugar to inhibit the agglutination of different types of cell, however, the inhibitory concentrations depend not only on $K_L$ (which is the same in each case), but also on the affinity/avidity of the agglutinin for the receptors on each type of cell as well as on the effective receptor concentration. The latter is a function of cell concentration, cell size and cell surface receptor density (Reynolds, 1979).

# CHAPTER 5

## Preliminary characterization of the haemagglutinins from *B. leachii* and *B. schlosseri* using radiolabelling techniques

## 5.1 Preamble

The sensitivity of the guinea pig erythrocyte haem-agglutinating activity of *B. leachii* haemolymph to reduction and alkylation and to protease digestion and of the sheep erythrocyte haemagglutinating activity to heating and incubation with urea suggested that these molecules were proteins. Based on the negligible optical density at 280 nm of the fractions containing haemagglutinating activity from Sephadex G-200 chromatography of haemolymph (Chapter 4), it appeared that the levels of each haemagglutinin in haemo-lymph was very low and that their purification in quantities sufficient for characterization by normal chemical methods would be difficult. In order to bypass this problem, an attempt was made to study the structure of the haemagglutinins from both *B. leachii* and *B. schlosseri* using radiolabelling techniques. This involved the radio-iodination of the proteins in haemolymph with iodine-125 and subsequent analysis of the purified labelled haemagglutinins by SDS-PAGE in non-reducing conditions. With the radiolabelling method used, the incorporation of iodine-125 is restricted mainly to the tyrosyl and histidyl residues of proteins (Bolton, 1977). Macromolecules other than proteins are not normally labelled to any significant extent, although in a mixture as complex as haemolymph it is conceivable that protein-associated lipids might also be labelled.

## Experimental

### 5.2 Isolation of purified radiolabelled haemagglutinins

The following experiments were carried out to isolate

radiolabelled haemagglutinins. Haemagglutinating positive fractions from the Sephadex G-200 chromatography runs of *B. leachii* haemolymph (Chapter 4) were pooled and concentrated by ultrafiltration (PM10 membrane). The proteins in the concentrate were labelled with iodine-125 as described in Chapter 2 (section 2.22). The binding activity of both agglutinin preparations appeared to be abolished by the labelling procedure since no radioactivity became associated with guinea pig erythrocytes during subsequent adsorption tests. On the other hand, a significant amount (4%) of radioactivity became associated with guinea pig erythrocytes using labelled haemolymph. This radiolabelled material could be eluted from the cells, and its binding inhibited, by lactose. These results indicated that the radiolabelled haemagglutinin(s) might be purified by adsorbing radiolabelled whole haemolymph with guinea pig erythrocytes and specifically eluting the cell bound agglutinins with lactose.

To reduce the volumes required, membrane vesicles rather than whole erythrocytes were used for the adsorption. These were prepared by sonication as described in Chapter 2 (section 2.7). Before use the vesicles were centrifuged (30,000 g, 15 min.) and resuspended twice in PBS (pH 7.4), once in 0.2 M propionic acid, twice in PBS and finally resuspended in PBS to yield a suspension of vesicles equivalent to a 35% v/v suspension of whole erythrocytes. Initially, the minimum amount of membrane material needed to quantitatively adsorb the iodine-125-labelled haemagglutinins from a known quantity of labelled *B. leachii* haemolymph

was determined. This was done by incubating a constant amount of haemolymph with varying quantities of vesicles as described in Figure 5.6. To minimise non-specific binding of haemolymph components to the vesicles, the PBS-diluent contained 1 mg/ml of BSA. The radioactivity associating with the vesicles plateaued at about 7% of the total amount added when vesicle doses higher than 100-150 µl of stock suspension were used.

To isolate radiolabelled haemagglutinins, the amounts used in the above procedure were amplified 50 fold. Thus, 5 ml of vesicle stock solution were centrifuged (27,000 g, 15 min.) and the vesicles resuspended in 4 ml of PBS-BSA diluent. One hundred microlitres of labelled haemolymph were clarified by centrifugation (27,000 g, 15 min.) and the supernatant (c.a. 95 µl) was added to the vesicle suspension. Centrifugation of haemolymph under these conditions was known from previous work to cause no reduction in the agglutinating activities of the fluid phase. The suspension was incubated for one hour at room temperature and the vesicles were then washed twice with PBS-BSA. The final vesicle pellet was resuspended in 4 ml of PBS containing 0.5 M lactose and 0.1 mg/ml of BSA (to act as a carrier protein), incubated for one hour at room temperature and centrifuged (27,000 g, 15 min.). The supernatant was dialysed overnight against 2 litres of PBS. The results are summarised in Figures 5.1 and 5.2. About 4% of the total radioactivity in *B. leachii* haemolymph remained bound to the washed vesicles and of this 49% was released by lactose. The overall recovery of radioactivity was 1.8%.

FIGURE 5.1

Recovery of radioactivity during the purification of labelled B. leachii haemagglutinins by adsorption to guinea pig erythrocyte vesicles.

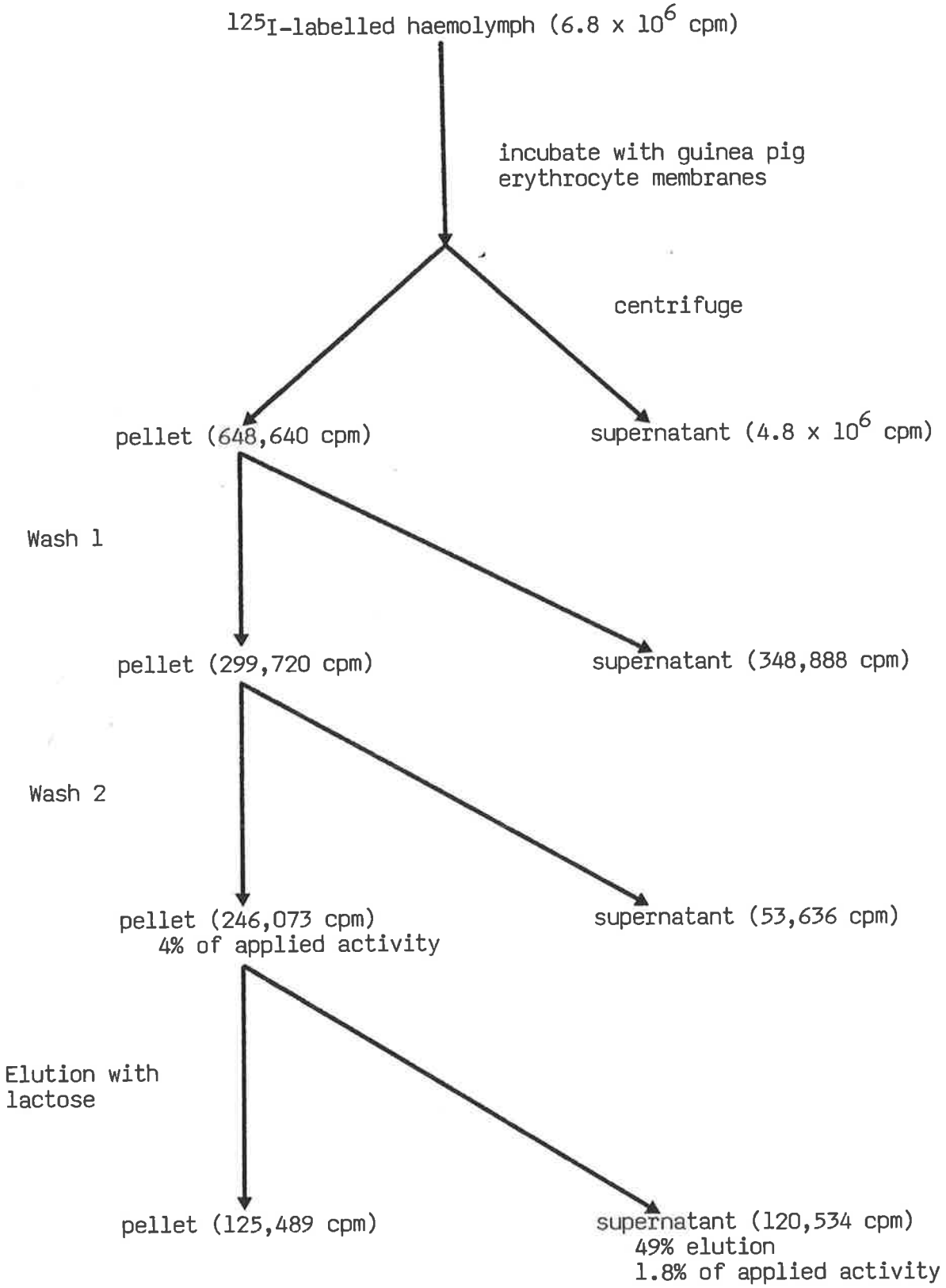
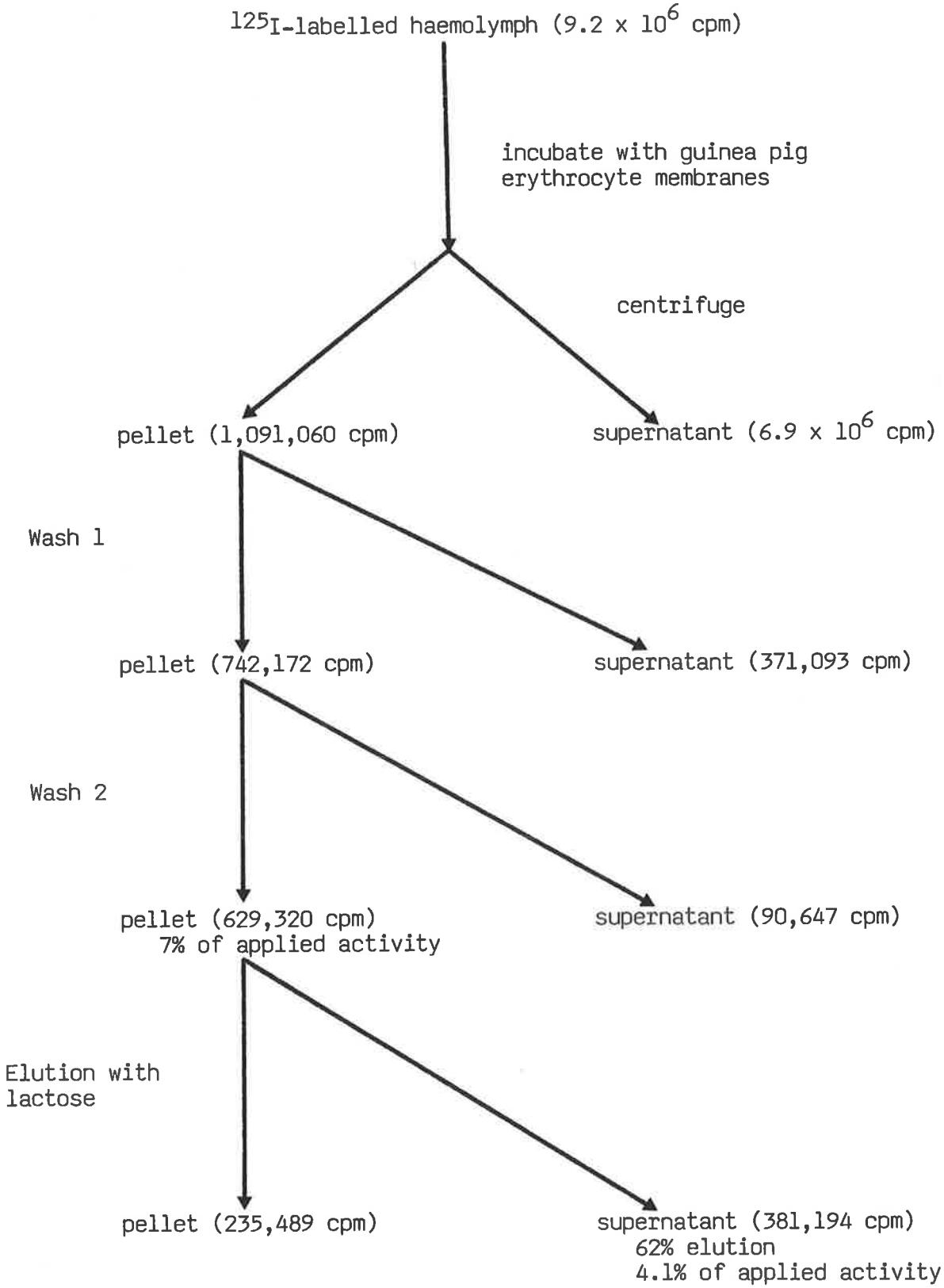


FIGURE 5.2

Recovery of radioactivity during the purification of labelled B. schlosseri haemagglutinins by adsorption to guinea pig erythrocyte vesicles.



The results for *B. schlosseri* haemolymph were similar. Of the 7% of radioactivity which remained associated with the vesicles, 62% was recovered by elution with lactose. This was an overall recovery of 4.1%.

## 5.3 Analysis of the radioactive material recovered in the lactose eluate

### 5.3.1 Binding activity of the material in the *B. leachii* lactose eluate

The binding activity of the labelled *B. leachii* lactose eluate was investigated by determining the proportion of radioactivity that could rebind to guinea pig erythrocytes in the presence or absence of lactose. Guinea pig erythrocytes (0.1 ml packed cells) were resuspended in 1 ml of each of the following iso-osmotic solutions:- PBS plus 0.1 mg/ml BSA; 0.135 M lactose/0.06 M NaCl/0.013 M phosphate (pH 8.0) plus 0.1 mg/ml BSA; dialysed *B. leachii* haemolymph diluted 1:1 with either PBS, or 0.135 M lactose solution (composition as above). Each suspension was mixed with 50 µl of the radiolabelled lactose eluate which had been dialysed against PBS. After incubation for 30 minutes at room temperature the cells were sedimented (900 g, 5 min.) and washed three times in PBS. They were then resuspended in 2 ml of 0.135 M lactose solution, incubated a further 30 minutes at room temperature and finally centrifuged (900 g, 5 mins.). The amount of radioactivity recovered in the various supernatants is presented in Table 5.1. In the absence of lactose nearly half of the labelled material bound firmly to the erythrocytes. There appeared to be

TABLE 5.1

Binding of radiolabelled *B. leachii* haemagglutinins to guinea pig erythrocytes*

| Fraction | Control | | Plus haemolymph | |
|---|---|---|---|---|
| | No lactose | Plus lactose | No lactose | Plus lactose |
| | radioactivity (cpm)§ | | | |
| Total added radioactivity | 5310 | 5078 | 3900 | 3925 |
| Third wash supernatant | 100 | 160 | 440 | 200 |
| Third wash cell pellet | 2025 (38%) | 508 (10%) | 448 (11%) | 333 (8%) |
| Lactose eluate | 1213 (23%) | 200 (4%) | 392 (10%) | 152 (4%) |

* Percent recoveries, relative to total label added, are shown in brackets.

§ Corrected for background (80 cpm).

little non-specific binding since in the presence of lactose the amount of cell-associated radioactivity was only a quarter of that observed in the absence of lactose. Sixty percent of the cell associated radioactivity was recovered by incubating the cells in lactose solution, but the recovery might have been greater if a longer incubation period had been used. It is noteworthy that the presence of unlabelled *B. leachii* haemolymph reduced the amount of radioactivity which bound to the cells in the absence of lactose from 38% to 11%, but had little affect on the amount of binding in the presence of lactose. These results showed that at least 50% of the radiolabel was associated with *B. leachii* lactose-binding proteins, and that the inhibitory effect of haemolymph was probably due to competition between labelled and unlabelled molecules for binding sites on the cells.

### 5.3.2 Analysis by continuous SDS-PAGE

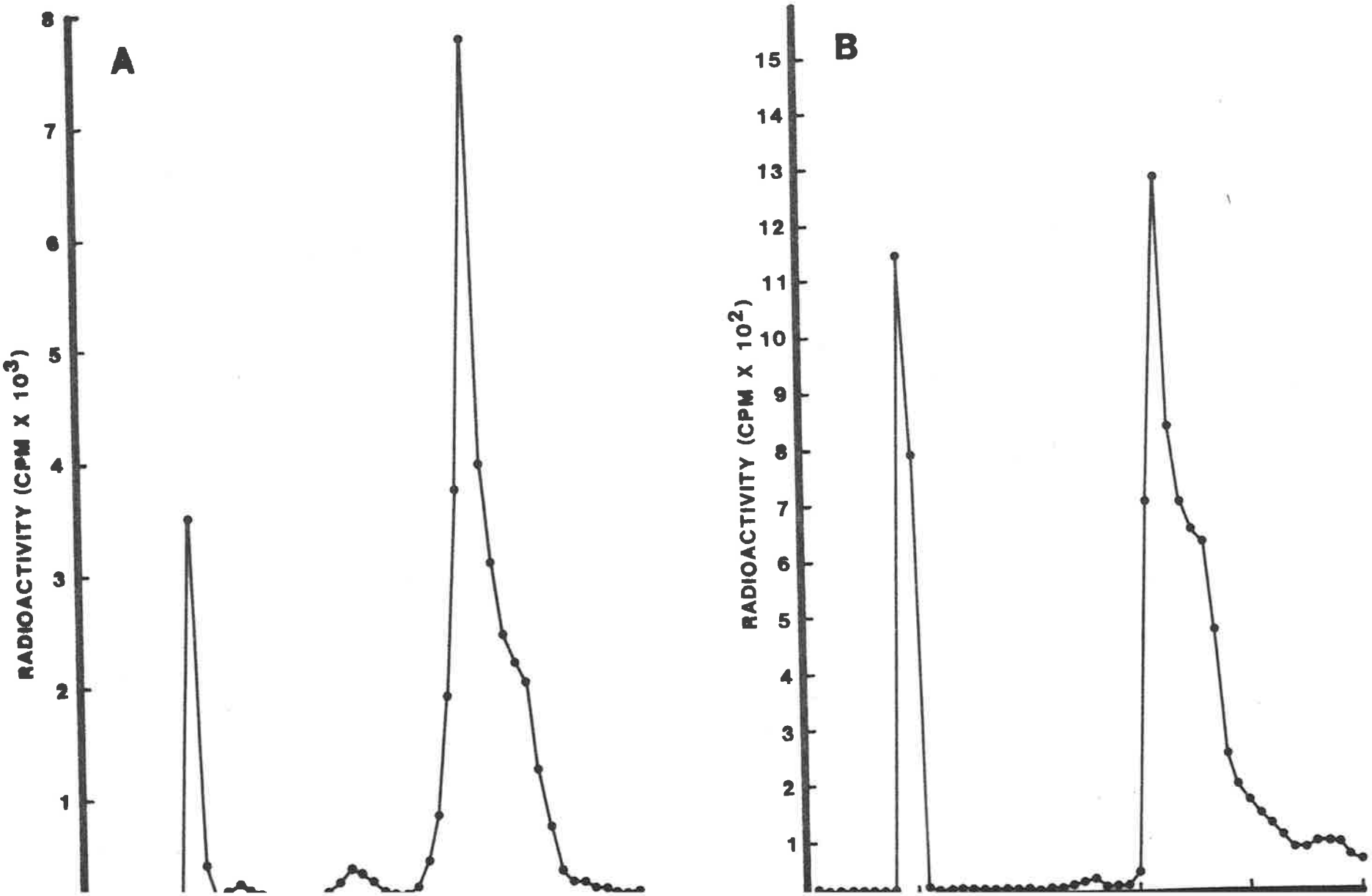
Samples of radiolabelled *B. leachii* and *B. schlosseri* lactose eluates were analysed by continuous SDS-PAGE (Chapter 2, section 2.21) under non-reducing conditions. The results are shown in Figure 5.3. Myoglobin, pepsin and bovine serum albumin were included as mol. wt. standards, relative mobility being plotted against log (mol. wt.). Both preparations yielded 2 major radioactive components. The *B. schlosseri* peak 1, which contained 9% of the applied radioactivity, had an apparent mol. wt. of 140,000 while the *B. leachii* peak 1 contained 18% of the applied activity and had an apparent mol. wt. of 145,000. Peak 2 contained 80% and 58% of the applied activities for each respective

FIGURE 5.3

Continuous SDS-PAGE analyses under non-reducing conditions of B. leachii and B. schlosseri lactose eluates (Figs. 5.1 & 5.2). Samples were dialysed against water, freeze dried, dissolved in 200 ul of SDS-PAGE sample buffer and 100 ul was loaded onto the gel. The gels were sectioned and counted for radioactivity.

A) B. schlosseri; 38,000 cpm loaded

B) B.leachii; 10,600 cpm loaded


A
B
RADIOACTIVITY (CPM X $10^3$)
RADIOACTIVITY (CPM X $10^2$)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

preparation and yielded mol. wts. of 27,000 and 33,000 respectively. The shoulder apparent on peak 2 from both preparations suggested that two overlapping peaks might be present.

## 5.4 Fractionation of radiolabelled *B. schlosseri* haemolymph by gel filtration

In order to determine whether the haemagglutinins in *B. schlosseri* haemolymph were related to the radiolabelled lactose binding components described in the previous sections, a mixture of labelled and unlabelled haemolymph was chromatographed on Sepharose 6B as described in the legend to Figure 5.4. The profile of total radioactivity (Fig. 5.4A) revealed significant heterogeneity in the size of the labelled material and several dominant peaks were apparent. It can be seen, however, that the guinea pig agglutinating activity associated with fractions 44-54 corresponded to a single, well resolved peak of radioactivity (Fig. 5.4A). These were the only fractions containing haemagglutinating activity. The amount of radiolabelled material in these fractions which could bind to guinea pig erythrocytes was also determined (Fig. 5.4B). It was found that there was a good correlation between the proportion of binding material present, ranging from 20% to 80%, and the haemagglutination titre. The binding of this material was completely inhibited by lactose. Various other fractions were also found to contain material which could bind to guinea pig erythrocytes (Fig. 5.4B), in proportions ranging from 20% to 90%, but none of this material could be inhibited by lactose.

FIGURE 5.4

Chromatography of B. schlosseri haemolymph on Sepharose 6B. Four millilitres of haemolymph (GPRBC titre = 1/3,200,000) containing 10 ul of radiolabelled haemolymph (1.1 x $10^6$ cpm) and 0.1 M lactose was loaded onto the column and eluted at 4° at 10 ml/hr with PBS / 50 mM lactose / 100 mM galactose. Fraction size was 4.6 ml. Two millilitre samples of each fraction were analysed for radioactivity. The remainder of each fraction was dialysed against TSA / 1 mM $CaCl_2$ and titrated for haemagglutinating activity against guinea pig and sheep erythrocytes. A number of the dialysed fractions were also analysed for the amount of radioactivity which bound to the erythrocytes both in the presence and absence of lactose. Thus, 0.1 ml samples were mixed with 0.3 ml of 2% erythrocytes suspended in PBS or PBS diluted 1:1 with 0.27 M (iso-osmotic) lactose, each supplemented with 1% foetal calf serum to provide carrier proteins. After incubation (37°, 45 min.), the cells were sedimented by centrifugation and 0.3 ml of each supernatant was counted for radioactivity. Tubes containing no erythrocytes were included as controls. The results are expressed as percent adsorption of label compared to the no cell control.

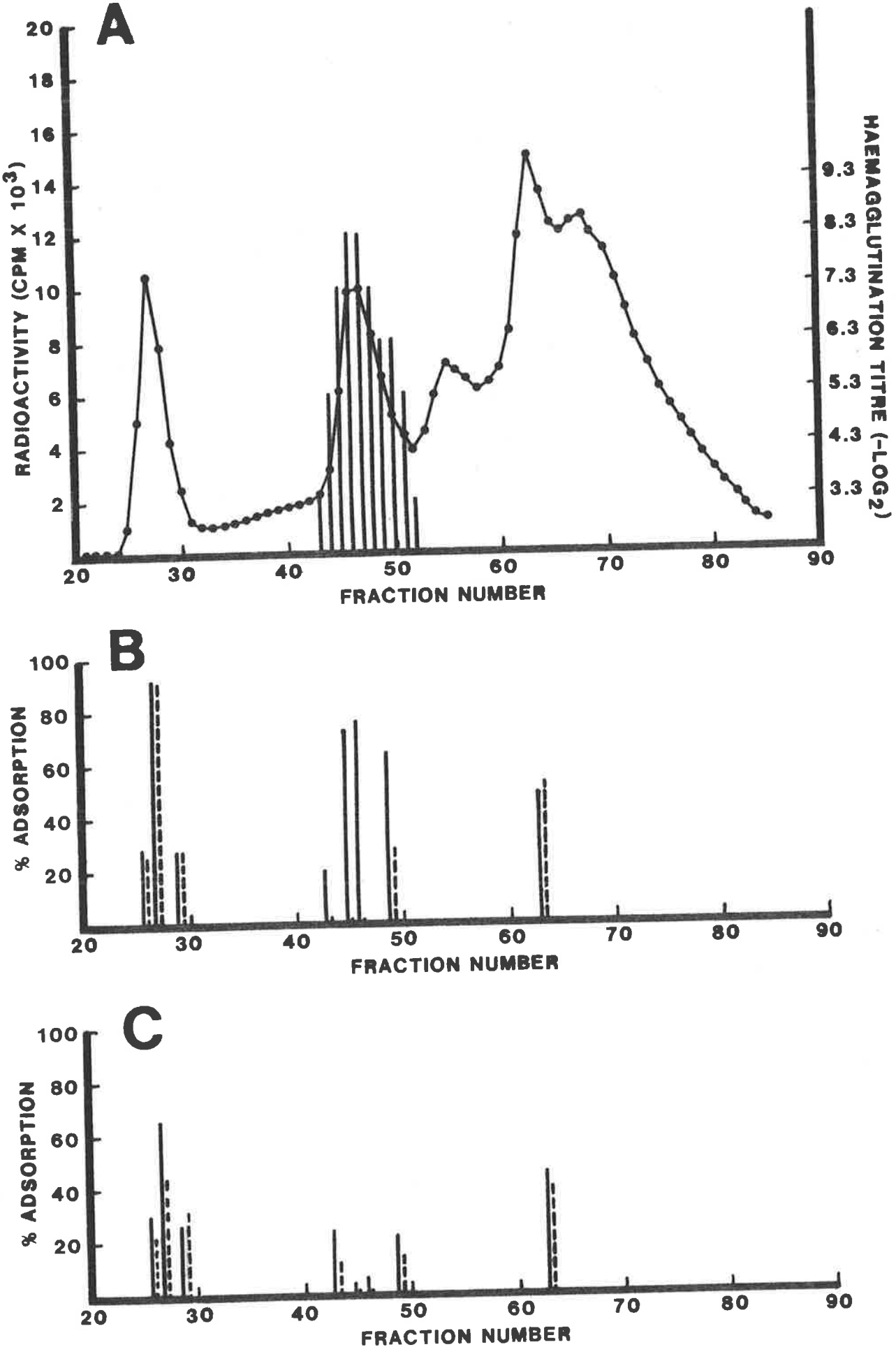
A. ●——● total radioactivity profile

| haemagglutination titre for guinea pig erythrocytes

B plus C Binding of $^{125}I$-labelled material to guinea pig (B) and sheep (C) erythrocytes in the presence or absence of lactose.

| no lactose

⋮ plus lactose


A
RADIOACTIVITY (CPM X $10^3$)
HAEMAGGLUTINATION TITRE ($-LOG_2$)
FRACTION NUMBER
B
% ADSORPTION
FRACTION NUMBER
C
% ADSORPTION
FRACTION NUMBER

No fractions agglutinated sheep erythrocytes and although the various fractions tested contained labelled material which could bind to sheep erythrocytes (20% to 60%), in no case was this binding inhibited by lactose (Fig. 5.4C).

## 5.5 Discussion

In this chapter an attempt was made to purify radiolabelled haemagglutinins by incubating labelled haemolymph with guinea pig erythrocyte membrane vesicles and specifically eluting with lactose the radiolabelled material which had adsorbed to the vesicles. It was considered, on the following grounds, that both the *B. leachii* and *B. schlosseri* eluted material consisted primarily of lactose-specific haemagglutinin(s):

1) When *B. schlosseri* haemolymph (labelled plus unlabelled) was chromatographed on Sephadex G-200 (Fig. 5.4), the only fractions which contained labelled material whose binding to guinea pig erythrocytes could be inhibited by lactose corresponded exactly to those fractions containing haemagglutinating activity. In the fraction containing the peak haemagglutinating activity, at least 80% of the radiolabelled material bound to guinea pig erythrocytes in a lactose specific fashion. These results suggested that the haemagglutinins in *B. schlosseri* haemolymph were indeed labelled with iodine-125. In contrast, lactose did not inhibit the binding of labelled material from other chromatography fractions. Thus, the material recovered in Section 5.2 by elution with lactose should have comprised reasonably pure labelled haemagglutinin(s).

FIGURE 5.5

Chromatography of B. leachii haemolymph on Sepharose 6B. Sample; 4 ml of haemolymph (GPRBC titre = 1/3,200, SRBC titre = 1/32) containing 10 ul of radiolabelled haemolymph ($6.8 \times 10^5$ cpm) and lactose (0.1 M). Eluant; PBS containing 50 mM lactose and 100 mM galactose. Fraction size = 4.6 ml. Flow rate = 10 ml/hr.

●——● radioactivity profile: cpm of 2 ml aliquots

haemagglutination titre for guinea pig erythrocytes: assayed after dialysis against TSA / 1 mM $CaCl_2$

haemagglutination titre for sheep erythrocytes: assayed after dialysis against TSA / 1 mM $CaCl_2$

Elution positions of blue dextran (Vo), bovine serum albumin (BSA), myoglobin, and paranitrophenol (Vt) are indicated.

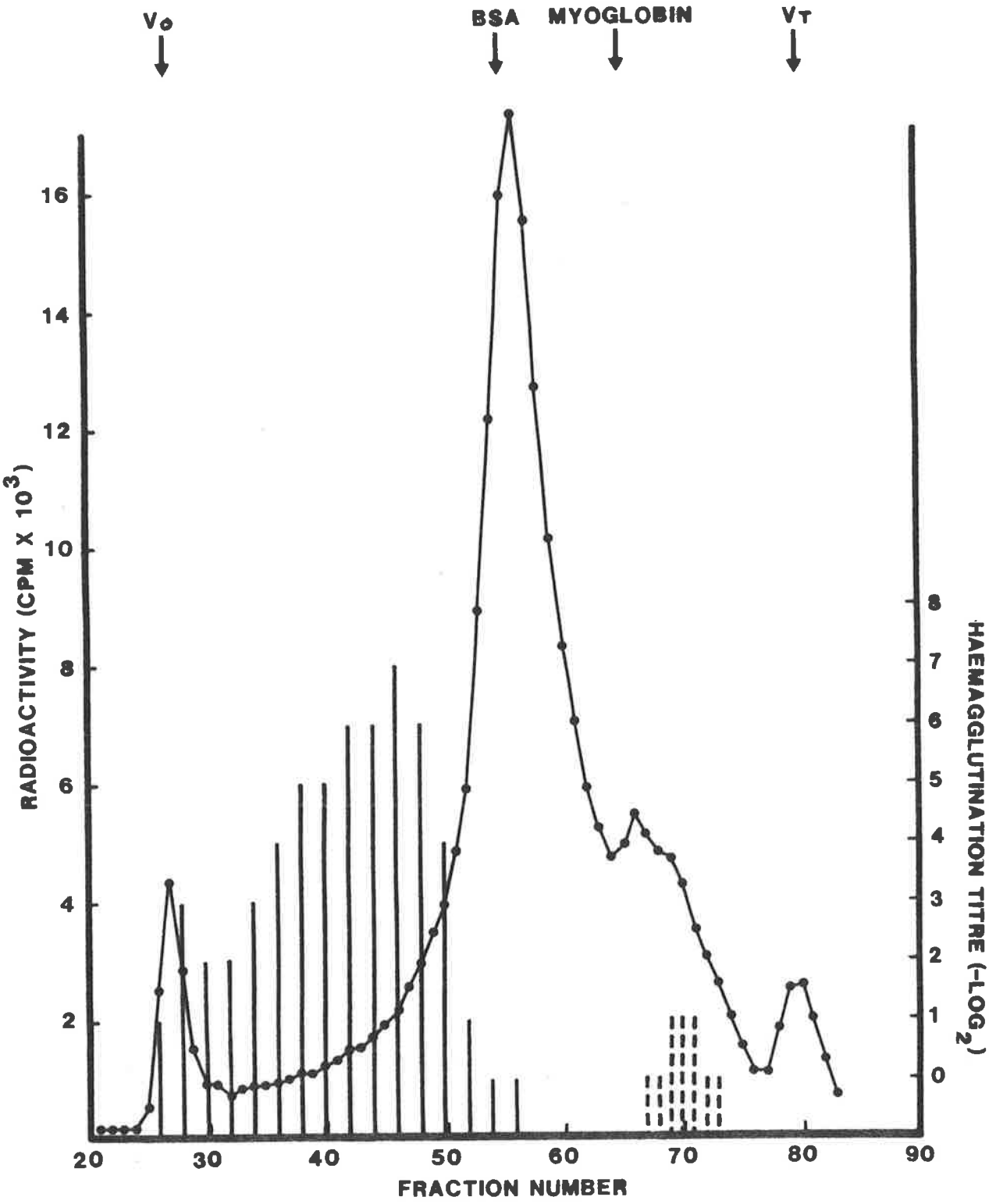
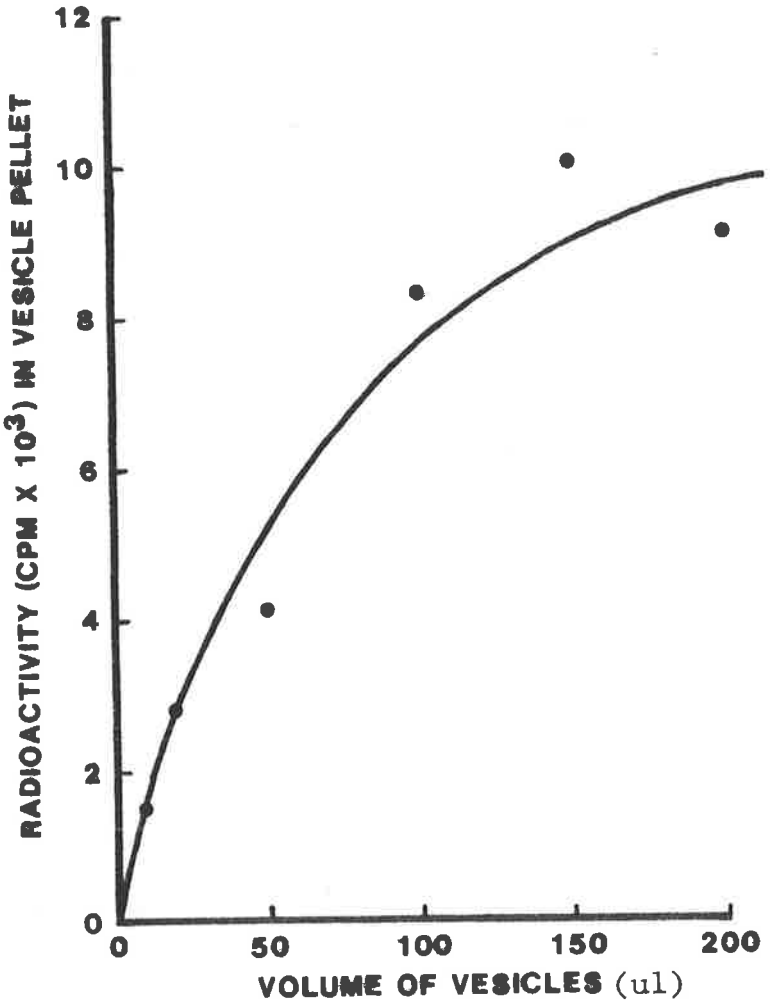

$V_o$
BSA
MYOGLOBIN
$V_T$
RADIOACTIVITY (CPM X $10^3$)
HAEMAGGLUTINATION TITRE ($-LOG_2$)
FRACTION NUMBER
16
14
12
10
8
6
4
2
8
7
6
5
4
3
2
1
0
20
30
40
50
60
70
80
90

FIGURE 5.6

Adsorption of radiolabelled *B. leachii* haemolymph with guinea pig erythrocyte vesicles. Radiolabelled haemolymph was diluted 1/15 in PBS containing 1 mg/ml BSA (PBS-BSA) and centrifuged at 27,000 *g* for 15 minutes. Fifty microlitre aliquots (containing 13,000 cpm) were mixed with 200 ul aliquots of PBS-BSA containing various amounts of guinea pig erythrocyte vesicles. After standing at room temperature for one hour, the supernatants were centrifuged (27,000 *g*, 15 min.) and the pellets were assayed for radioactivity. The values shown are corrected for background radioactivity determined from tubes containing no vesicles (5,615, 5,558 cpm).


RADIOACTIVITY (CPM X $10^3$) IN VESICLE PELLET
12
10
8
6
4
2
0
0
50
100
150
200
VOLUME OF VESICLES (ul)

2) Tests of the guinea pig erythrocyte binding activity of the *B. leachii* lactose eluate showed that at least 50% of the label was associated with lactose binding proteins whose binding to cells could be competitively inhibited with unlabelled *B. leachii* haemolymph. This was consistent with the hypothesis that the labelled proteins present in the eluate were haemagglutinins.

Inspection of the data presented in this Chapter and in Chapter 4 reveals that *B. schlosseri* haemolymph possesses a haemagglutinin that is very similar to *B. leachii* HA-1. Thus, in the sugar inhibition experiments (Tables 4.2 and 4.3) it was found that the order of the potency of the sugars was the same for both haemagglutinins (i.e., lactose >D-galactose >L-arabinose >D-glucose >D-xylose >L-fucose) and that the concentration of any given sugar required for 50% inhibition was in each case very similar. The binding specificity of these haemagglutinins must therefore be very similar. Also, as shown by their identical behaviour during sedimentation velocity (Fig. 4.4) and gel filtration chromatography on Sepharose 6B (Figs. 5.4 and 5.5), the apparent size of the native haemagglutinins from each species is similar, if not identical. Finally, SDS-PAGE analysis in non-reducing conditions of both the *B. leachii* and *B. schlosseri* lactose eluates revealed two labelled bands with apparent mol. wts. of approximately 140,000 and 30,000. In subsequent work (Chapters 6 and 7), it was found the *B. leachii* HA-1 and HA-2 haemagglutinins purified by affinity chromatography resolved into major polypeptide components with mol. wts. of approximately 140,000 and 30,000, respectively, when subjected to SDS-PAGE in non-reducing conditions. By

comparison with this later work, it would appear that the *B. leachii* SDS-PAGE peak 1 (Fig. 5.3B) (mol. wt. = 145,000) represents HA-1 and that, therefore, the apparently equivalent *B. schlosseri* peak 1 (Fig. 5.3A) (mol. wt. = 140,000) probably represents the HA-1 like haemagglutinin in *B. schlosseri* haemolymph. It would also appear that the *B. leachii* SDS-PAGE peak 2 (Fig. 5.3B) (mol. wt. = 33,000) represents HA-2.

No agglutinins similar to the *B. leachii* HA-2 molecules were positively detected in *B. schlosseri* haemolymph, although the latter contained a low level of haemagglutinating activity for sheep erythrocytes (titre = 1/4 - 1/8). No data were obtained on the binding specificity of this agglutinin and dilution effects prevented its detection in fractions obtained by gel filtration. However, it is possible that the second band (mol. wt. = 27,000) seen in the SDS-PAGE gels may represent this sheep erythrocyte agglutinin.

While the work presented in this chapter was in progress, a method by which larger quantities of the haemagglutinins might be purified using affinity chromatography was published (Uy and Wold, 1977). In view of the expanded opportunities this would create for the characterization of these molecules, efforts were directed away from the radiolabelling experiments, which were clearly limited in scope, to the affinity chromatography experiments described in the next chapter. Since the expectations for this method were fully realised, the line of work described in this chapter was not completed as rigorously as one would have liked. Notwithstanding this fact, the results presented here are consistent with the assertion that the

lactose eluate preparations did in fact consist mostly of radiolabelled haemagglutinins. For this to have been conclusively demonstrated, however, the erythrocyte binding activity of both eluates should have been analysed in a fashion similar to that done for the *B. leachii* preparation and both labelled haemolymph preparations fractionated by gel filtration and analysed as was done for *B. schlosseri* haemolymph. It would also have been important to assess the homogeneity of the labelled material in the lactose eluates by gel filtration chromatography in Sephadex G-200 and to determine whether this material did in fact correspond in size to the haemagglutinin(s). This would also have constituted a further step in the purification of the labelled haemagglutinin(s).

## 5.6 Summary

1) Material isolated from radiolabelled *B. leachii* and *B. schlosseri* haemolymph by adsorption to guinea pig erythrocyte membranes followed by elution with lactose was judged by several criteria to be pure labelled haemagglutinins.

2) The haemagglutinins from both *B. leachii* and *B. schlosseri* were shown by SDS-PAGE analysis in non-reducing conditions to consist of two poly-peptide chains with mol. wts. of approximately 140,000 and 30,000.

3) A comparison of the results obtained in this and the previous chapter revealed that *B. schlosseri* haemolymph contains a haemagglutinin that is very similar to *B. leachii* HA-1.

# CHAPTER 6

## Purification of the haemagglutinins in *B. leachii* haemolymph by affinity chromatography

## 6.1 Preamble

The classical methods of separating proteins rely on the physicochemical properties of size (e.g. gel chromatography, ultracentrifugation), charge (e.g. ion exchange, electrophoresis) or solubility. After the processing of a heterogeneous mixture of proteins by one of these methods it is most likely that several proteins with properties similar to that of the protein of interest will be isolated. It can be seen, therefore, that to purify a protein to homogeneity using these methods several stages need to be applied, each relying on different physicochemical properties for separation. As there are losses at each stage, the overall yields can be low and large amounts of starting material are needed. When, as in the present case, there is only a limited amount of material available and this is present in solution at low concentration, a purification protocol based on these procedures is usually impractical. In contrast, affinity chromatography methods in which the protein of interest is immobilised on solid phase support material, usually through specific interactions (e.g. antibody-antigen, enzyme-substrate, lectin-carbohydrate) with a ligand covalently attached to the solid phase support, are ideal for these situations. The protein can be purified several fold and concentrated from a large volume in a single step with high yield. It seemed from previous results (Chapters 4 and 5) that an affinity column could be constructed to purify the *B. leachii* haemagglutinins using Sepharose gel to which lactose had been coupled (Uy and Wold, 1977). The development of a purification procedure using such an affinity column is described in this chapter.

Experimental

## 6.2 Interaction of *B. leachii* haemagglutinins with Sepharose 4B containing covalently-bound lactose

To apply affinity chromatography to the purification of *B. leachii* haemagglutinins, a solid phase support to which the haemagglutinins could bind was needed. Sepharose and Sephadex were initially tested as it had previously been shown that some interaction occurred between the haemagglutinins and these materials (Chapters 4 and 5). In these experiments, 10 ml aliquots of haemolymph were passed at a flow rate of 5 ml/hr down a small column containing about 5 ml (packed volume) of Sepharose 4B or Sephadex G-200. A significant proportion of the sheep erythrocyte haemagglutinating activity was adsorbed by Sepharose, but most of the activity for guinea pig erythrocytes passed unretarded through the column. Neither activity was retarded to any detectable degree by Sephadex G-200.

Because lactose was known to bind to both *B. leachii* agglutinins, this sugar was covalently coupled to Sepharose 4B by the method of Uy and Wold (1977) and an affinity column prepared as described in Chapter 2, section 2.14.1. To test whether the haemagglutinins would bind to this column, haemolymph previously dialysed against TSA was applied as shown in Figure 6.1A. Only 31% of the sheep erythrocyte agglutinating (HA-2) activity was recovered in the effluent (fractions 2-8), indicating that about 70% of the HA-2 molecules had bound to the column. Guinea pig erythrocyte agglutinating activity was detected in later fractions (7-18) indicating that the HA-1 agglutinin interacted weakly with

FIGURE 6.1

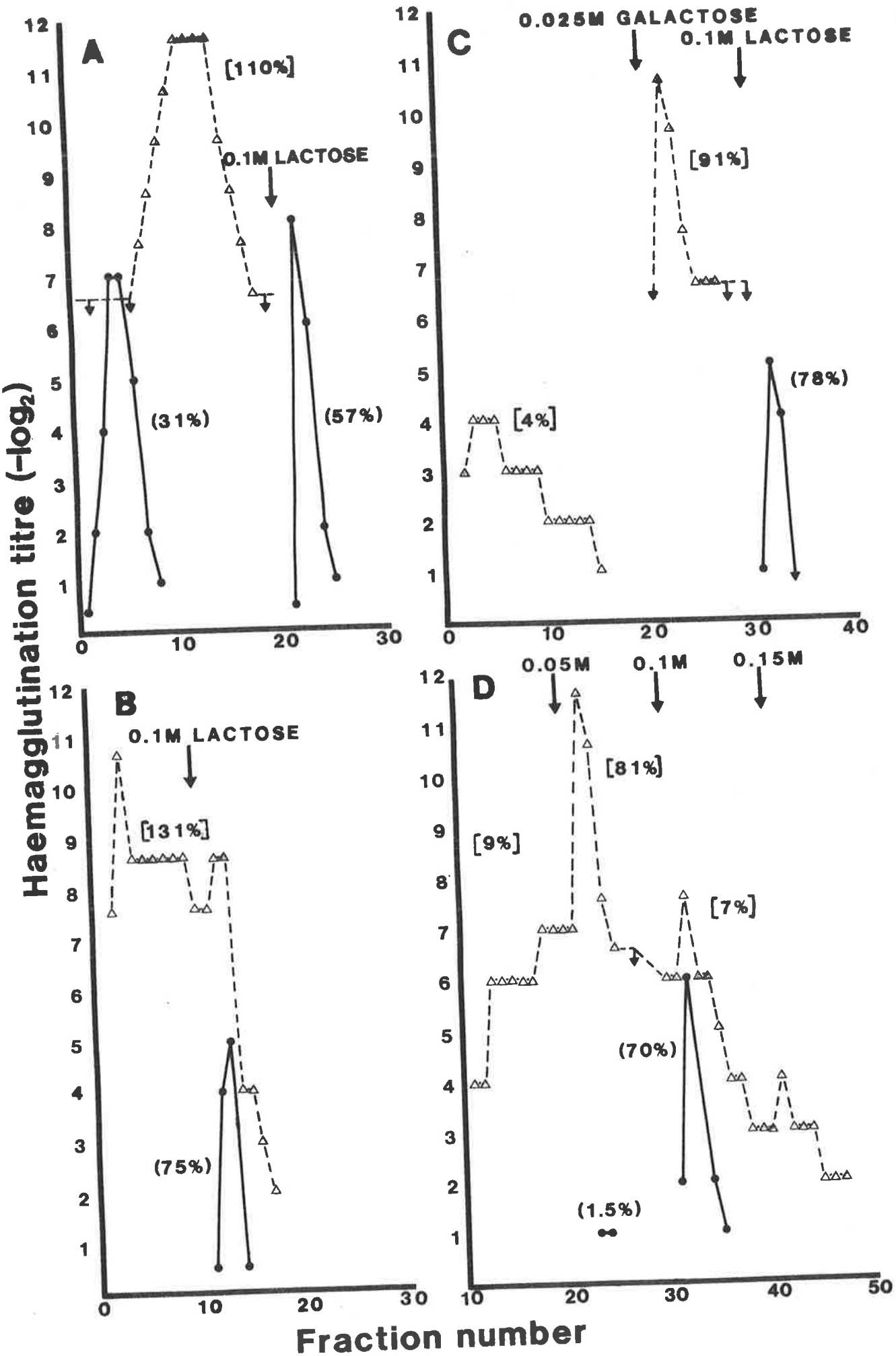
Adsorption of B. leachii haemagglutinins from haemolymph (dialysed against TSA) under various conditions by the lactose-substituted Sepharose affinity column. Elution was at 50 ml/hr and 5 ml fractions were collected. Fractions were assayed without dialysis for agglutinins against sheep (●——●) and guinea pig erythrocytes (△-----△). The overall percentage haemagglutinating activity recovered with each of the sugar solutions is shown in parentheses: sheep erythrocytes ( ); guinea pig erythrocytes [ ].

A. Ten millilitres of haemolymph (SRBC titre = 1/512; GPRBC titre = 16,400) was applied at a flow rate of 5 ml/hr at room temperature (c.a. 25°) The guinea pig erythrocyte haemagglutinating activity in the lactose eluate was not tested.

B. Five millilitres of haemolymph (SRBC titre = 1/64; GPRBC titre = 1/3,200) was applied at room temperature at a flow rate of 10 ml/hr.

C. Five millilitres of haemolymph (SRBC titre = 1/64; GPRBC titre = 1/3,200) was applied at 4° at 5 ml/hr.

D. Ten millilitres of haemolymph (SRBC titre = 1/64; GPRBC titre = 1/3,200) was applied at 4° at 5 ml/hr.


A
[110%]
0.1M LACTOSE
(31%)
(57%)
B
0.1M LACTOSE
[131%]
(75%)
C
0.025M GALACTOSE
0.1M LACTOSE
[91%]
[4%]
(78%)
D
0.05M
0.1M
0.15M
[81%]
[9%]
[7%]
(70%)
(1.5%)
Haemagglutination titre (-log2)
Fraction number

the column such that its passage was retarded. In an effort to recover the bound HA-2 molecules, the column was washed with 0.1 M lactose in TSA. This treatment caused immediate elution of the sheep erythrocyte agglutinins from the column in nearly 100% yield (Fig. 6.1A). It therefore appeared that the HA-2 molecules had indeed bound to the gel matrix by specific interaction with the immobilised lactose residues.

It seemed from the above results that the binding capacity of the column for HA-2 had been exceeded. The amount of haemolymph applied to the column was therefore reduced to 5 ml, and the flow rate was increased to 10 ml/hr. Under these conditions (Fig. 6.1B) all of the HA-2 activity was firmly bound and could be recovered only by elution with lactose. Most, if not all, of the HA-1 activity was not bound, although its passage appeared to be somewhat retarded.

These results indicated that the HA-1 molecules interacted weakly with the column. In an attempt to facilitate this interaction and thereby increase the binding of the agglutinins, the flow rate was reduced to 5 ml/hr and the experiment was conducted at $4^{o}$ rather than at room temperature. Under these conditions, at least 95% of the agglutinating activity to both types of erythrocytes bound firmly to the column (Fig. 6.1C). Both agglutinins could be eluted by 0.1 M lactose (results not shown).

The results given in Chapter 4 had shown that the HA-2 and HA-1 molecules had different binding site specificities. In particular, galactose was shown to be highly inhibitory for HA-1 activity but inactive against HA-2. On the basis of this finding, it was anticipated that galactose could be

utilised to preferentially elute the HA-1 activity. Thus 5 ml of haemolymph was applied to the column which was then washed consecutively with TSA, 25 mM galactose and 0.1 M lactose (Fig. 6.1C). Nearly all the applied guinea pig erythrocyte haemagglutinating activity bound to the column and, as expected, was eluted by the galactose solution. The HA-2 activity remained bound to the column and was recovered by elution with lactose.

## 6.3 Optimisation of the galactose concentration for the differential elution of HA-1 from the affinity column

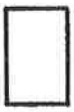
The results presented thus far had shown that the HA-1 and HA-2 haemagglutinins could be isolated from *B. leachii* haemolymph by adsorption to a Sepharose-lactose affinity column and recovered separately by elution with different sugars. Further experiments were carried out to determine the highest concentration of galactose that could be used to elute the HA-1 molecules, thereby ensuring the highest possible yield, without releasing HA-2 molecules. Ten millilitres of haemolymph were loaded onto the column at a flow rate of 5 ml/hr at $4^{o}$. In contrast to the results obtained at room temperature (Fig. 6.1A), both agglutinins were completely adsorbed to the column, a result which must be attributed to the lower temperature. The column was washed successively with 50 mM and 100 mM solutions of galactose in TSA, resulting in the recovery of about 1.5% and 70% of the sheep erythrocyte (HA-2) activity respectively (Fig. 6.1D). A final wash with 150 mM galactose released no further HA-2 activity. Most of the HA-1 activity was recovered with 50 mM galactose.

The upper limit of galactose concentrations which could be expected to have no effect on HA-2 binding appeared from these results to be below 50 mM. The experiment was therefore repeated using 30, 40, 50, 60, 70 and 80 mM solutions of galactose (Fig. 6.2). For each sugar concentration, the recovery of HA-1 and HA-2 activity is expressed as a percent of the total recovered (Fig. 6.2). For precise quantitation, each fraction was dialysed before assay in order to ensure that the galactose had no inhibitory effect on the titrations. However, dialysis appeared to reduce the recovery of both activities. This conclusion was based on the findings that the total yield relative to that applied was about 50% for both activities and that dialysis caused a marked reduction in the titre of those fractions in which the agglutinating activity was high enough to be measured before dialysis. This was probably a result of loss through non-specific binding to the dialysis tubing. In comparison, dialysis of haemolymph, which contains many other proteins, had no effect on the titre of either agglutinin.

A small amount (3%) of the HA-2 activity was eluted with 30 mM galactose while most of the remaining activity was eluted with 40 mM and 50 mM galactose. In contrast, 91% of the HA-1 activity was eluted with 30 mM galactose. The small amount of guinea pig erythrocyte agglutinating activity recovered at higher galactose concentrations was almost certainly due to HA-2 molecules since it could not be inhibited by 67 mM galactose (c.f. Fig. 6.2). On the basis of these results (Figs. 6.1C and 6.2), it was decided to use a concentration of 25 mM galactose in order to optimally separate the agglutinins in subsequent work.

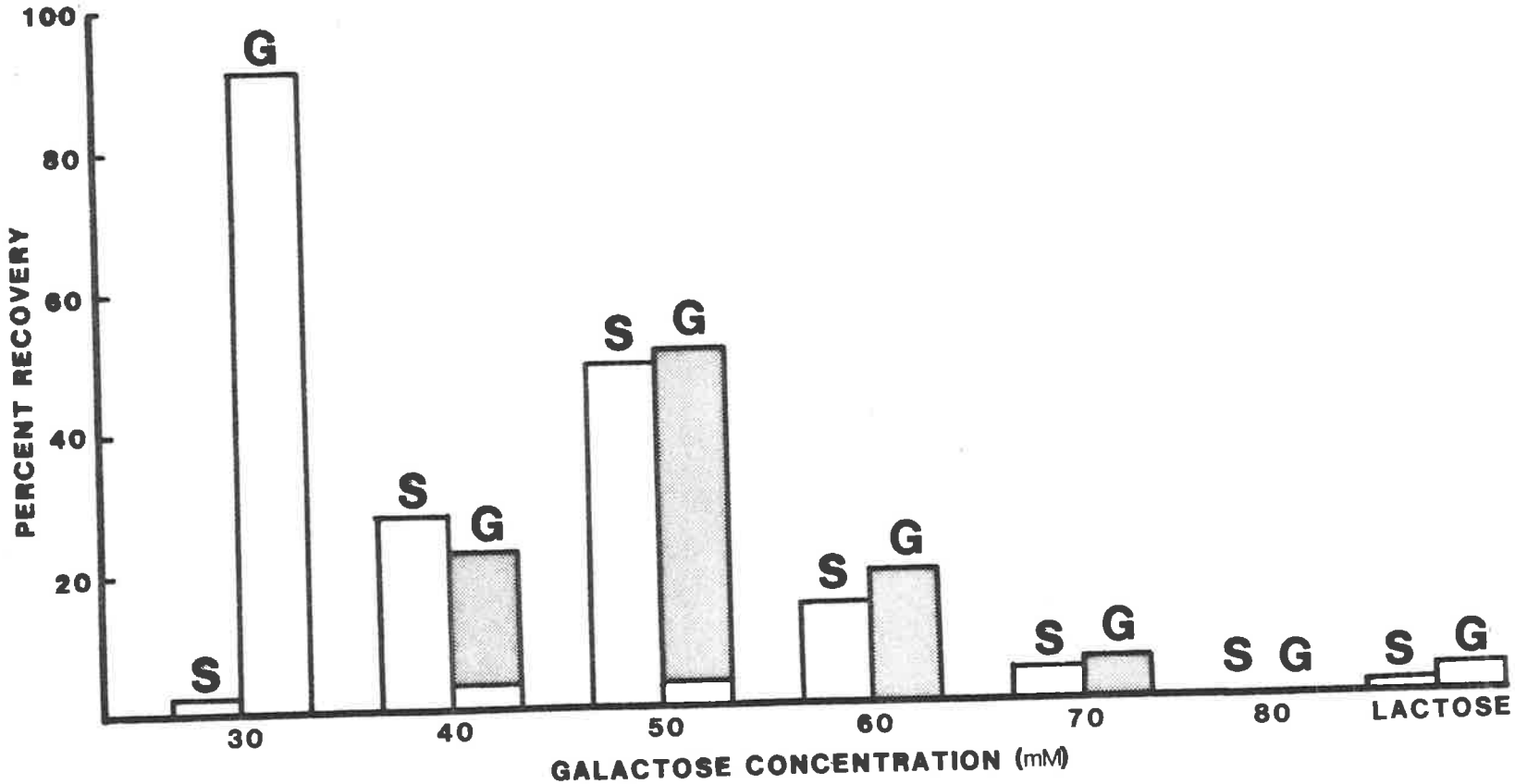
FIGURE 6.2

Recovery of applied haemagglutinating activity from the Lactose-Sepharose affinity column by elution with various concentrations of galactose. The column was loaded (5 ml/hr) with 10 ml of B. leachii haemolymph, washed with 50 ml of TSA and then washed (50 ml/hr) with 20 ml of each sugar solution. Haemagglutinating activity for sheep erythrocytes (S) and guinea pig erythrocytes (G) was measured after dialysis. The results are expressed as the percent of the total activity recovered in each case.



assayed in the absence of galactose

assayed in the presence of 67 mM galactose


100
80
60
40
20
PERCENT RECOVERY
S
G
S
G
S
G
S
G
S
G
S
G
S
G
30
40
50
60
70
80
LACTOSE
GALACTOSE CONCENTRATION (mM)

## 6.4 Large scale purification of the *B. leachii* haemagglutinins

In view of the apparent low concentrations of the *B. leachii* haemagglutinins in haemolymph (Chapters 4 and 5), it was important that the affinity chromatography procedure be able to process haemolymph in at least 50 ml batches if this procedure was to be of any practical use. To assess whether this was possible, an attempt was made to purify the haemagglutinins from 60 ml of haemolymph using the conditions established as optimal for this purpose, namely, application of the haemolymph to the column at a flow rate of $\leq$5 ml/hr at 4$^{o}$ and elution of HA-1 and HA-2 with 25 mM galactose and 0.1 M lactose, respectively. The details are given in Figure 6.3.

The overall yield as measured by haemagglutinating activity was 17% for HA-1 and 10% for HA-2, based on the total activity in the haemolymph applied to the affinity column. However, only about 30% of each activity bound to the column, indicating that the column was probably saturated. The unbound activity, which was recovered in the column effluent, was not included in the subsequent purification steps. Thus, the final yields based on the material which bound to the affinity column were highly satisfactory, being 17% x (100/27%) = 63% for HA-1 and 10% x (100/33%) = 30% for HA-2. When subjected to Sephadex G-200 chromatography, the preparations obtained by elution with the galactose and lactose each gave one major haemagglutinating peak corresponding to HA-1 and HA-2, respectively. No activity (fractions dialysed or undialysed) was found outside these peaks. This showed

FIGURE 6.3

Outline of the procedure used to purify the haemagglutinins from 60 ml of haemolymph using the Lactose-Sepharose affinity column. All steps were performed at 4°. The haemolymph was applied to the affinity column at a flow rate of 5 ml/hr and the sugar solutions at a flow rate of 50 ml/hr. Five millilitre fractions were collected. Samples were concentrated using an Amicon Model 52 ultrafiltration cell (YM10 membrane for HA-2 and YM30 membrane for HA-1). Sephadex G-200 chromatography was performed as in Figure 4.4 and was included as a final purification step to remove any contaminating species that may still have been present in the affinity column eluates. Fractions were assayed without dialysis for agglutinins against sheep (●——●) and guinea pig (▽-----▽) erythrocytes.

STEP 1. APPLY HAEMOLYMPH (60ml) TO AFFINITY COLUMN

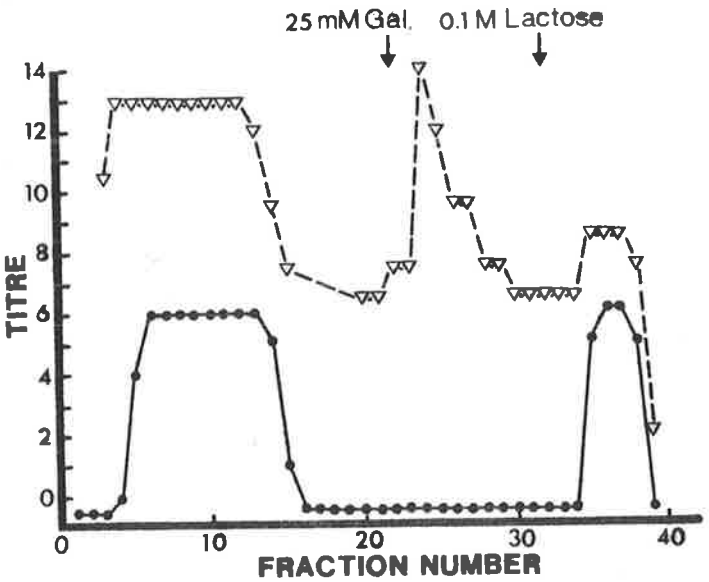
HAU = 480,000 (GPRBC)

HAU = 3,840 (SRBC)

Protein = 0.45mg/ml

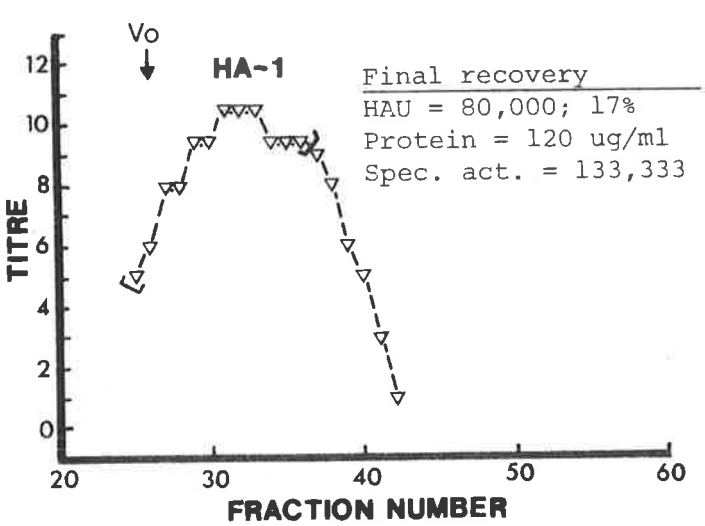
Spec. act. = 17,778(GPRBC)

Spec. act. = 142 (SRBC)



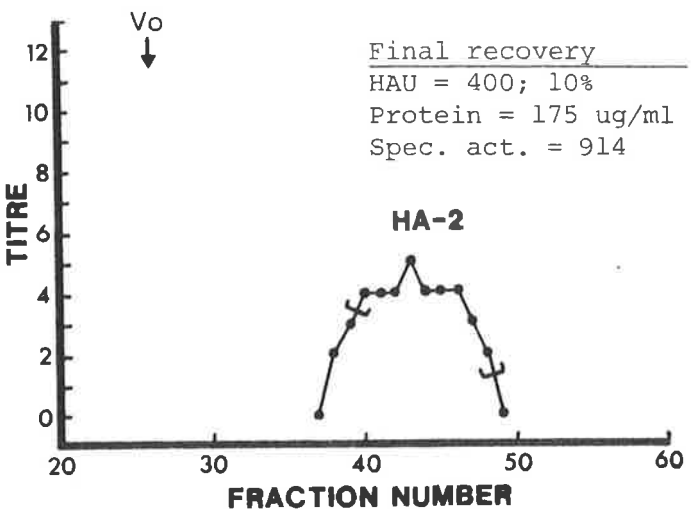

STEP 2. PROCESS GALACTOSE ELUATE

1) Pool affinity column fractions 24-27
   HAU = 128,000; 27%
2) Concentrate to 5 ml
   HAU = 160,000; 33%
3) Run on Sephadex G-200
4) Pool fractions 25-36
   HAU = 48,000; 10%
5) Concentrate to 5 ml




STEP 3. PROCESS LACTOSE ELUATE

1) Pool affinity column fractions 35-38
   HAU = 1,280; 33%
2) Concentrate to 5 ml
   HAU = 800; 21%
3) Run on Sephadex G-200
4) Pool fractions 40-48
   HAU = 800; 21%
5) Concentrate to 5 ml



that the galactose and lactose solutions did indeed separately elute HA-1 and HA-2 with little cross contamination. As indicated by the increase in specific haemagglutinating activity (HAU/mg protein), HA-1 was purified 7.5 fold and the HA-2 about 6 fold.

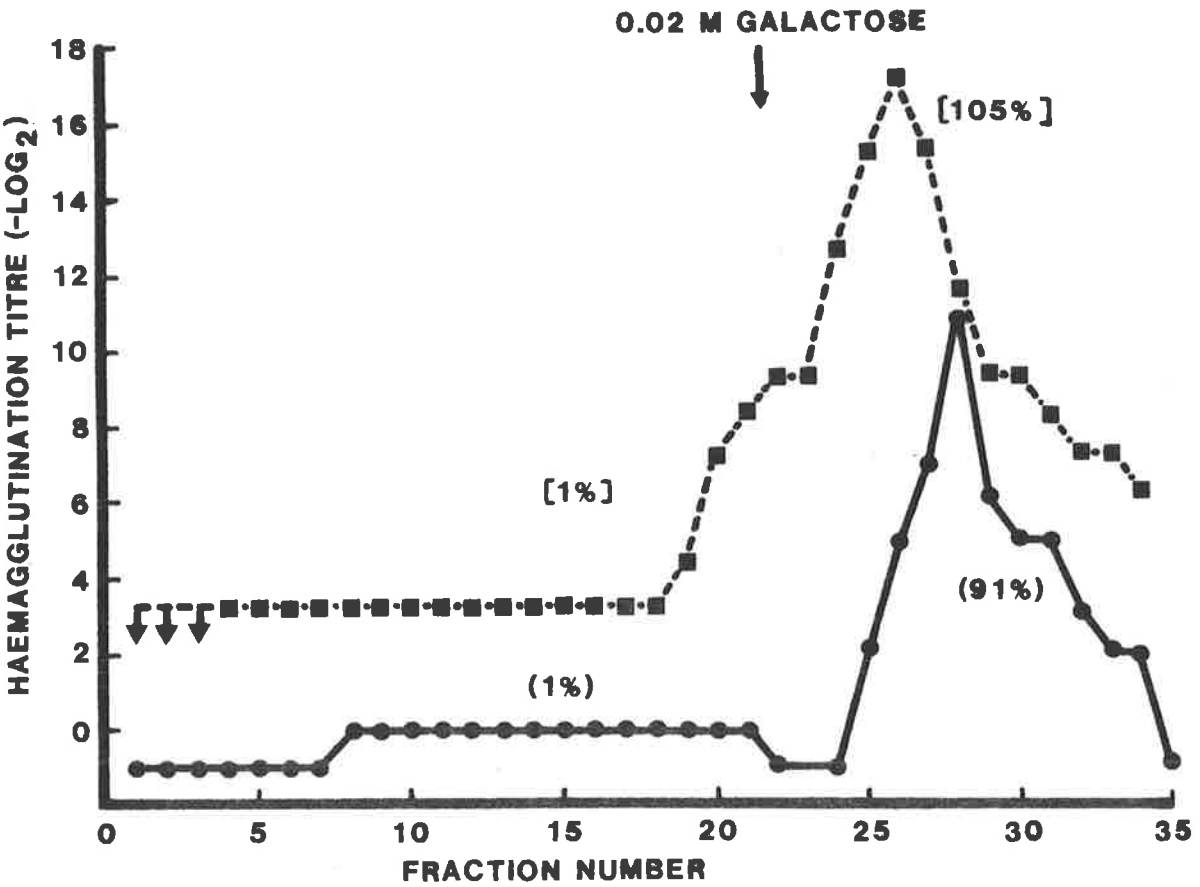
## 6.5 Interaction of the haemagglutinins with acid treated Sepharose 4B

The preceding results showed that the lactose affinity column had a very limited binding capacity. A means of increasing this capacity was suggested from work done by Baldo, Sawer, Stick and Uhlenbruck (1978) on the agglutinin from the clam *Tridacna maxima*. This is a galactosyl binding protein which normally interacts only weakly with Sepharose. However, the agglutinin will bind strongly to Sepharose which has been pretreated with acid (Baldo *et al.*, 1978). Since this situation seemed analogous to that of the *B. leachii* agglutinins, it appeared that pretreating Sepharose with acid might also increase its binding capacity for the *B. leachii* agglutinins, in particular the HA-1.

Accordingly, a sample of Sepharose 4B (8 ml settled volume) was treated with acid (Chapter 2, section 2.14.2) and loaded into a 20 ml glass syringe. Seventy five millilitres of *B. leachii* haemolymph, which had been dialysed against TSA, was passed through the column at 5 ml/hr at $4^{o}$. As shown in Figure 6.4, only 1% of both the sheep and guinea pig erythrocyte agglutinating activities appeared in the effluent, indicating that both HA-1 and HA-2 bound firmly to the gel. This column therefore appeared to have a binding capacity for both

FIGURE 6.4

Behavior of B. leachii haemagglutinins on the acid-treated Sepharose affinity column at 4°. Sample: 75 ml of haemolymph (SRBC titre = 1/128, GPRBC titre = 1/16,000), flow rate = 5 ml/hr. Elution flow rate = 50 ml/hr. Fraction size = 5 ml. All fractions were assayed without dialysis for agglutinins against sheep (●——●) and guinea pig (■-----■) erythrocytes. The overall percentage haemagglutinating activity recovered with each of the eluant sugar solutions is shown in parentheses: sheep erythrocytes ( ); guinea pig erythrocytes [ ].


0.02 M GALACTOSE
[105%]
[1%]
(91%)
(1%)
HAEMAGGLUTINATION TITRE (-LOG2)
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25
30
35

haemagglutinins of at least three times that of the earlier lactose-Sepharose column. Both agglutinins were recovered by elution at 50 ml/hr with 20 mM galactose, indicating that the binding was sugar specific. The HA-2 appeared to be bound less firmly than it was to lactose-Sepharose, since in the latter case galactose concentrations of >40 mM were necessary to elute the agglutinin. To see whether HA-1 and HA-2 could be eluted separately from the acid treated Sepharose column, the experiment was repeated using 5, 10 and 20 mM solutions of galactose in TSA as the eluant (see Fig. 6.5A). More than 90% of the applied HA-1 activity was eluted by 5 mM galactose, although a significant amount (6%) of the HA-2 activity was also eluted. Small amounts of HA-2 were recovered in the 10 mM and 20 mM galactose eluant fractions. Despite the greater binding capacity of acid-treated Sepharose for both agglutinins these results showed that lactose-Sepharose was superior in allowing resolution of the two types of agglutinins.

## 6.6 Interaction of the haemagglutinins with acid treated Sepharose conjugated with lactose

It seemed likely that lactose-substituted, acid treated Sepharose would have the advantages of both the previously tried adsorbents, i.e., it should have high binding capacity for both haemagglutinins and bind HA-2 molecules with high affinity, such that these are not eluted by low concentrations of galactose.

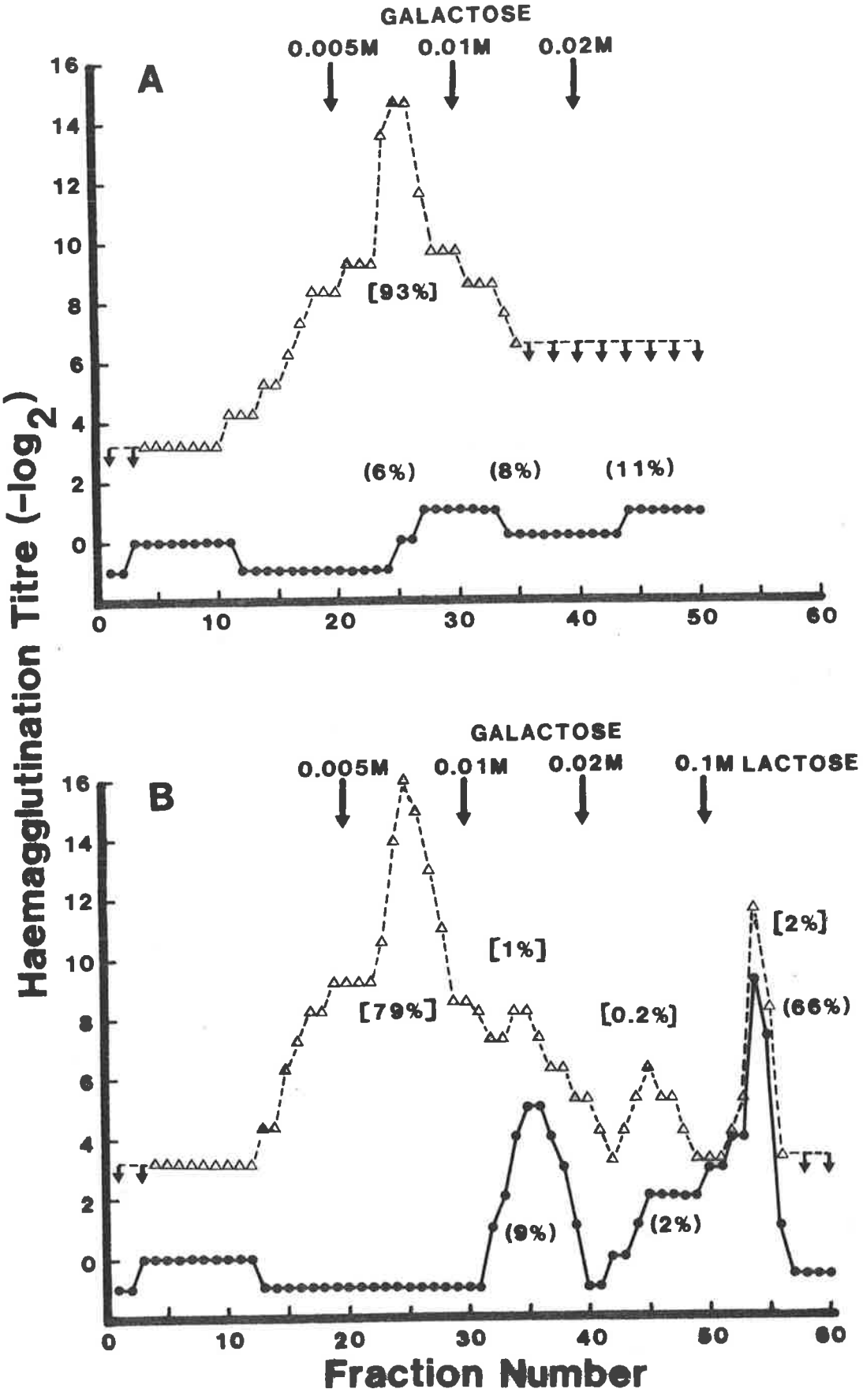
The gel was prepared by coupling lactose to acid-treated Sepharose as described in Chapter 2. Fifty millilitres of

FIGURE 6.5

Behavior of B. leachii haemagglutinins on different affinity columns at 4°. Haemolymph was applied at 4 ml/hr. Elution was performed at 50 ml/hr, 5 ml fractions being collected. All fractions were assayed without dialysis for agglutinins against sheep (●——●) and guinea pig (△----△) erythrocytes. The overall percentage haemagglutinating activity recovered with each of the eluant sugar solutions is shown in parentheses: sheep erythrocytes ( ); guinea pig erythrocytes [ ].

A. Acid-treated Sepharose. Sample: 50 ml of heamolymph; SRBC titre = 1/32, GPRBC titre = 1/8,000.

B. Lactose-substituted, Acid-treated Sepharose. Sample: 50 ml of haemolymph; SRBC titre = 1/128, GPRBC titre = 1/16,000.


GALACTOSE
0.005M
0.01M
0.02M
A
[93%]
(6%)
(8%)
(11%)
B
GALACTOSE
0.005M
0.01M
0.02M
0.1M LACTOSE
[79%]
[1%]
[0.2%]
[2%]
(66%)
(9%)
(2%)
Haemagglutination Titre (-log2)
Fraction Number

haemolymph (dialysed against TSA) was passed through the column at 3 ml/hr at 4°. The column was then washed at 50 ml/hr with 50 ml of TSA followed by TSA containing 5, 10 and 20 mM galactose and finally 0.1 M lactose. The results are shown in Figure 6.5B. Less than 0.5% of the sheep erythrocyte haemagglutinating activity and only 4% of the applied guinea pig activity was detected in the column effluent, indicating that both agglutinins were almost fully retained by the gel matrix. Elution with 5 mM galactose released approximately 79% of the guinea pig erythrocyte agglutinating activity and, significantly, these fractions contained no detectable sheep erythrocyte agglutinating activity. Only small amounts of HA-1 activity were eluted by the subsequent sugar solutions (10 mM galactose,1%; 20 mM galactose,0.2%; 100 mM lactose,2%). A small proportion (11%) of the sheep erythrocyte haemagglutinating activity was detected in the 10 mM and 20 mM galactose eluate fractions (9% and 2% respectively), but most (66%) was eluted by the lactose. These results showed that the lactose-substituted, acid-treated Sepharose affinity column had a satisfactory binding capacity and that HA-1 and HA-2 molecules could be differentially eluted from this column with little cross-contamination.

## 6.7 Purification of *B. leachii* haemagglutinins using the lactose-substituted, acid-treated Sepharose affinity column

In light of the results obtained in the previous section, an attempt was made to purify the haemagglutinins from 50 ml of *B. leachii* haemolymph using the lactose-substituted, acid-

treated Sepharose column (Lac-acid-Sepharose). The procedure was identical to that used earlier for the lactose-Sepharose column (Fig. 6.3). The results of the affinity column step are shown in Figure 6.6. After the haemolymph (50 ml) had been applied, the column was washed with TSA. More than 99% of both agglutinating activities was retained by the column, in contrast to the results with lactose-Sepharose (Fig. 6.3) where only about 30% of each activity was bound. Upon elution with 5 mM galactose, 90% of the HA-1 activity was recovered. No HA-2 activity was detected in these fractions. In contrast, subsequent elution with 0.1 M lactose released all (120%) of the sheep erythrocyte activity, together with 1.5% of the guinea pig erythrocyte activity which was shown later by gel chromatography to be due to HA-2. Each eluate was pooled and concentrated in order to continue the purification by chromatography on Sephadex G-200 (data not shown). The overall yields of HA-1 and HA-2 activity (35% and 50%, respectively; Table 6.1) were increased 3 to 5 fold in comparison to those obtained previously (Fig. 6.3). This was due mostly to the greater binding capacity of the affinity column. Fractionation of the HA-1 and HA-2 preparations on Sephadex G-200 indicated that each agglutinin was eluted from the affinity column with little cross contamination. As judged by specific haemagglutination activities, it was estimated that the HA-1 had been purified 17-fold and the HA-2 6-fold.

## 6.8 The effect of EDTA on the binding of HA-1 to lactose-substituted, acid-treated Sepharose

In Chapter 4 it was shown that the haemagglutinating

FIGURE 6.6

Purification of B. leachii haemagglutinins using the Lac-acid-Sepharose affinity column. Sample: 50 ml of haemolymph (SRBC titre = 1/320, GPRBC titre = 1/23,000), flow rate = 3 ml/hr. Elution flow rate = 50 ml/hr. Fraction size = 5 ml. All fractions were assayed without dialysis for agglutinins against sheep (●——●) and guinea pig (■------■) erythrocytes. The overall percentage haemagglutinating activity recovered with each of the eluant sugar solutions is shown in parentheses: sheep erythrocytes ( ); guinea pig erythrocytes [ ].

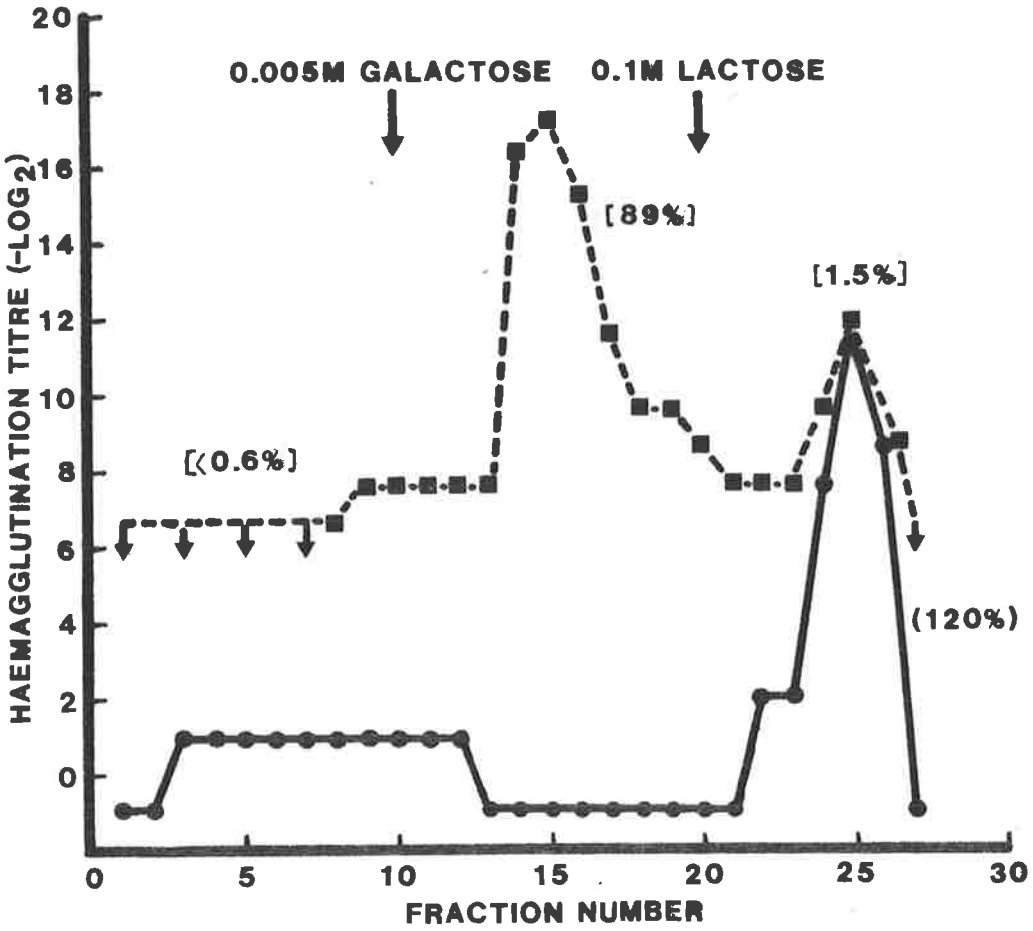

0.005M GALACTOSE
0.1M LACTOSE
[89%]
[1.5%]
[<0.6%]
(120%)
HAEMAGGLUTINATION TITRE (-LOG2)
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
25
30

TABLE 6.1

The recoveries of the B. leachii haemagglutinins during purification from 50 ml of haemolymph using the acid-treated, lactose-substituted Sepharose affinity column*

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* The protocol used was as outlined in Figure 6.3.

@ Assayed using guinea pig erythrocytes.

\+ HAU per mg of protein.

£ The results for this step are shown in Figure 6.6.

§ Figure 6.6, fractions 13-19 inclusive.

\# Results not shown but profile obtained was very similar to that shown in Figure 6.3.

¶ Assayed using sheep erythrocytes.

ß Figure 6.6, fractions 22-28 inclusive.

TABLE 6.1

The recoveries of the B. leachii haemagglutinins during purification from 50 ml of haemolymph using the lactose-substituted, acid-treated Sepharose affinity column*

A) Recovery of HA-1@

| Stage of purification | Volume (ml) | Titre$^{-1}$ | Total HAU | Percent recovery | Protein (mg) | Specific activity+ |
|---|---|---|---|---|---|---|
| AFFINITY CHROMATOGRAPHY£ | | | | | | |
| Haemolymph | 50 | 32,000 | $1.6 \times 10^6$ | 100 | 0.45 | 71,111 |
| Pool of galactose eluate fractions§ | 35 | 32,000 | $1.1 \times 10^6$ | 69 | - | - |
| Concentrate of pooled fractions | - | - | - | - | - | - |
| SEPHADEX G-200 CHROMATOGRAPHY# | | | | | | |
| Pool of agglutinating peak fractions | 65 | 8,000 | 520,000 | 33 | - | - |
| Concentrate of pooled fractions | 3.5 | 160,000 | 560,000 | 35 | 0.135 | $1.2 \times 10^6$ |

B) Recovery of HA-2¶

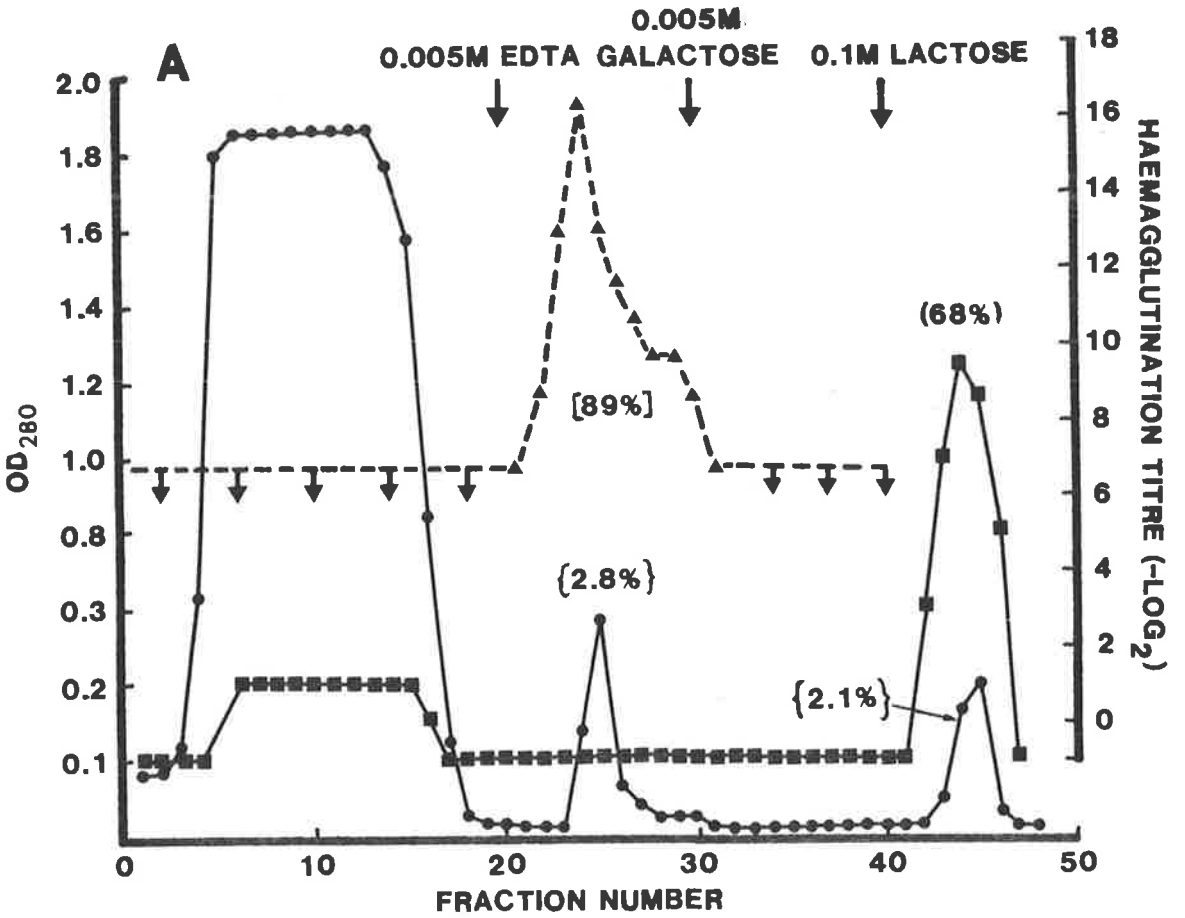
| Stage of purification | Volume (ml) | Titre$^{-1}$ | Total HAU | Percent recovery | Protein (mg) | Specific activity+ |
|---|---|---|---|---|---|---|
| AFFINITY CHROMATOGRAPHY£ | | | | | | |
| Haemolymph | 50 | 320 | 16,000 | 100 | 0.45 | 711 |
| Pool of lactose eluate fractionsβ | 35 | 320 | 11,200 | 70 | - | - |
| Concentrate of pooled fractions | 3 | 4,500 | 13,500 | 84 | - | - |
| SEPHADEX G-200 CHROMATOGRAPHY# | | | | | | |
| Pool of agglutinating peak fractions | 65 | 128 | 8,320 | 52 | - | - |
| Concentrate of pooled fractions | 5 | 3,200 | 8,000 | 50 | 0.8 | 4,000 |

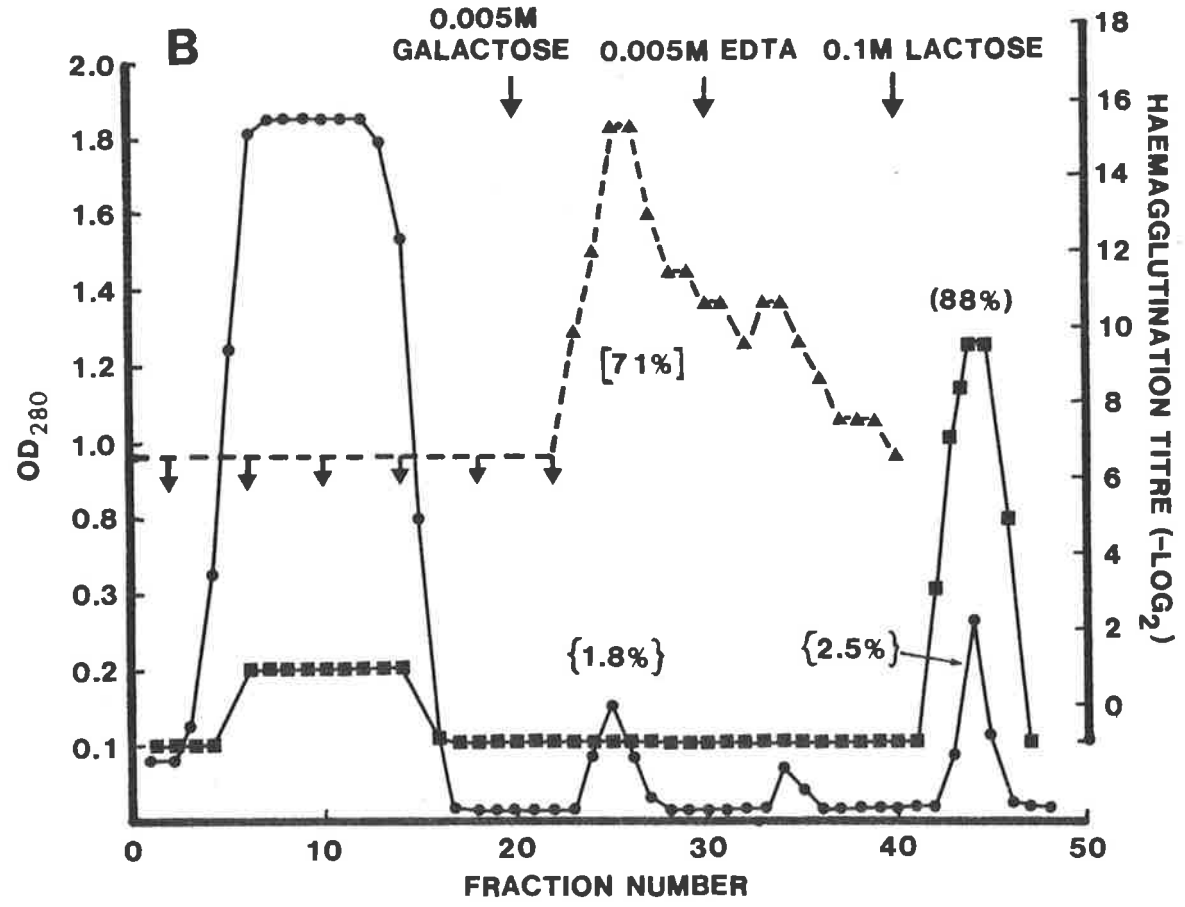
activity of HA-1 could be inhibited by EDTA. The inhibition was reversible, since the subsequent addition of $Ca^{++}$ ions restored full agglutinating activity. EDTA had no effect on HA-2 activity and it therefore seemed probable that the binding of HA-1 to the column could be prevented *in toto* without affecting the binding of HA-2.

To test whether this was possible, the affinity column (Lac-acid-Sepharose) was loaded with 50 ml of dialysed haemolymph and washed with TSA followed by TSA containing 5 mM EDTA, 5 mM galactose and finally 0.1 M lactose in that order (Fig. 6.8A). Essentially all of the HA-1 activity (>99%) was retained by the column and of this, approximately 89% was eluted by EDTA. This activity coincided with a sharp $OD^{280}$ peak which represented 2.8% of the $OD^{280}$ units applied to this column. No additional material (haemagglutinating activity or $OD^{280}$ units) was eluted by galactose. The column also retained nearly all of the HA-2 activity (>98%), but in contrast with the HA-1, none of the HA-2 was released by EDTA or galactose. This activity was released by lactose as illustrated in Figure 6.8A. An $OD^{280}$ peak containing approximately 2.1% of the total $OD^{280}$ units applied to the column corresponded to the HA-2 activity. When the reverse procedure was tried, i.e. eluting first with galactose and then with EDTA, two $OD^{280}$ peaks representing recoveries of 1.8% and 0.8% respectively, were obtained (Fig. 6.8B). These peaks coincided with guinea pig erythrocyte specific haemagglutinating activity totalling approximately 71% (galactose) and 1% (EDTA) of the applied activity. These results indicated that initial elution with EDTA released

FIGURE 6.8

Effect of EDTA on elution of *B. leachii* haemagglutinins from Lac-acid-Sepharose. Sample: 50 ml of haemolymph, $OD_{280}$ = 2.2, GPRBC titre = 1/11,314, SRBC titre = 1/160, flow rate = 3 ml/hr. Elution flow rate = 50 ml/hr. Fraction size = 4 ml. All fractions were assayed without dialysis for agglutinins against sheep (■——■) and guinea pig (▲------▲) erythrocytes and for absorbance at 280 nm (●——●). The overall percentage haemagglutinating activity and OD(280 nm) units recovered with each of the eluant sugar solutions is shown in parentheses: sheep erythrocytes ( ); guinea pig erythrocytes [ ]; OD(280 nm) units { }. The experiments were identical except that in (A) EDTA elution preceded galactose elution whereas in (B) the procedure was reversed.


A
0.005M EDTA
0.005M GALACTOSE
0.1M LACTOSE
OD280
HAEMAGGLUTINATION TITRE (-LOG2)
FRACTION NUMBER
[89%]
{2.8%}
{2.1%}
(68%)


B
0.005M GALACTOSE
0.005M EDTA
0.1M LACTOSE
OD280
HAEMAGGLUTINATION TITRE (-LOG2)
FRACTION NUMBER
[71%]
{1.8%}
{2.5%}
(88%)

bound material (including some HA-1 agglutinin) not released by galactose. This is considered in more detail later in this chapter. The recovery of HA-2 by lactose was similar in both experiments.

These results showed that EDTA did prevent the binding of HA-1 *in toto* and suggested that it should be possible to completely deplete haemolymph of HA-2 without any risk of contamination with HA-1 by passage through the affinity column in the presence of EDTA. It seemed that this procedure would also allow much larger amounts of haemolymph than had been previously used to be processed at a single time since the prevention of HA-1 adsorption should decrease competition for ligand receptor sites on the affinity column and thus increase the binding capacity of the column for HA-2 agglutinin. Similarly, after removal of the HA-2 molecules from the column and reconstitution of the haemolymph with $Ca^{++}$ ions, the column should be capable of retaining a larger quantity of HA-1 molecules.

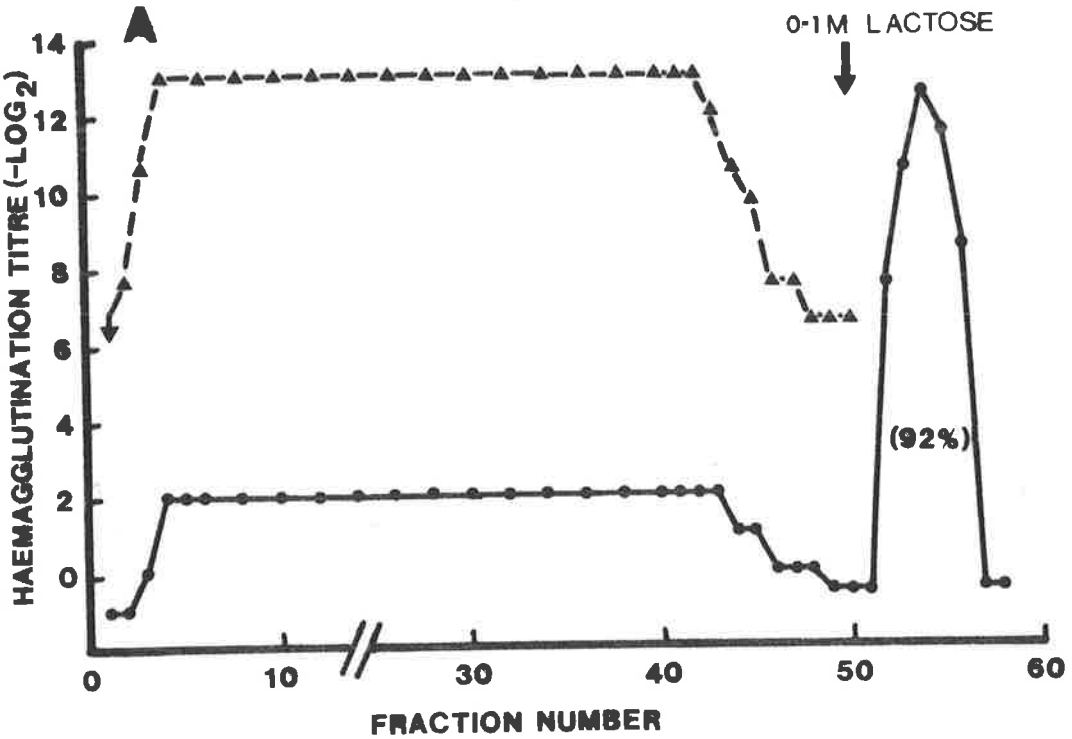
The experiment utilised a fresh column comprising 15 ml of Lac-acid-Sepharose. The sample of haemolymph (200 ml) was dialysed twice against two litres of TSA and made 4 mM with respect to EDTA before application. The results are illustrated in Figure 6.9. As expected, more than 95% of the HA-2 bound to the column whereas the HA-1 was fully recovered in the effluent (Fig. 6.9A). After washing the column with TSA containing 4 mM EDTA, 92% of the HA-2 activity was recovered by elution with 0.1 M lactose. The column was then washed with TSA supplemented with 1 mM $CaCl_2$. The effluent haemolymph fractions from the EDTA passage were pooled (214 ml) and made 8 mM with respect to $CaCl_2$ before

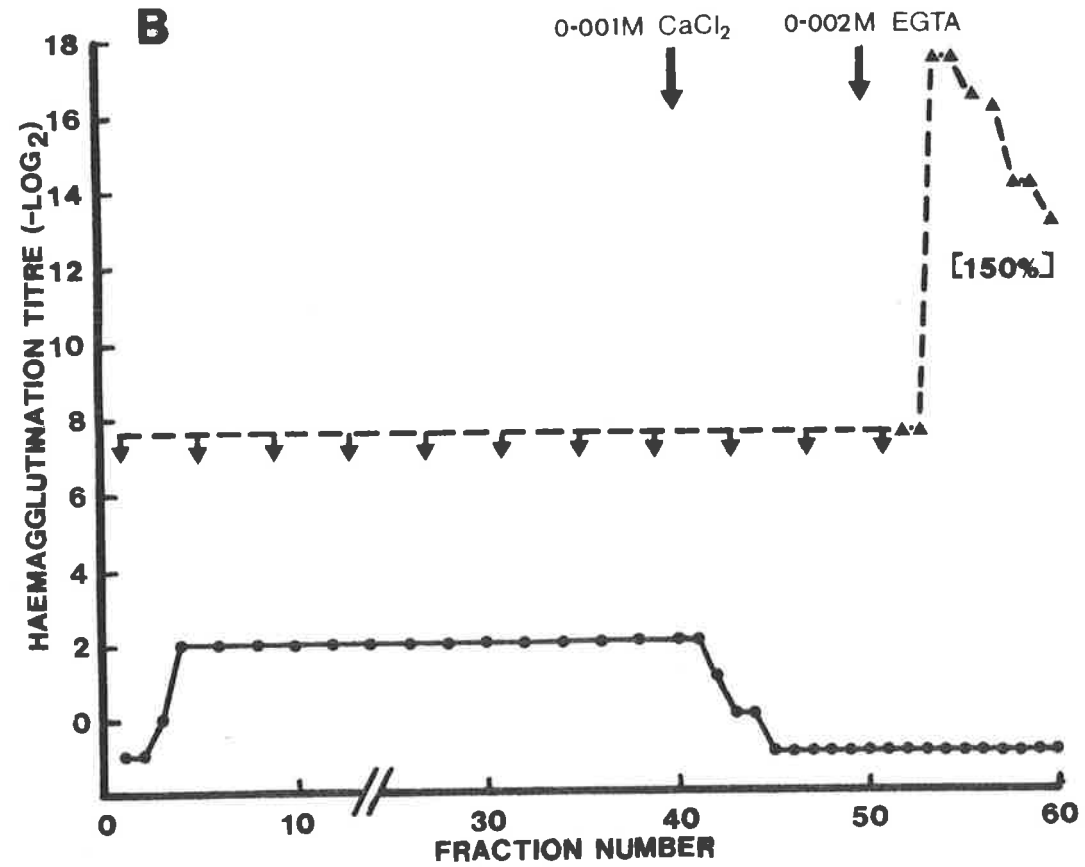
FIGURE 6.9

Two stage purification of the B. leachii haemagglutinins using affinity chromatography in EDTA. Sample flow rate = 15 ml/hr. Fraction size = 5 ml. All fractions were assayed without dialysis for agglutinins against sheep (●——●) and guinea pig (▲------▲) erythrocytes. The overall percentage haemagglutinating activity recovered with each of the eluant sugar solutions is shown in parentheses: sheep erythrocytes ( ); guinea pig erythrocytes [ ].

A. Sample: 200 ml of haemolymph containing 4 mM EDTA, (SRBC titre = 1/320, GPRBC titre = 1/8,000). The column was washed (50 ml/hr) with TSA and then with TSA containing 0.1 M lactose.

B. Sample: fractions 3 - 44 (214 ml) from stage (A) reconstituted with 8 mM $CaCl_2$. The column was washed (50 ml/hr) with TSA/$Ca^{++}$ and then with TSA/EGTA.


A
0·1M LACTOSE
(92%)
HAEMAGGLUTINATION TITRE (-LOG2)
FRACTION NUMBER
0
2
4
6
8
10
12
14
0
10
30
40
50
60


B
0·001M CaCl2
0·002M EGTA
[150%]
HAEMAGGLUTINATION TITRE (-LOG2)
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
0
10
30
40
50
60

reapplication to the affinity column (Fig. 6.9B). No HA-1 was detected in the effluent, indicating complete retention of the agglutinin by the column. The column was washed with TSA containing 1 mM $CaCl_2$ and then with TSA containing 2 mM EGTA. Approximtely 99% of the HA-1 was recovered in the EGTA eluate.

## 6.9 Analysis of the lactose eluate (HA-2) fractions from the affinity column

The protein content of the eluate fractions was assayed using the Lowry method (Chapter 2). Aliquots containing 10 to 40 µg of protein were then taken from relevant fractions for discontinuous SDS-PAGE analysis. The protein was precipitated in trichloroacetic acid and sodium deoxycholate (Chapter 2, section 2.16.3) and the precipitates were washed in ether/ethanol (1:1) before being dissolved in the SDS sample buffer and applied to 7% and 11% gels as described in Chapter 2, section 2.16. As shown in Figure 6.10, the HA-2 material appeared to be fairly homogeneous in the denaturing conditions of the gel. Only one major and 2 or 3 very minor bands were evident.

Eluate fractions 51 to 57 were pooled, concentrated by ultrafiltration to 5 ml (YM10 membrane) and subjected to chromatography on Sephadex G-200. In common with the previous results, only one haemagglutinating peak was obtained using both dialysed and undialysed fractions (results not shown). This peak was in the position expected for HA-2. The haemagglutinin-positive fractions were pooled and concentrated. SDS-PAGE analysis (Fig. 7.5) revealed only one major band and one very minor band. The percent recoveries

FIGURE 6.10

Analyses by SDS-PAGE in non-reducing conditions of fractions recovered from the affinity column in Figure 6.9

A. Analysis in a 7% polyacrylamide gel.

A whole haemolymph, 10 ug

Lactose eluate

B fraction 54, 40 ug

C fraction 54, 20 ug

D fraction 54, 10 ug

E fraction 55, 40 ug

F fraction 55, 20 ug

G fraction 55, 10 ug

EGTA eluate

H fraction 54, 40 ug

I fraction 54, 20 ug

J fraction 54, 10 ug

K fraction 55, 40 ug

L fraction 55, 20 ug

M fraction 55, 10 ug

B. Analysis in an 11% polyacrylamide gel.

A whole haemolymph, 10 ug

EGTA eluant

B fraction 54, 40 ug

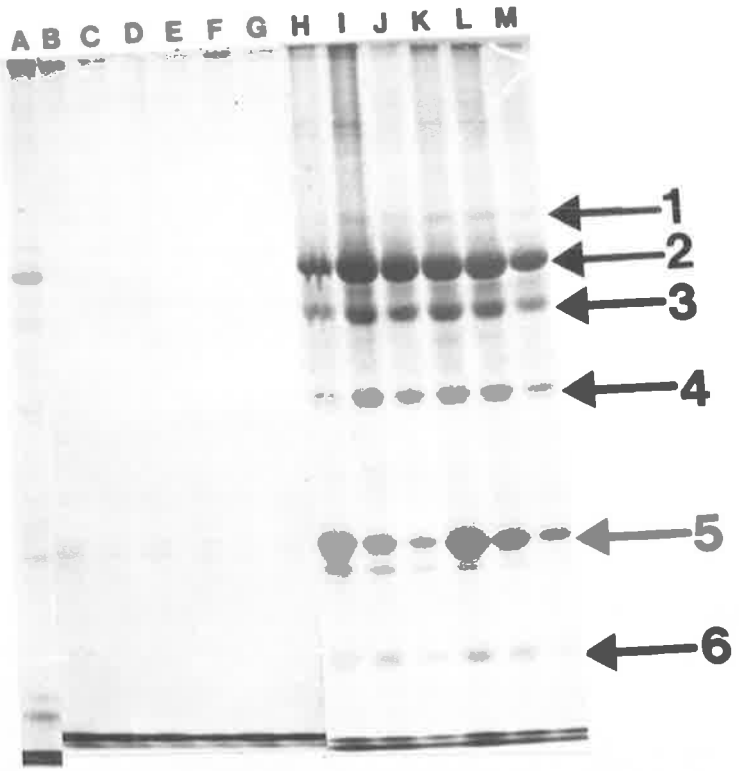
C fraction 55, 40 ug

Lactose eluate

D fraction 54, 40 ug

E fraction 55, 40 ug

A




B

at each step of the purification were very similar to those shown in Table 6.1, the overall recovery being 50%. The final preparation had a protein concentration of 1 mg/ml and a haemagglutinating titre (sheep erythrocytes) of 1/6,400. From its specific activity (6,400 HAU per mg of protein), it was estimated that the HA-2 had been purified approximately 16-fold compared to haemolymph.

## 6.10 Analysis of the EGTA eluate (HA-1) fractions from the affinity column

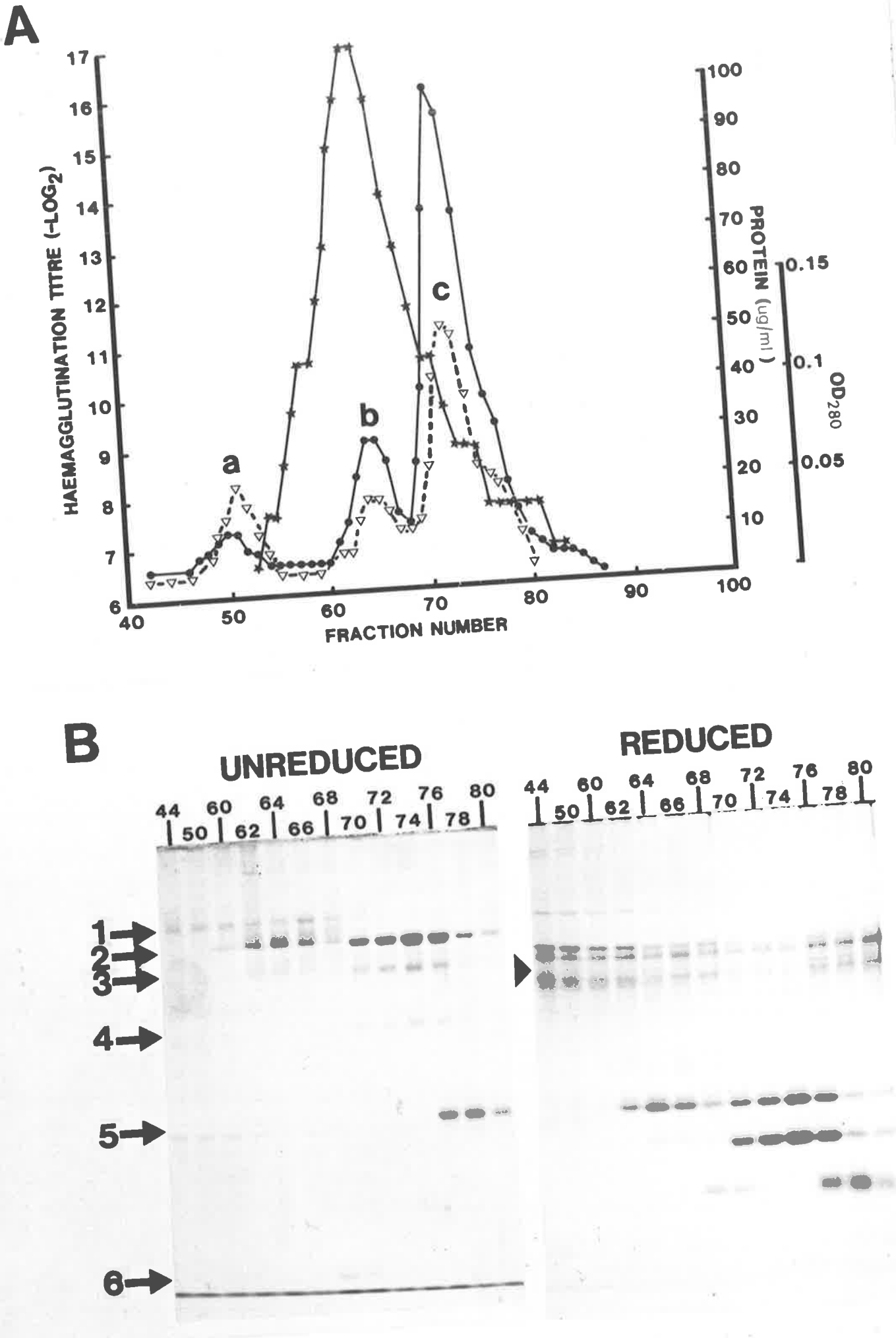
The EGTA eluate fractions were analysed by (unreduced) SDS-PAGE as described above and the results are presented in Figure 6.10. Under the denaturing conditions employed four to five major polypeptide bands and several minor bands were detected, indicating the presence of a number of different sized components.

The agglutinin was further purified by pooling the EGTA eluate fractions and subjecting the concentrate to chromatography on Sephacryl S300. This gel, which had only recently become available, was used instead of Sephadex G-200 since it appeared to give better resolution of high molecular weight material. The eluant buffer (TSA) contained 0.5 mM EGTA plus 0.5 mM EDTA to prevent interaction of the haemagglutinins with the Sephacryl (see Chapter 2 for full details). Besides assaying their haemagglutinating activity, the fractions were analysed for protein by $OD^{280}$ measurements and by the Lowry method. The results are shown in Figure 6.11A. It can be seen that three protein peaks a, b, and c were resolved, but only peak (b) corresponded with the haemagglutinating activity. Nearly 100% of the applied

FIGURE 6.11

A. Sephacryl S-300 chromatography of the EGTA (HA-1) eluate fractions from the Lac-acid-Sepharose affinity column. Fractions 53-58 inclusive from Figure 6.9B were pooled and concentrated to 6 ml (Amicon Model 52 ultrafiltration cell) and then chromatographed (Chapter 2, section 2.11). The protein content of the fractions was measured by absorbance at 280 nm (▽-----▽) and by the Lowry method (Chapter 2, section 2.15.1, part ii) (●——●). All fractions were assayed without dialysis for agglutinins against guinea pig (✶——✶) and sheep (no activity detected) erythrocytes.

B. SDS-PAGE analyses in reducing (11% gel) and non-reducing (7% gel) conditions of the Sephacryl S-300 fractions. Approximately 2-3 ug of protein from the indicated fractions was precipitated in trichloroacetic acid and sodium deoxycholate (Chapter 2, section 2.16.3) and the precipitate was dissolved in sample buffer and loaded onto the gels. The cluster of bands marked by the arrow for reducing conditions would appear to be artifacts since they were also obtained from fraction 44 which is before the void volume of the column (fraction 50). These bands were not obtained in SDS-PAGE analyses of material prepared subsequently (see Chapter 7).


A
HAEMAGGLUTINATION TITRE (-LOG2)
PROTEIN (ug/ml)
OD280
FRACTION NUMBER
a
b
c
B
UNREDUCED
REDUCED
1
2
3
4
5
6

HA-1 activity was recovered in this peak. No HA-2 activity was detected in any fractions, either dialysed or undialysed.

The fractions comprising the major protein peaks (b and c) were analysed by discontinuous SDS-PAGE under non-reducing conditions. The results are presented in Figure 6.11B. Many of the bands observed in the analysis of the affinity column eluates (Fig. 6.10) were identified in the Sephacryl S-300 fractions. Thus, peak (b) material yielded 2 bands corresponding to bands 1 and 2 of the earlier analysis, whilst peak (c) material contained bands 2, 3 and 4. The shoulder seen on peak (c) appeared to contain band 5 polypeptides. Band 6 could be seen in the fractions between peaks (b) and (c). When the fractions were analysed by SDS-PAGE under reducing conditions, only one polypeptide band could be seen in the fractions corresponding to peak (b) and 2 bands in those representing peak (c). Reduction of bands 5 (peak (c)) and 6 (peaks (b)-(c)) gave rise to no additional bands.

The final HA-1 preparation was obtained by pooling fractions 60-72 inclusive. After concentration, the protein content of the solution was 0.34 mg/ml and the haemagglutination titre (guinea pig erythrocyte) was 1/160,000, giving a specific activity of 470,000 HAU per mg of protein. This represented a 30-fold purification of HA-1 from the haemolymph with a yield of approximately 50% based on haemagglutinating activity.

## 6.11 Production of antisera

Antisera were raised in rabbits as described in Chapter 2 (section 2.20.1) using the preparations whose purification

is described above. Two hundred micrograms of HA-2 and 300 µg of HA-1 were used for each immunisation, respectively. The HA-1 preparation contained approximately equal amounts of protein from peak (b) (HA-1) and peak (c) (Fig. 6.11A). As will be seen later (Chapter 7), the resulting antiserum contained activity to both of these components.

## 6.12 Further studies on the proteins eluted from the Lac-acid-Sepharose column by galactose and EGTA

As shown in the preceding section the material eluted from the affinity column by EGTA appeared to contain other proteins besides the HA-1 agglutinin. These were either other carbohydrate binding proteins (like HA-1) or proteins which bound non-specifically, perhaps through $Ca^{++}$-dependent interactions, with the affinity column. To investigate this point further, a second 200 ml batch of haemolymph was processed exactly as before (Fig. 6.9) except that 5 mM galactose was used to elute the HA-1. The column was subsequently washed with TSA containing 2 mM EGTA. The $OD^{280}$ elution profile and the SDS-PAGE analyses of relevant unreduced fractions is depicted in Figure 6.12. As found earlier (Fig. 6.8B), the bulk of the eluted material (68%) was recovered using galactose, the remaining 32% being eluted with EGTA. The recovery of haemagglutinating activity (not shown) was identical to that of Figure 6.8B. The results of the SDS-PAGE analyses of these fractions (Fig. 6.12B) show that bands 1, 2, 3 and 4 (c.f. Fig. 6.10) were present in the galactose eluates, whereas only band 5 could be detected in the subsequent EGTA eluate. However, when

FIGURE 6.12

A. Elution of HA-1 from the Lac-acid-Sepharose affinity column with galactose. Haemolymph (200 ml) from which the HA-2 had been removed by affinity chromatography in EDTA (as in Fig. 6.9A) was reconstituted with $CaCl_2$ (8 mM) and reapplied to the affinity column. The column was then eluted with 5 mM galactose followed by 2 mM EGTA. Experimental details were as described in Figure 6.9. The protein content of each fraction was measured by absorbance at 280 nm. The percentage of the total eluted material recovered in each peak is shown in parentheses.

B. SDS-PAGE analyses in non-reducing conditions (performed as described in Fig. 6.11) of the material eluted from the affinity column by galactose and EGTA.

Galactose eluate

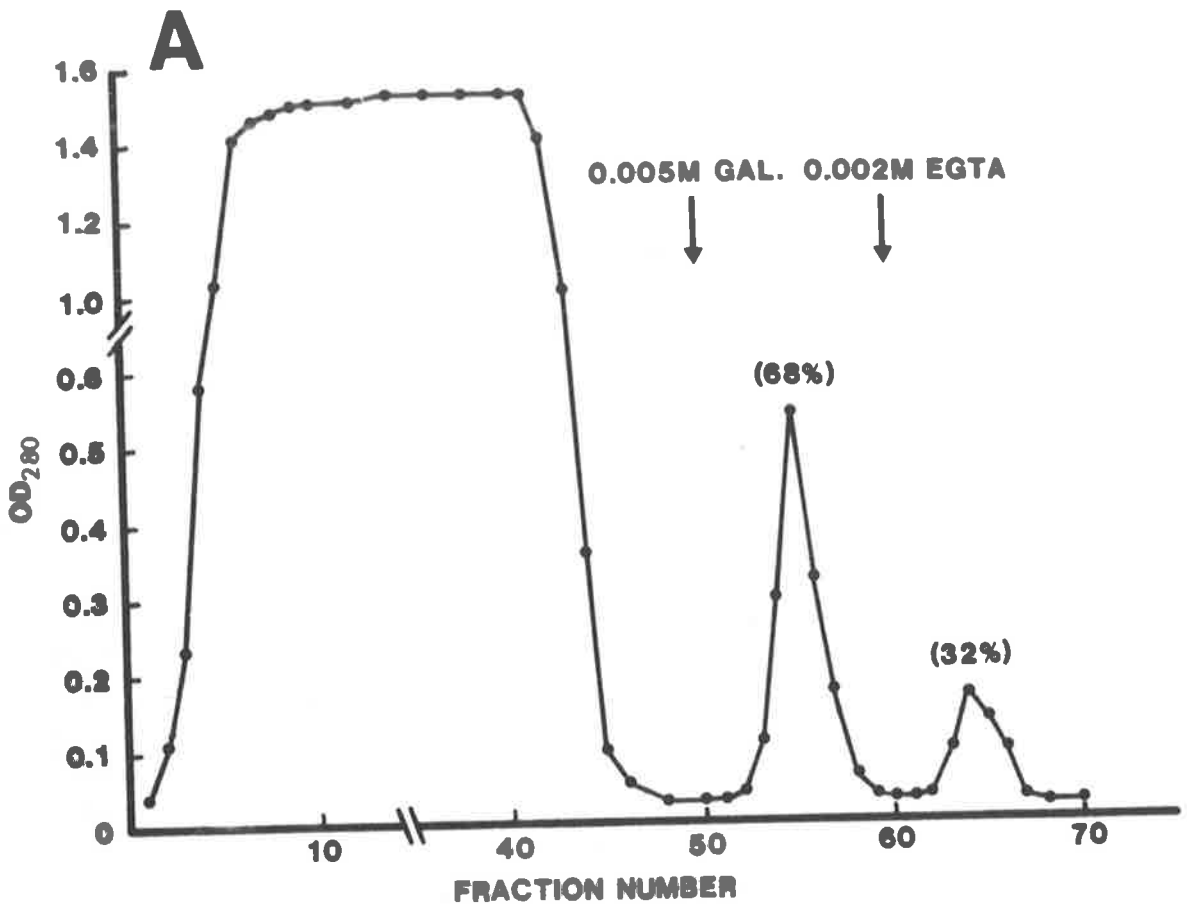
| | |
|---|---|
| A | fraction 4, 10 ug |
| B | fraction 4 2 ug |
| C | fraction 5, 10 ug |
| D | fraction 5, 2 ug |
| E | fraction 6, 10 ug |
| F | fraction 6, 2 ug |

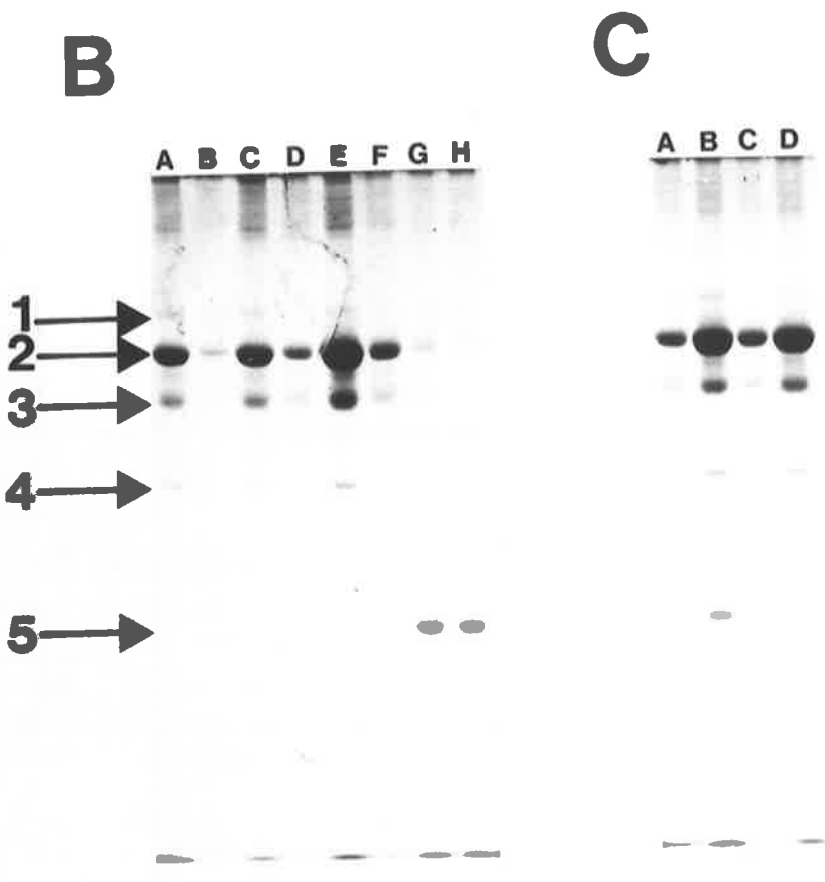
EGTA eluate

| | |
|---|---|
| G | fraction 5, 5 ug |
| H | fraction 4, 5 ug |

C. SDS-PAGE analyses (non-reducing conditions) of material eluted from the Lac-acid-Sepharose affinity column solely by EGTA. Haemolymph (200 ml from same batch as above) was treated exactly as in (A) except that the column was eluted first with EGTA rather than galactose.

| | |
|---|---|
| A | EGTA eluate fraction 4, 2 ug |
| B | EGTA eluate fraction 4, 10 ug |
| C | EGTA eluate fraction 5, 2 ug |
| D | EGTA eluate fraction 5, 10 ug |


A
1.6
1.4
1.2
1.0
0.6
0.5
0.4
0.3
0.2
0.1
0
OD280
0.005M GAL.
0.002M EGTA
(68%)
(32%)
10
40
50
60
70
FRACTION NUMBER


B
A B C D E F G H
1
2
3
4
5
C
A B C D

the experiment was repeated but the column eluted first with EGTA rather than with galactose (data not shown), the EGTA eluate comprised bands 1-4 as well as band 5 as can be seen in Figure 6.12C. These results indicated that the proteins represented by bands 1-4 are eluted by both EGTA and galactose, whereas those comprising band 5 can be eluted by EGTA only. Band 6 (Fig. 6.10) was not evident in any fractions, possibly because it was not present in this particular batch of haemolymph or because the samples loaded onto the gel contained less protein (10 µg maximum).

The material eluted by galactose (Fig. 6.12A, fractions 53-58) was concentrated and chromatographed on Sephacryl S-300. The elution profile (Fig. 6.13A) was very similar to that obtained earlier for the EGTA eluted material (Fig. 6.11A), the three protein peaks a, b and c being apparent but in different relative amounts. Only peak (b) material contained haemagglutinating activity (data not shown). SDS-PAGE analyses (Fig. 6.13B) indicated that the proteins constituting peak (b) contained polypeptide bands 1 and 2 while the protein in peak (c) comprised bands 2 and 3. The yield of peak (b) (HA-1) was approximately 0.5 mg of protein and that of peak (c) approximately 3.5 mg of protein, from 200 ml of haemolymph.

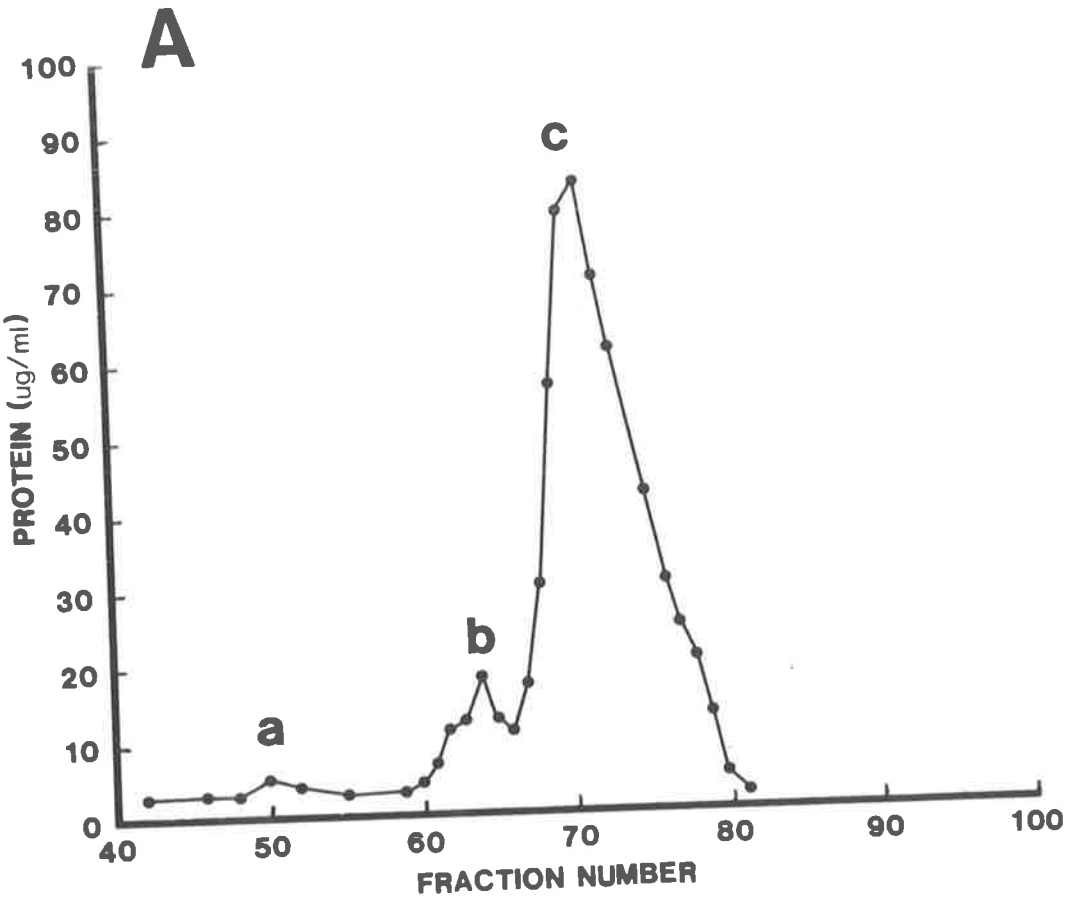
It was clear from the results of the preceding experiments that, in addition to the HA-1 agglutinin, a relatively large amount of protein bound to the Lac-acid-Sepharose column during the adsorption step in the presence of $Ca^{++}$ and was eluted together with the HA-1 by galactose. The bulk of this protein appeared to be homogeneous in size as judged by its behaviour on Sephacryl S-300 ((peak (c), Figs. 6-11 and

FIGURE 6.13

Analyses of the galactose eluate (HA-1) fractions from the Lac-acid-Sepharose affinity column.

A. Sephacryl S-300 chromatography. The material eluted by galactose (Fig. 6.12A, fractions 53-58) was concentrated to 6 ml and chromatographed on Sephacryl S-300 at 4° (Chapter 2, section 2.11). The protein content of the fractions was measured using the Lowry method (Chapter 2, section 2.15.1, part ii).

B. SDS-PAGE analyses in non-reducing conditions (7% gel) of the material contained in peaks (b) and (c) from (A). Approximately 2-3 ug of protein from the indicated fractions was precipitated in trichloroacetic acid and sodium deoxycholate (Chapter 2, section 2.16.3) and the precipitate was dissolved in sample buffer and loaded onto the gels.


A
100
90
80
70
60
50
40
30
20
10
0
PROTEIN (ug/ml)
a
b
c
40
50
60
70
80
90
100
FRACTION NUMBER


B
60
62
64
66
68
70
74
76
78
80
1
2
3

6.13). From its behaviour during affinity chromatography in the presence or absence of EDTA, $Ca^{++}$ and galactose (and lactose, data not shown) it seemed that this protein was, like HA-1, a $Ca^{++}$-dependent, lactose binding protein. It was accordingly called Lactose Binding Protein Three (LBP-3), the HA-1 and HA-2 being the other two lactose binding proteins in *B. leachii* haemolymph.

From the results of Figure 6.11A, the LBP-3 appeared unable to agglutinate guinea pig erythrocytes, or at least to do so very inefficiently compared with HA-1. Nonetheless, it was considered that the LBP-3, like the HA-1 or HA-2, might be involved in self-nonself recognition in *B. leachii* and it therefore seemed relevant (and important) to study this protein in more detail. Accordingly, a large quantity of the haemolymph (500 ml) was fractionated by affinity chromatography and Sephacryl S-300 chromatography using the protocol described in Figures 6.12 and 6.13 (i.e., removal of HA-2 in EDTA followed by adsorption of HA-1/LBP-3 in $Ca^{++}$ and elution with galactose). The results of the chromatography of the galactose eluate on Sephacryl S-300 are depicted in Figure 6.14A and are indistinguishable from those of Figure 6.13A. Upon rechromatography (Fig. 6.14B and 6.14C), the HA-1 and LBP-3 each ran as homogeneous proteins, with no apparent cross-contamination. It can be seen from these figures that the LBP-3 was virtually inactive in terms of agglutinating activity (peak fraction 89; $OD^{280}$ = 0.35, haemagglutination titre = 1/64, specific activity = 266 HAU/mg) compared with the HA-1 (peak fraction 80; $OD^{280}$ = 0.08, haemagglutination titre = 1/512,000, specific activity = $9.4 \times 10^6$ HAU/mg). The small amount of activity associated with

FIGURE. 6.14

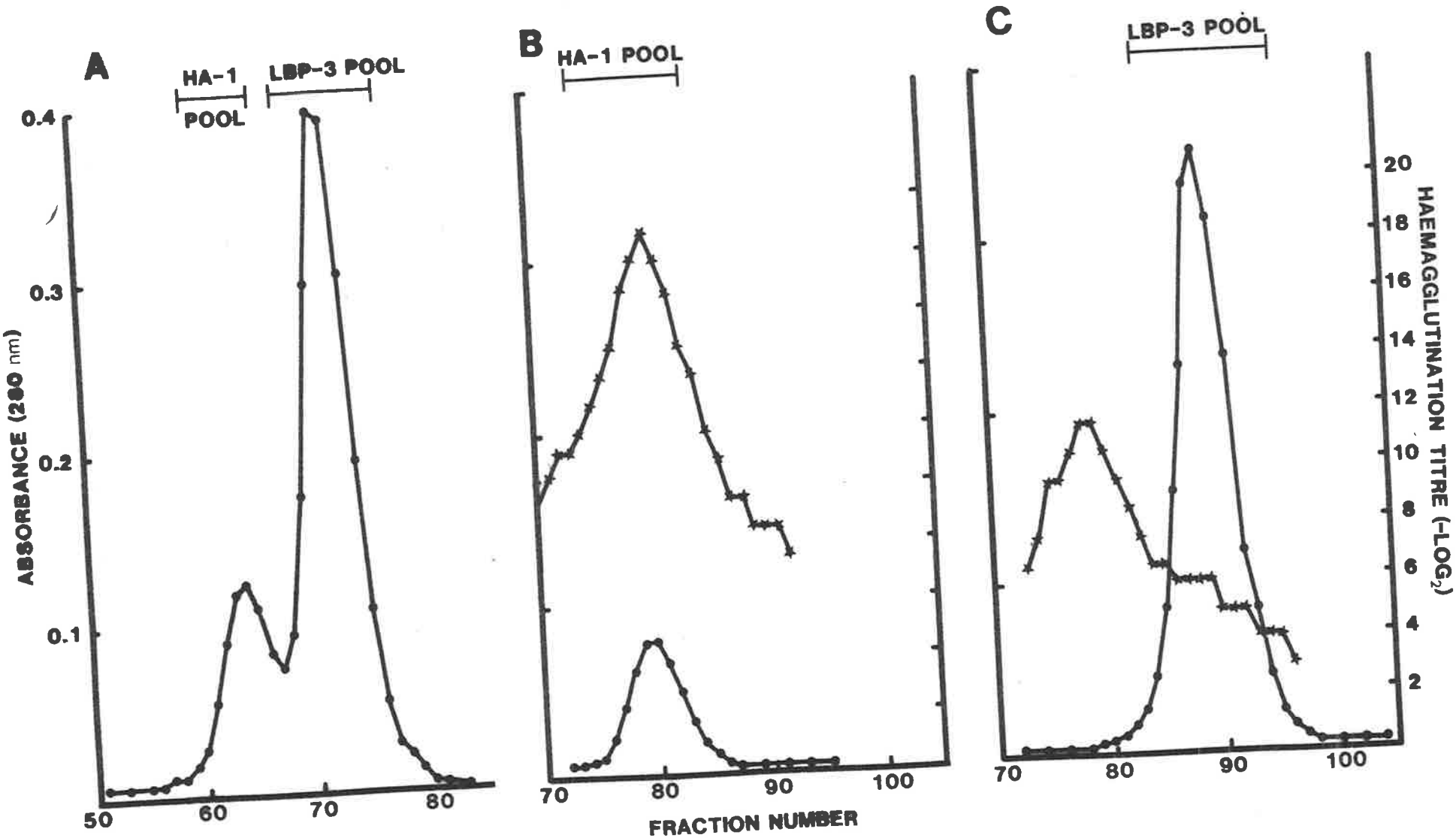
Purification of HA-1 and LBP-3 from 500 ml of haemolymph: Sephacryl S-300 chromatography at 4° of the galactose eluate from the affinity column (Fig. 6.16) (Chapter 2, section 2.11). Fractions were assayed for agglutinating activity against guinea pig erythrocytes (*——*) and for protein by absorbance at 280 nm (●——●).

A. The galactose eluate fractions (Fig. 6.16, fractions 67-75) were pooled, concentrated to 6 ml (Amicon Model 52 ultrafiltration cell) and then chromatographed. Fraction size was 6.1 ml. The HA-1 and LBP-3 peaks were separately pooled as indicated and concentrated to 6 ml for rechromatography.

B. Rechromatography of HA-1. Fraction size was 4.8 ml.

C. Rechromatography of LBP-3. Fraction size was 4.8 ml.

To obtain the final preparations of purified HA-1 and LBP-3, the HA-1 and LBP-3 peaks in (B) and (C) were pooled as indicated and concentrated to 5 ml.


A
HA-1 POOL
LBP-3 POOL
B
HA-1 POOL
C
LBP-3 POOL
ABSORBANCE (280 nm)
0.4
0.3
0.2
0.1
50
60
70
80
70
80
90
100
FRACTION NUMBER
70
80
90
100
HAEMAGGLUTINATION TITRE (-LOG2)
20
18
16
14
12
10
8
6
4
2

the LBP-3 fractions could, in fact, be accounted for by the very small quantity of HA-1 which overlapped the LBP-3 peak (Fig. 6.14C).

Because the LBP-3 could not be detected by haemagglutinating activity, a rocket immunoelectrophoresis assay was developed using the rabbit anti-serum raised in section 6.11. This serum contained antibodies for both the HA-1 and LBP-3 (see Chapter 7). To determine whether the affinity column step had removed all of the LBP-3 from the haemolymph, both the EDTA containing effluent and the $Ca^{++}$-supplemented effluent fractions were, together with the galactose eluate, assayed for LBP-3 by this technique. The fractions were also assayed for HA-2 by the single radial immunodiffusion method of Mancini (Mancini, Carbonara and Heremans, 1965) using the rabbit anti-HA-2 serum. The results are illustrated in Figures 6.15 and 6.16.

In accordance with the results from haemagglutination assays, the concentration of HA-2 measured by immunodiffusion in the effluent fractions (<4 µg/ml) was significantly lower than that of the haemolymph which had been applied to the affinity column (70 µg/ml) showing that at least 94% of the HA-2 had been adsorbed to the column. At least 98% of this was recovered in the lactose eluate. In contrast to the HA-2 results, but consistent with the HA-1 haemagglutination data (Fig. 6.15), there was no detectable difference in the concentration of HA-1/LBP-3 reactive material in the EDTA effluent of the affinity column compared to the original haemolymph (90 µg/ml) as measured by rocket immunoelectrophoresis (Fig. 6.15). Upon rechromatography of the $Ca^{++}$-reconstituted EDTA effluent, however, the first 240 ml of

FIGURE 6.15

Adsorption of HA-2 from 500 ml of haemolymph by the Lac-acid-Sepharose affinity column at 4°. The haemolymph (SRBC titre = 1/640) was made 10 mM with respect to EDTA and applied to the column at a flow rate of 15 ml/hr. The column was then washed at 50 ml/hr with TSA followed by TSA containing 0.1 M lactose. Fractions 1-42 were 12 ml and 43-70 were 5 ml. The fractions were assayed without dialysis for agglutinins against sheep (●——●) and guinea pig (■——■) erythrocytes. In addition, the fractions were assayed for HA-1 content (▼——▼) by rocket immunoelectrophoresis (see Fig. 6.16 for details) and for HA-2 content (□-----□) by the single radial immunodiffusion method (Mancini, Carbonara and Hereman, 1965). The single radial immunodiffusion assays were performed as follows. Six millilitres of 1% agarose (dissolved in immunoelectrophoresis buffer) containing 50 mM lactose and 0.17 mg/ml anti-(HA-2) rabbit IgG (the IgG was purified from rabbit antiserum raised in section 6.11 as described in Chapter 2, section 2.20.2) was poured onto a 5 x 7.6 cm glass plate. Rows of 2.5 mm diameter wells at 14 mm spacing were then cut in the agarose. Eight microlitres of each fraction were loaded into the wells and the plate was incubated at 37° for 24 hours. Washing and staining of the plates were performed as for rocket immunoelectrophoresis (Chapter 2, section 2.20). The HA-2 concentrations were estimated from the diameters of the precipitin rings by reference to a standard curve. This standard curve was constructed by plotting $(\text{diameter})^2$ against protein concentration for various concentrations of purified HA-2 (section 6.9).

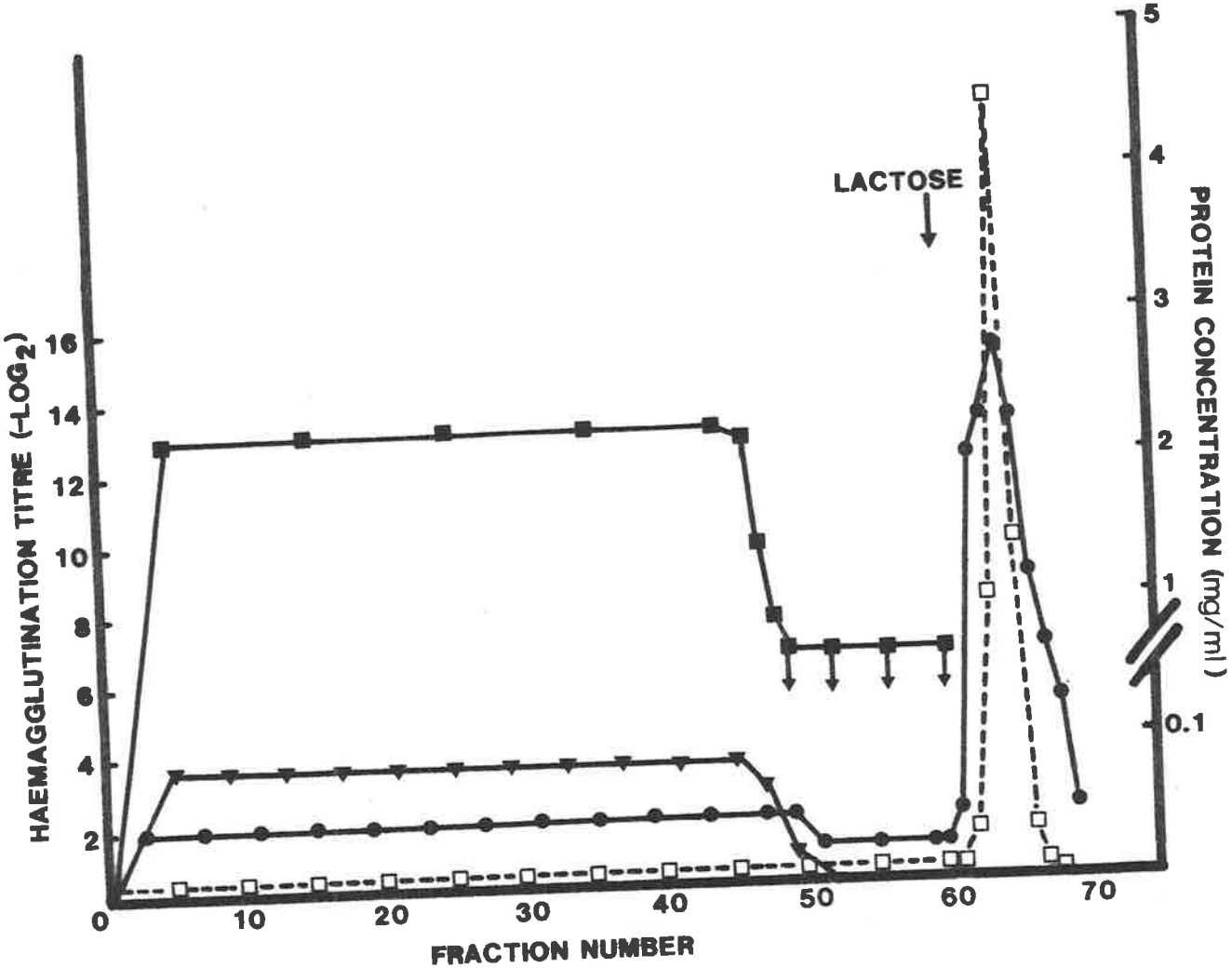
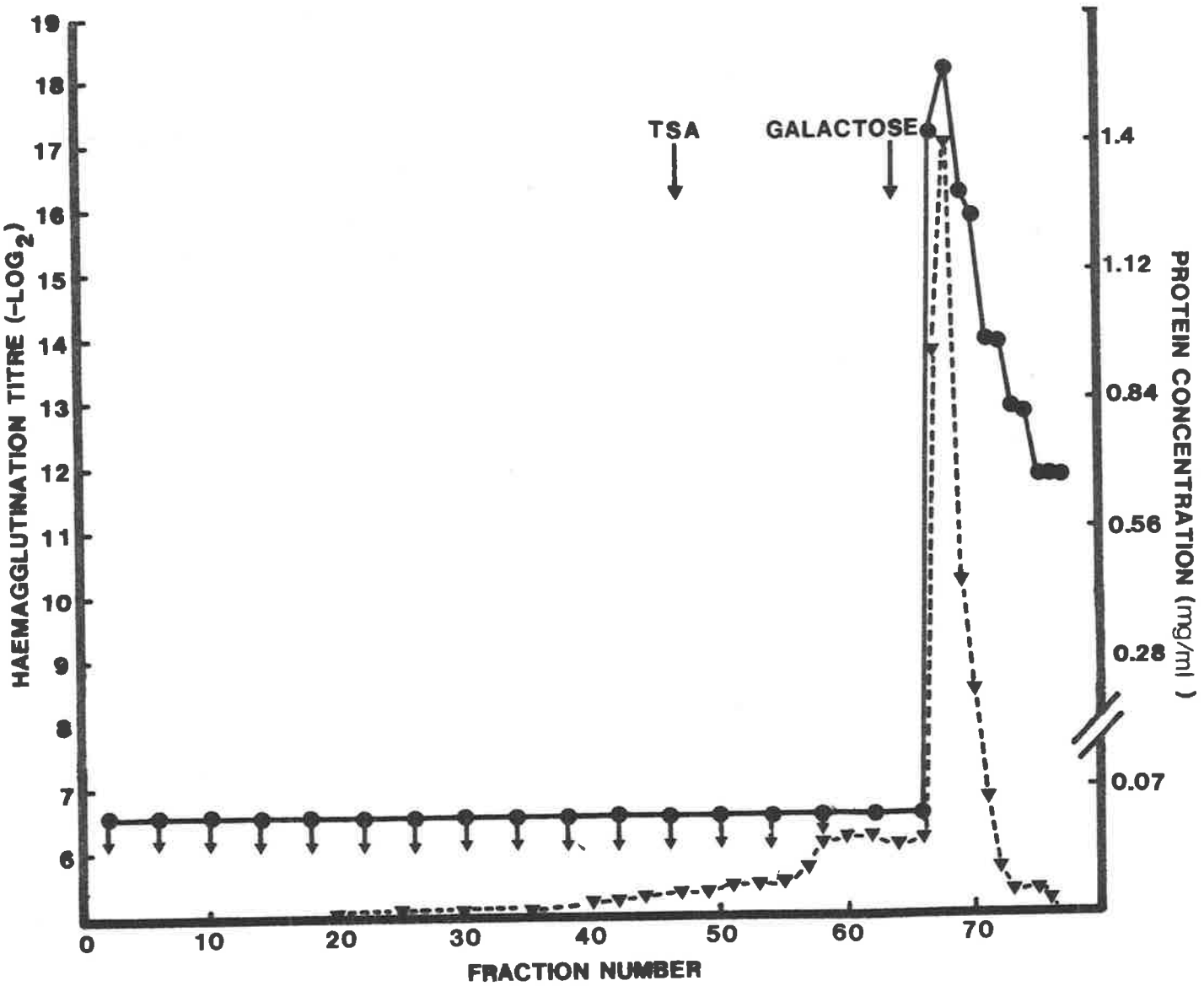

LACTOSE
HAEMAGGLUTINATION TITRE (-LOG2)
PROTEIN CONCENTRATION (mg/ml)
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
10
20
30
40
50
60
70
0.1
1
2
3
4
5

FIGURE 6.16

Adsorption of HA-1 and LBP-3 from 500 ml of haemolymph by the Lac-acid-Sepharose affinity column at 4°. The haemolymph effluent fractions (2-49) from Figure 6.15 were pooled, made 10 mM with respect to $CaCl_2$ and applied to the column at a flow rate of 15 ml/hr. The column was then washed at 50 ml/hr with TSA followed by 5 mM galactose in TSA. Fractions 1-41 were 12 ml and 42-75 were 5 ml. The fractions were assayed without dialysis for agglutinins against guinea pig erythrocytes (●———●) and were also assayed for total HA-1 and LBP-3 content by rocket immunoelectrophoresis (▼-----▼) (Chapter 2, section 2.20.3) using a rabbit antiserum raised in section 6.11. The concentration of HA-1/LBP-3 was estimated from the heights of the rockets by reference to a standard curve. This standard curve was constructed by plotting rocket height against protein concentration for various concentrations of purified LBP-3 (fraction 72, Fig. 6.11).


TSA
GALACTOSE
HAEMAGGLUTINATION TITRE (-LOG2)
PROTEIN CONCENTRATION (mg/ml)
FRACTION NUMBER
19
18
17
16
15
14
13
12
11
10
9
8
7
6
1.4
1.12
0.84
0.56
0.28
0.07
0
10
20
30
40
50
60
70

effluent (fractions 1-20) contained undetectable amounts of HA-1/LBP-3, whilst fractions 20-40 contained barely detectable amounts (7 µg/ml) (Fig. 6.16). The concentration began to rise slowly (8 µg/ml - 10 µg/ml) in the last few haemolymph fractions (40-43) and particularly in the TSA wash fractions (44-65), the concentration in fractions 60-65 being 40 µg/ml which represented 44% of the original haemolymph concentration. Only about 9% of the total HA-1/LBP-3 reactive material was recovered in these fractions. Since none of these fractions exhibited any detectable haemagglutinating activity, it seemed probable that this antigenic material which began to slowly leach off the column was LBP-3. About 35% of the total HA-1/LBP-3 reactive material applied to the column was recovered in the galactose eluate.

Selected fractions from the affinity column effluent were also analysed by discontinuous SDS-PAGE in non-reducing conditions (as in section 6.9). The results are shown in Figure 6.17. The polypeptide composition of the material recovered in the lactose and galactose eluate fractions was very similar to that seen previously (Figs. 6.10 and 6.12B). The band detected in the HA-2 rich lactose eluate fractions could be correlated with a major band in whole haemolymph (Fig. 6.17B). Similarly, a band(s) present in samples of whole haemolymph could be correlated with the major band seen in the galactose (HA-1/LBP-3 rich) eluate fractions. The HA-2 band was not present in analyses of the EDTA effluent fractions, whereas the HA-1/LBP-3 band was readily apparent. In contrast, the HA-1/LBP-3 band was not present in $Ca^{++}$ effluent fractions 1-40 (Fig. 6.17A) but was present in fraction 60.

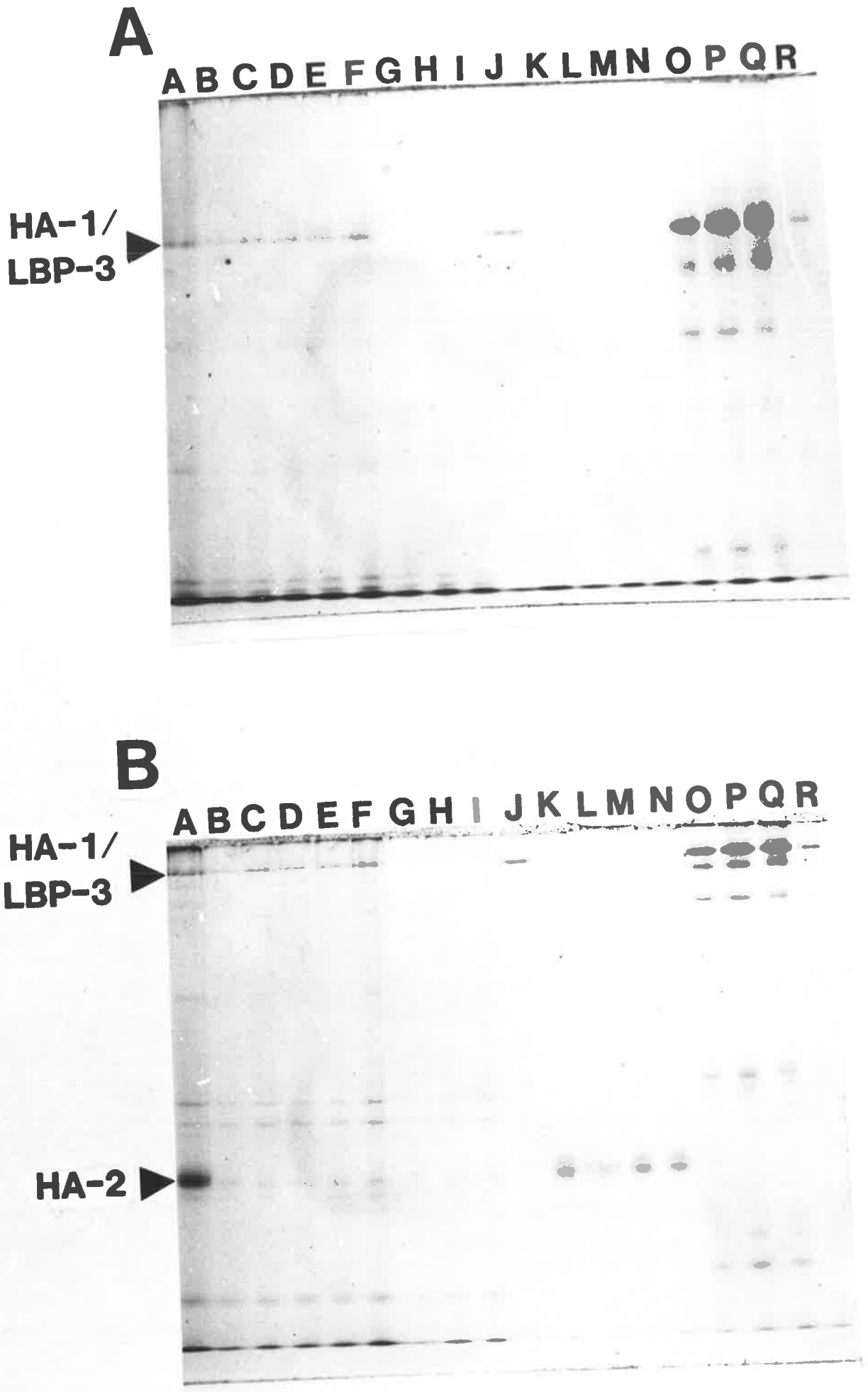
FIGURE 6.17

Analyses by SDS-PAGE in non-reducing conditions of the affinity column effluent fractions obtained in Figures 6.15 and 6.16. Approximately 3-5 ug of protein from each fraction was precipitated with trichloroacetic acid and deoxycholate (Chapter 2, section 2.16.3) for analysis.

| | |
|---|---|
| A | unadsorbed haemolymph |
| <u>Haemolymph effluent, adsorbed in the presence of EDTA (Fig. 6.15)</u> | |
| B | fraction 5 |
| C | fraction 15 |
| D | fraction 25 |
| E | fraction 35 |
| F | fraction 45 |
| <u>Haemolymph effluent, adsorbed in the presence of $Ca^{++}$ (Fig. 6.16)</u> | |
| G | fraction 15 |
| H | fraction 30 |
| I | fraction 40 |
| J | fraction 60 |
| <u>Lactose eluate (Fig. 6.15)</u> | |
| K | fraction 62 |
| L | fraction 63 |
| M | fraction 64 |
| N | fraction 65 |
| <u>Galactose eluate (Fig. 6.16)</u> | |
| O | fraction 64 |
| P | fraction 65 |
| Q | fraction 66 |
| R | fraction 67 |

A. 7% polyacrylamide gel.

B. 11% polyacrylamide gel.


A
A B C D E F G H I J K L M N O P Q R
HA-1/
LBP-3
B
A B C D E F G H I J K L M N O P Q R
HA-1/
LBP-3
HA-2

These results show that the Lac-acid-Sepharose column had sufficient binding capacity to adsorb all of the HA-1 and HA-2 from 500 ml of haemolymph. However, the binding capacity of the column for LBP-3 appeared to be just exceeded.

The final purified preparations of HA-1 and LBP-3 were obtained by pooling and concentrating fractions from the Sephacryl S-300 fractionations as indicated in Figures 6.14B and 6.14C. The recoveries in terms of haemagglutinating units (HA-1) and $OD^{280}$ units (HA-1 and LBP-3) at each stage of the purification procedure are shown in Table 6.2. For both proteins the loss during each concentration step was approximately 20-30%. The final yields, relative to the amount released by galactose from the affinity column (20.1 $OD^{280}$ units) was 44% (8.7 $OD^{280}$ units) and 9% (1.76 $OD^{280}$ units) for LBP-3 and HA-1 respectively. As judged by haemagglutinating activity (guinea pig erythrocytes), the overall recovery of HA-1 from the haemolymph was 57%. The total yield of protein (Folin-phenol) was 1.5 mg for HA-1 and 7.5 mg for LBP-3. The purified HA-1 exhibited a specific haemagglutinating activity ($1.2 \times 10^6$ HAU/mg of protein) 55 fold higher than that of unfractionated haemolymph (21,600 HAU/mg of protein).

## 6.13 Discussion

In this chapter the purification using affinity chromatography of the *B. leachii* haemagglutinins was described. To achieve this, a material suitable for use as a solid phase support had to be found and initially untreated Sepharose 4B was tested. This is composed of polymers of galactose and

TABLE 6.2

A) The recovery of HA-1 during purification from 500 ml of haemolymph*

| Stage of Purification | Volume (ml) | Titre$^{-1}$ | Total HAU | Percent recovery of HAU | $OD_{280}$ | Total OD units | Percent recovery of OD units |
|---|---|---|---|---|---|---|---|
| | | | AFFINITY CHROMATOGRAPHY@ | | | | |
| Haemolymph | 500 | 11,300 | $5.6 \times 10^6$ | 100 | - | - | - |
| Pool of galactose eluate fractions+ | 30 | 160,000 | $4.8 \times 10^6$ | 86 | 0.67 | 20.1 | 100 |
| concentrate of pooled fractions | 6 | $1.28 \times 10^6$ | $7.7 \times 10^6$ | 140 | - | - | - |
| | | | SEPHACRYL S-300 CHROMATOGRAPHY£ | | | | |
| 1st Run | | | | | | | |
| Pool of HA-1 peak fractions§ | 43 | 128,000 | $5.5 \times 10^6$ | 98 | 0.091 | 3.9 | 19.4 |
| Concentrate of pooled fractions | 6 | $1.28 \times 10^6$ | $7.7 \times 10^6$ | 140 | 0.490 | 2.9 | 14 |
| 2nd Run | | | | | | | |
| Pool of HA-1 peak fractions# | 53 | 64,000 | $3.4 \times 10^6$ | 60 | 0.048 | 2.5 | 12 |
| Concentrate of pooled fractions | 5 | 640,000 | $3.2 \times 10^6$ | 57 | 0.353 | 1.76 | 9 |

Continued over page

TABLE 6.2 (Continued)

B) The recovery of LBP-3 during purification from 500 ml of haemolymph

| Stage of purification | Volume (ml) | $OD_{280}$ | OD units | Percent recovery |
|---|---|---|---|---|
| AFFINITY CHROMATOGRAPHY@ | | | | |
| Pool of galactose eluate fractions+ | 30 | 0.67 | 20.1 | 100 |
| Concentrate of pooled fractions | 6 | - | - | - |
| SEPHACRYL S-300 CHROMATOGRAPHY£ | | | | |
| 1st Run | | | | |
| Pool of LBP-3 peak fractions¶ | 61 | 0.204 | 12.44 | 62 |
| Concentrate of pooled fractions | 6 | 1.708 | 10.25 | 51 |
| 2nd Run | | | | |
| Pool of LBP-3 peak fractionsß | 62 | 0.158 | 9.8 | 49 |
| Concentrate of pooled fractions | 5 | 1.736 | 8.68 | 44 |

* HA-1 was assayed using guinea pig erythrocytes.

@ Results for this stage are shown in Figure 6.16.

+ Figure 6.16, fractions 67-75 inclusive.

£ Results for this stage are shown in Figure 6.14.

§ Figure 6.14A, fractions 60-66.

# Figure 6.14B, fractions 74-84.

¶ Figure 6.14A, fractions 68-77.

ß Figure 6.14C, fractions 84-96.

3, 6-anhydro-L-galactose held together by 1→4 and 1→3 linkages (Fischer, 1980). The HA-2 haemagglutinins, which presumably recognise the areas around the 1→4 linkages in the gel, bound weakly to untreated Sepharose. The binding of HA-1 molecules was very much weaker.

Uy and Wold (1977) covalently coupled a number of simple sugars, including lactose, to Sepharose via ether linkages and used the resulting conjugates to purify a number of lectins by affinity chromatography. The primary site of coupling for glucose was shown to be the primary alcohol at the C6 position. The attachment site(s) of other sugars (e.g. lactose, fucose, galactose) which were successfully conjugated to Sepharose were not determined. However, it would seem on the basis of their results that lactose could link through either its glucose or galactose components. Since HA-1 appeared to recognise the non-reducing end of galactose (Chapter 4), there seemed a good chance that lactose coupled to Sepharose through its glucose C6 would still be recognised by the HA-1. It was also hoped that the attached lactosyl groups would also be recognised by the HA-2. Both haemagglutinins bound well to lactose-Sepharose and binding was apparently specific since each could be eluted with lactose. However, since the capacity of this gel for the two agglutinins was unsatisfactorily low, a number of experiments were conducted in which acid-treated Sepharose (both substituted with lactose and unsubstituted) was tested for its capacity to bind both haemagglutinins.

Treating the Sepharose with acid (based on the work of Baldo, Sawyer, Stick and Uhlenbruck, 1978) greatly increased

its binding capacity for both agglutinins such that its capacity was even greater than that of lactose-Sepharose. Again, this binding was not due to nonspecific interactions since the haemagglutinins could be eluted by a low concentration of galactose. The acid treatment may have partially hydrolysed the Sepharose, exposing terminal galactose residues which could be recognised by the agglutinins. The value of attached lactose groups in stabilising HA-2 binding was demonstrated by the finding that higher concentrations of galactose were required to elute HA-2 from lactose-substituted-acid treated Sepharose as compared to unsubstituted-acid-treated Sepharose. The binding capacity for HA-1 appeared to be undiminished by the coupling procedure. Based on these findings, lactose-substituted-acid treated Sepharose was adopted for routine use as the solid phase support.

The hope that the affinity column could be used to purify the haemagglutinins was achieved. The material recovered from the affinity column was already highly purified as judged by SDS-PAGE analyses. The few minor bands seen in addition to the major band for the HA-2 preparation disappeared after chromatography on Sephadex G-200. Most of the bands seen in SDS-PAGE gels (unreduced) of the galactose eluate could also be identified in the relevant Sephacryl S-300 fractions and in the final purified HA-1 and LBP-3 preparations. These were judged (by two dimensional SDS-PAGE and immunochemical analysis, see next chapter) not to be contaminants as such but to be different forms of the HA-1 and LBP-3 molecules. Up to 500 ml of haemolymph could be processed at a single time giving total protein yields of

1.5 mg for HA-1, 15 mg for HA-2 and 7.5 mg for LBP-3. As judged by haemagglutinating activity, the recoveries of HA-1 and HA-2 were at least 50% and the degree of purification from haemolymph was 55 and 16 fold respectively. The quantities recovered were more than adequate to perform physico-chemical and chemical analyses on these proteins and these experiments are described in the next chapter.

The proteins recovered from the affinity column by elution with galactose were comprised of two distinct proteins, the HA-1 agglutinin (peak (b), Fig. 6.11) and a non-agglutinating, $Ca^{++}$ dependent, lactose binding protein (LBP-3; peak (c), Fig. 6.11). Like the HA-1 agglutinin, LBP-3 was eluted from the affinity column by both EGTA or galactose. This protein, which clearly has a binding specificity very similar to the HA-1, possessed a subunit very similar in size to that comprising the HA-1 molecule (Fig. 6.11B).

In Chapter 3 it was reported that the haemagglutinating activity of HA-1 could be abolished by treatment with EDTA and restored by the addition of $Ca^{++}$ ions. Two modes of action for the $Ca^{++}$ ions can be envisaged. Firstly, $Ca^{++}$ ions may be necessary for maintaining the polymeric structure of the haemagglutinin, as has been suggested to occur in the case of the crayfish haemagglutinin (Jenkin and Hardy, 1975) and sponge aggregation factors (Jumblatt, Schlup and Burger, 1980). Thus the removal of $Ca^{++}$ might cause the haemagglutinin to depolymerise while having no effect on the binding sites. Secondly, $Ca^{++}$ ions may play a role in stabilising the conformation of the HA-1 molecule, as has been proposed for the plant lectin Concanavilin A which has been shown to undergo conformational changes and to lose

carbohydrate binding site activity upon removal of $Ca^{++}$ ions (Lis and Sharon, 1977). The later explanation appears to best fit the data for the HA-1 haemagglutinin. Thus, the apparent size of HA-1 as measured by gel filtration on Sephadex G-200 in the presence of sugars or on Sephacryl S-300 in the presence of EGTA is the same, indicating that removal of $Ca^{++}$ has no depolymerising effect on the haemagglutinin. Moreover, the binding of the HA-1 agglutinin to the affinity column was prevented by EDTA/EGTA, indicating that the removal of $Ca^{++}$ ions abolished binding site activity.

The results of this chapter reinforce the conclusions of Chapter 4 that the HA-2 haemagglutinin is a homogeneous lactose-specific protein and that it does not comprise a family of different molecules with a range of different specificities. Were this not the case, it would be expected that adsorption of haemolymph on the lactose-affinity column would not remove all HA-2 molecules. The results show quite clearly, however, that all material that was antigenically related to HA-2 (Fig. 6.15) or possessed similar sized polypeptide chains as HA-2 (Fig. 6.17) was completely removed from haemolymph by adsorption to the affinity column. The same results were also obtained for both HA-1 and LBP-3 (Figs. 6.16 and 6.17) indicating that these proteins were also homogeneous in their binding specificity for lactose.

There are a number of ways by which an estimate of the haemagglutinin levels in haemolymph can be made. For instance, as judged by haemagglutinating activity, the total yield of HA-2 was 50%. Therefore, since the protein yield was 5 mg,

the amount in the original 200 mls should have been approximately 10 mg. This gives 0.1 mg/ml of HA-2 in haemolymph. Similarly for HA-1, the haemagglutinating yield was 60%, the protein yield was 1.5 mg and, therefore, the amount in the original 500 ml of haemolymph should have been approximately 2.5 mg. This gives 0.005 mg of HA-1 per ml of haemolymph. Alternatively, estimates of haemagglutinin levels can be made from the specific activity measurements of the purified preparations and the haemagglutinin titres in haemolymph. Using this method, the estimate for HA-2 (titre = $\frac{1}{320}$, specific activity = 6400 HAU/mg) is 0.05 mg/ml and for HA-1 (titre = $\frac{1}{11,300}$, specific activity = $2.1 \times 10^6$ HAU/mg) is 0.005 mg/ml. The estimates using immunochemical methods (section 6.12) were 0.07 mg/ml for HA-2 and 0.09 mg/ml for HA-1 and LBP-3 combined.

## 6.14 Summary

In this chapter the haemagglutinins in *B. leachii* haemolymph were purified using an affinity column made from acid treated Sepharose 4B which contained conjugated lactose. This material was chosen after several different types had been tested for high binding affinity and capacity. Several different protocols for purifying the haemagglutinins using this column were tried before the following was adopted. Firstly, HA-2 was selectively adsorbed to the column in the presence of EDTA. The HA-2 was then eluted with lactose and subjected to chromatography on Sephadex G-200. The haemolymph was reconstituted with $Ca^{++}$ and the HA-1 was then adsorbed to the column. After elution with galactose, the

HA-1 preparation was chromatographed on Sephacryl S-300. During this step a third protein was isolated. Like HA-1, this protein bound lactose and required $Ca^{++}$ ions for activity. It was designated Lactose Binding Protein Three (LBP-3).

Immunochemical and SDS-PAGE analyses of the affinity column effluents showed that all HA-1, HA-2 and LBP-3 molecules were adsorbed from haemolymph by the affinity column.

# CHAPTER 7

## Physicochemical characterization of the *B. leachii* haemagglutinins

## 7.1 Preamble

This chapter describes the results of experiments designed to further characterize the *B. leachii* HA-1 and HA-2 haemagglutinins and the LBP-3, which had been purified in milligram quantities as described in the previous chapter. These proteins, especially the HA-1 and LBP-3 appeared at this stage of the investigation to share certain features; all were lactose-binding molecules, both the HA-1 and LBP-3 required $Ca^{++}$ ions for binding activity and it appeared from SDS-PAGE analysis that these two molecules might share a common subunit. The type of information sought included physicochemical data (e.g. detailed mol. wt. estimates, amino acid composition and subunit composition), the binding site specificity of the LBP-3 (studied by adsorption techniques) and the antigenic cross-reactivity of the proteins.

## Experimental

## 7.2 Determination of native molecular weights

The behaviour of a protein during gel chromatography is frequently only dependent on its mass and, therefore, mol. wts. can usually be directly estimated using empirically derived plots of elution position versus log mol. wt. (Pharmacia gel filtration handbook, 1979; Rodbard, 1976). However, the results obtained with HA-1, which appeared in gel chromatography to be larger than IgG and in sedimentation velocity to be smaller than IgG (Chapter 4), indicated that this method was probably not applicable in this case.

Both gel chromatography and sedimentation velocity ultracentrifugation are transport processes and represent

systems which are not at thermodynamic equilibrium (Fish, 1975). The behaviour of molecules in these systems depends not only on the mass of the molecules but is also influenced by their shape. Thus, in order to estimate mol. wt. directly from gel filtration data, the effect of shape on chromatographic behaviour must be the same for both the unknown proteins and the calibration standards, a condition that seems to be approximated only by globular proteins. It is clear from its behaviour that HA-1 is not a globular protein.

The only methods in which the behaviour of macromolecules is dependent only on their mass and which, therefore, can be used to directly determine anhydrous mol. wt. are those in which the measurements are made when a system is at thermodynamic equilibrium (e.g. sedimentation equilibrium ultra-centrifugation, light scattering). Unfortunately, the equipment needed for such methods was not available. Therefore, the mol. wt. estimates had to be made using transport methods. To do this, additional information about molecular shape was required and this was derived by combining the results from both gel chromatography and sedimentation velocity ultracentrifugation. The parameters of Stoke's radius and sedimentation coefficient for a protein were estimated using these methods and used to calculate mol. wt. by substitution in an equation derived by classical mechanics or non-equilibrium thermodynamics theory (Bowen, 1970; Fish, 1975; this chapter, section 7.2.3).

### 7.2.1 Estimation of the Stoke's radii of HA-1, HA-2 and LBP-3 by chromatography in Sephadex G-200

The Stoke's radius of a molecule is a measure of its effective hydrodynamic size and is derived by defining the molecule in terms of a sphere with equivalent hydrodynamic properties, e.g., having the same frictional coefficient or diffusion coefficient. Siegel and Monty (1966) have shown that for chromatography in Sephadex G-200 there is a linear relationship between the Stoke's radius (defined in terms of the diffusion coefficient) of a protein and its elution position, Kav.

The preparation of the column, the chromatography running conditions and the calculation of the Stoke's radii of the standard proteins are described in Chapter 2, sections 2.10 and 2.13. The elution profiles of the standards, the haemagglutinins and LBP-3 are shown in Figure 7.1. All the proteins eluted as well defined, symmetrically shaped peaks, presenting no problems for the determination of their elution positions. The calibration standards were chromatographed several times and their elution positions never changed by more than one fraction. To check that column behaviour remained constant between runs, sodium iodide-125 was always included as a bed volume marker.

As was observed earlier with Sephacryl S-300 (Chapter 6), the HA-1 eluted first, well ahead of catalase, whereas the LBP-3 eluted nearly coincidentally with catalase. HA-2 eluted just after bovine serum albumin. Increasing the concentration of the test proteins 5-40 fold had no effect on their elution behaviour. This eliminated any possibility

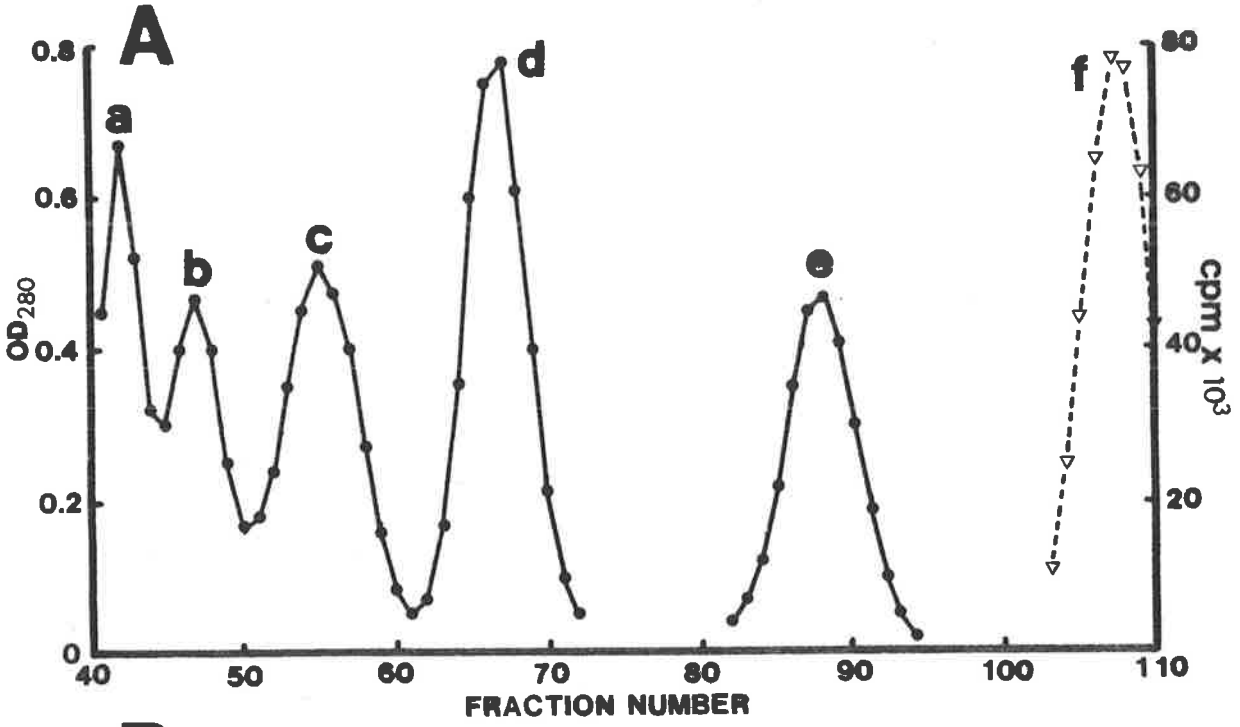
FIGURE 7.1

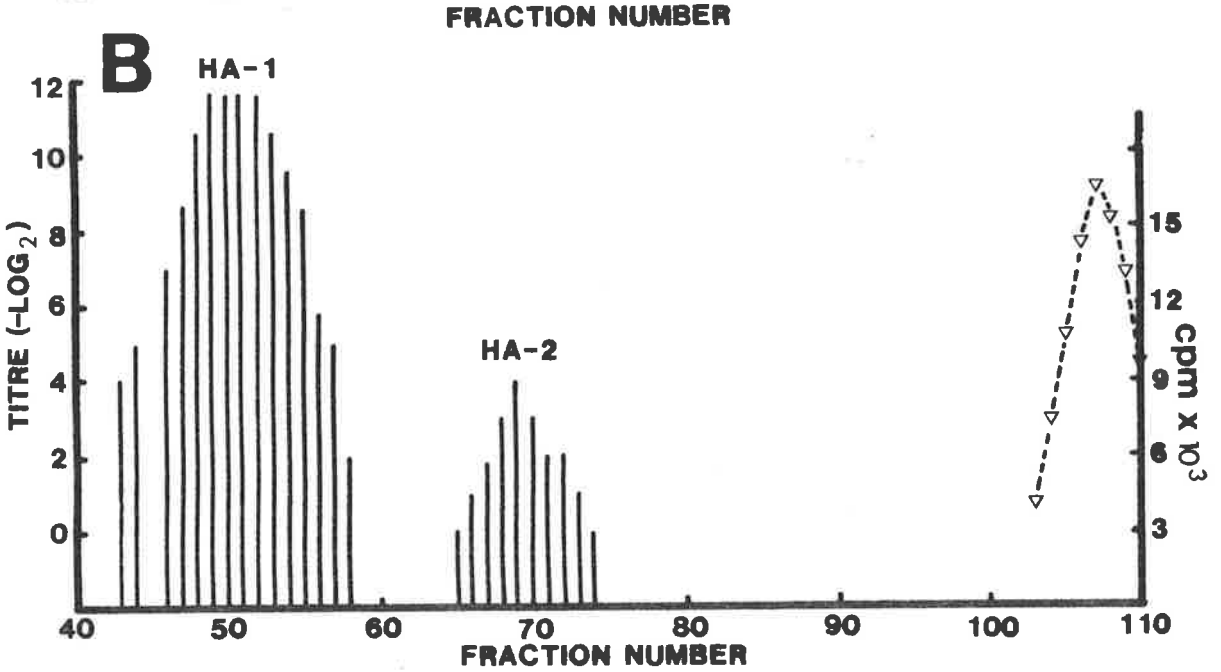
Estimation of Stoke's radii by chromatography in Sephadex G-200. Sample volume = 5 ml, flow rate = 16 ml/hr, fraction size = 4.8 ml, temperature = 4°, eluant buffer = PBS containing 50 mM lactose, 100 mM D-galactose and 0.5% v/v azide.

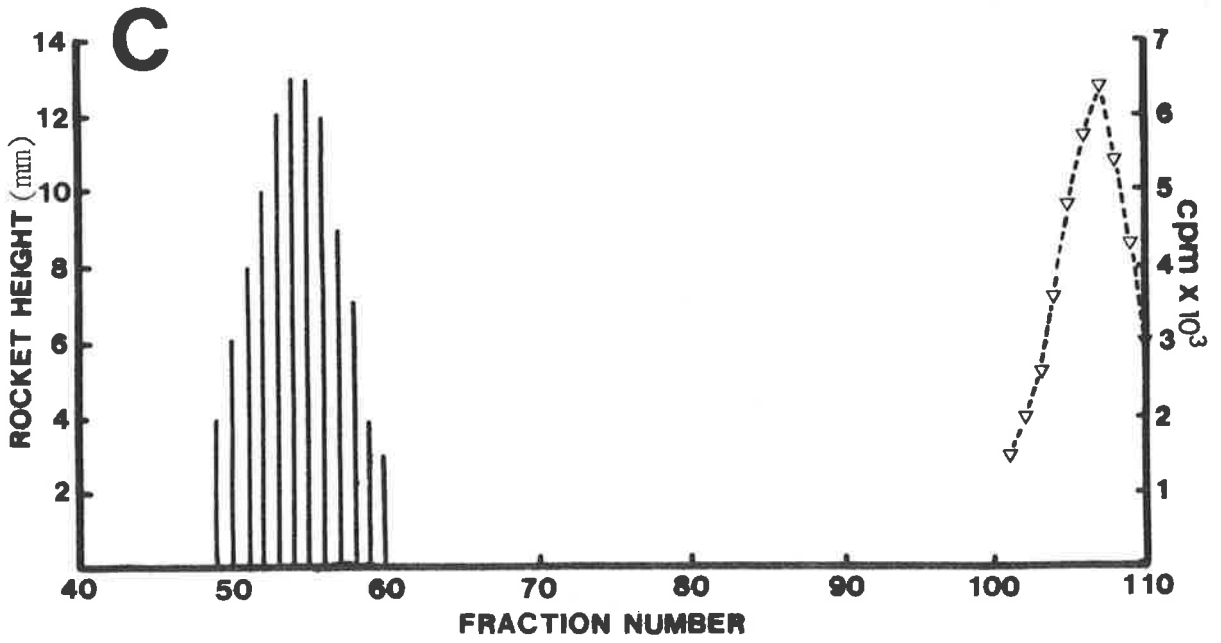
A. Calibration of the column: The calibration standards were a) thyroglobulin (10 mg), b) ferritin (10 mg), c) catalase (10 mg), d) bovine serum albumin (20 mg), e) myoglobin (10 mg) and f) sodium iodide-125 (470,000 cpm). The absorbance at 280 nm (●——●) and the radioactivity (▽----▽) of the fractions were measured.

B. Chromatography of HA-1 and HA-2. The sample was 5 ml of haemolymph containing sodium iodide-125 (90,000 cpm) as a bed volume marker. HA-1 and HA-2 were detected by haemagglutination titration against guinea pig and sheep erythrocytes respectively.

C. Chromatography of LBP-3. The sample was purified LBP-3 (0.3 mg/ml) containing sodium iodide-125 (42,000 cpm) as a bed volume marker. LBP-3 was detected by rocket immunoelectrophoresis (Chapter 2, section 2.20) using a rabbit antiserum (Chapter 6, section 6.11).


A
a
b
c
d
e
f
$OD_{280}$
cpm x $10^3$
FRACTION NUMBER


B
HA-1
HA-2
TITRE ($-LOG_2$)
cpm x $10^3$
FRACTION NUMBER


C
ROCKET HEIGHT (mm)
cpm x $10^3$
FRACTION NUMBER

that they may have been part of a protein-protein interacting system (Fish, 1975) and confirmed that their true behaviour had been measured.

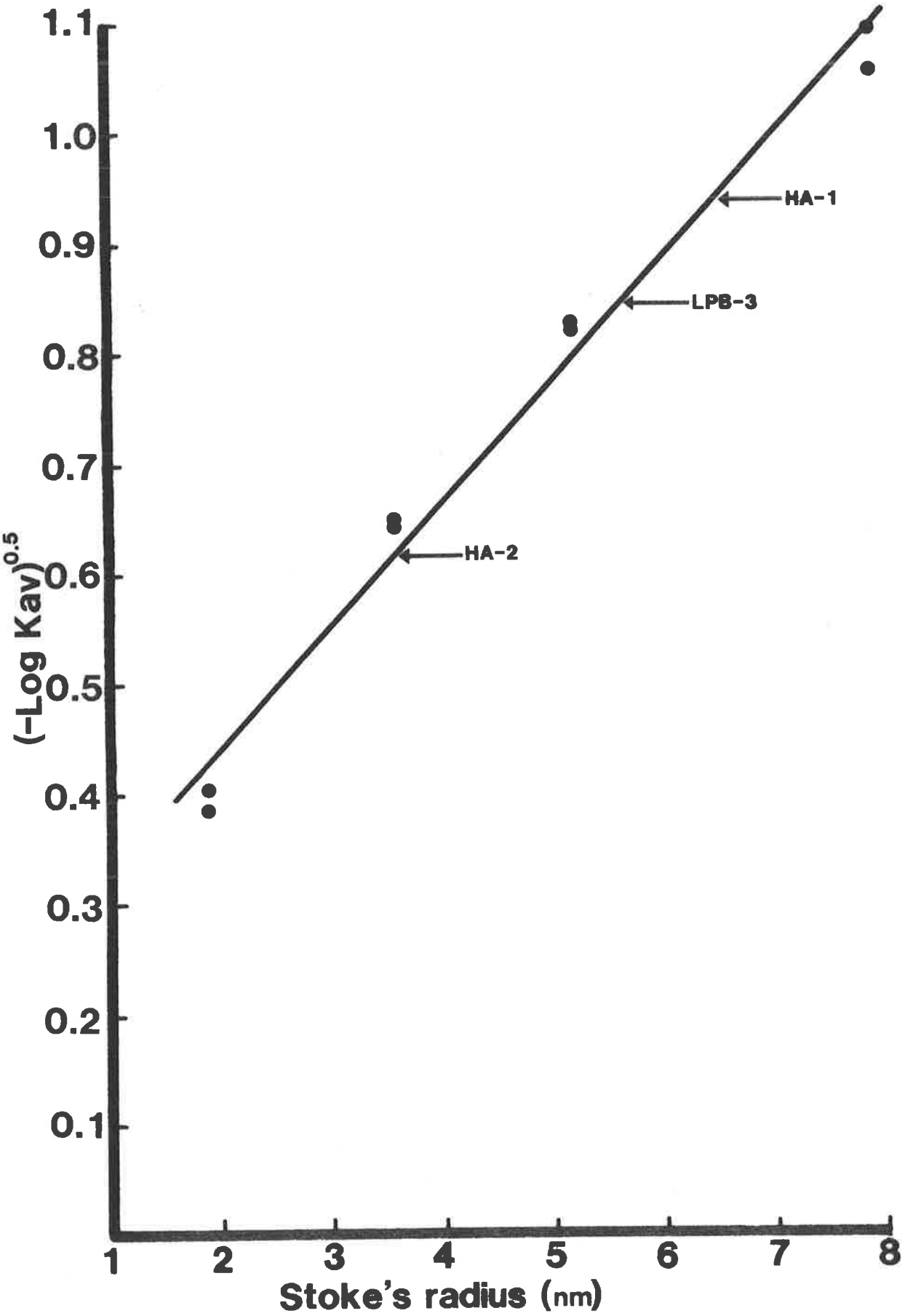
The Stoke's radii were derived from the measured Kav values from a calibration graph (Fig. 7.2) which was constructed by plotting the Stoke's radii of the standard proteins against $(-\log (Kav))^{\frac{1}{2}}$ (Siegel and Monty, 1966) using the method of least-squares linear regression. The correlation coefficient was 0.99. The Stoke's radius estimated for HA-1, HA-2 and LBP-3 was 6.5 nm, 3.6 nm and 5.6 nm respectively.

### 7.2.2 Determination of the sedimentation coefficients of HA-1 and HA-2

The sedimentation coefficient is a measure of the rate of movement of a molecule through a solvent under the influence of a centrifugal field. It is best measured using an analytical ultracentrifuge in boundary sedimentation velocity experiments but, in the absence of such equipment, it can be measured using a preparative ultracentrifuge in zonal sedimentation velocity experiments in which the rate of sedimentation of a zone of protein through a density gradient (usually sucrose) is measured. It is possible to measure sedimentation coefficients using linear sucrose gradients, but fairly complicated calculations are involved (Martin and Ames, 1961; McEwen, 1967; Dingman, 1972). However, if isokinetic sucrose gradients are used, the sedimentation velocity of a particle is constant and, therefore, the sedimentation coefficient for a protein can

FIGURE 7.2

Relationship between $K_{av}$ (Sephadex G-200, Fig. 7.1) and Stoke's radius for the marker proteins (Chapter 2, Table 2.2). The correlation coefficient was 0.99. The positions corresponding to HA-1, HA-2 and LBP-3 are indicated.


1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
(-Log Kav)^0.5
HA-1
LPB-3
HA-2
1
2
3
4
5
6
7
8
Stoke's radius (nm)

be easily and directly calculated by comparison with an internal standard. In the following experiments isokinetic gradients were used to calculate the sedimentation coefficients for HA-1 and HA-2.

The construction of the gradients and details of the sedimentation velocity experiments are described in Chapter 2, section 2.12. To achieve a statistically accurate estimate of the sedimentation coefficients, twelve gradients were run; six each for HA-1 and HA-2. Purified preparations of each protein under investigation were used, with radiolabelled BSA as the internal standard. The *B. leachii* proteins were detected by haemagglutination. The data depicted in Figure 7.3 show typical results. It can be seen that all components moved as sharp, well defined, symmetrically shaped zones, allowing accurate determination of sedimentation distances. These were calculated as the distance (number of fractions) between the centre of the sample at the start of the experiment and the centre of the sedimented zone at the end of the experiment. Table 7.1 summarises the results from all twelve gradients and shows the calculated sedimentation coefficients. There was very little variation between the gradients as shown by the low standard deviations. The sedimentation coefficients, with 95% confidence limits (Bailey, 1973), for HA-1 and HA-2 were 5.68 ± 0.23 and 4.29 ± 0.11 respectively.

### 7.2.3 Calculation of the molecular weights and frictional ratios of HA-1 and HA-2

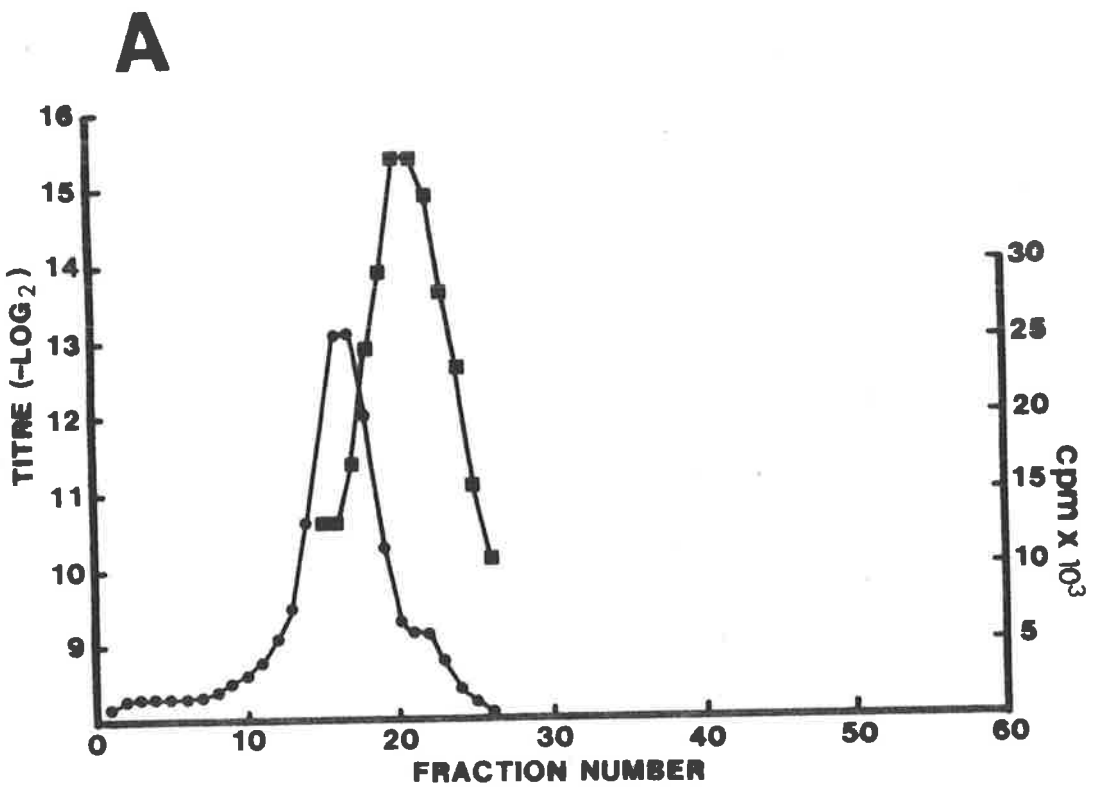
There are a number of forces that act on a sedimenting molecule. These are 1) the centrifugal force which depends on the mass of the molecule, ii) an opposing force due to the

FIGURE 7.3

Sedimentation velocity ultracentrifugation in isokinetic sucrose gradients. Gradients (12 ml) were centrifuged for 16 hr at 39,000 rpm and fractionated into 0.2 ml aliquots.

A. Sedimentation of HA-1. Sample was 0.2 ml of purified HA-1 (titre = 1/320,000) containing radiolabelled bovine serum albumin (200,000 cpm). Fractions were assayed for GPRBC haemagglutinating activity (■——■) and for radioactivity (●——●).

B. Sedimentation of HA-2. Sample was 0.2 ml of purified HA-2 (titre = 1/2,000) containing radiolabelled bovine serum albumin (150,000 cpm). Fractions were assayed for SRBC haemagglutinating activity (■——■) and for radioactivity (●——●).


A
TITRE (-LOG2)
16
15
14
13
12
11
10
9
cpm x 10³
30
25
20
15
10
5
0
10
20
30
40
50
60
FRACTION NUMBER

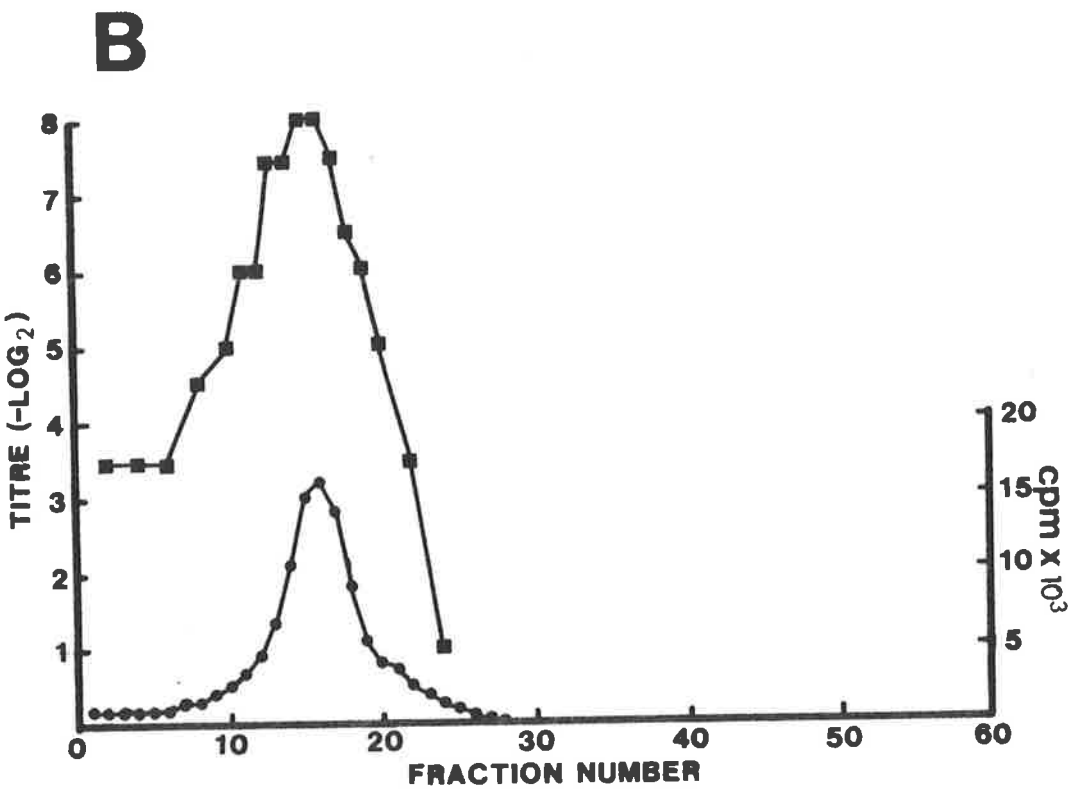

B
TITRE (-LOG2)
8
7
6
5
4
3
2
1
cpm x 10³
20
15
10
5
0
10
20
30
40
50
60
FRACTION NUMBER

TABLE 7.1

Determination of sedimentation coefficients by sedimentation velocity ultracentrifugation in sucrose density gradients.

A) Sedimentation coefficient of HA-2.

| Tube | No of fractions collected | Distance sedimented | | (HA-1/BSA) ratio§ | Estimated sedimentation coefficient# |
|---|---|---|---|---|---|
| | | BSA* | HA-2 | | |
| 1 | 60 | 15 | 15 | 1 | 4.41 |
| 2 | 60 | 15 | 15 | 1 | 4.41 |
| 3 | 60 | 15 | 14.5 | 0.967 | 4.26 |
| 4 | 59.5 | 15.5 | 14.5 | 0.935 | 4.125 |
| 5 | 60 | 15 | 14.5 | 0.967 | 4.26 |
| 6 | 63 | 15 | 14.5 | 0.967 | 4.26 |
| | | | | Mean ± S.D. = | 4.29 ± 0.108 |

B) Sedimentation coefficient of HA-1

| Tube | No of fractions collected | Distance sedimented | | (HA-1/BSA) ratio§ | Estimated sedimentation coefficient# |
|---|---|---|---|---|---|
| | | BSA* | HA-2 | | |
| 1 | 60 | 13.5 | 18.5 | 1.37 | 6.04 |
| 2 | 60 | 14.5 | 19 | 1.31 | 5.78 |
| 3 | 60 | 15 | 19 | 1.266 | 5.58 |
| 4 | 59.5 | 14.5 | 18.5 | 1.27 | 5.626 |
| 5 | 60 | 15.5 | 20 | 1.29 | 5.69 |
| 6 | 60 | 16 | 19.5 | 1.22 | 5.39 |
| | | | | Mean ± S.D. = | 5.68 ± 0.217 |

* Bovine serum albumin.

§ Distance sedimented by the haemagglutinin divided by the distance sedimented by BSA.

# Calculated using the equation: sedimentation coefficient = (HA/BSA) ratio x sedimentation coefficient of BSA. The sedimentation coefficients are expressed in Svedberg units ($S_{20,w}$). The value used for BSA was 4.41 S (Phelps and Putnam, 1960; Squire, Moser and O'Konski, 1968).

buoyancy of the molecule and iii) the frictional drag of the molecule which is influenced by the shape of the molecule. An analysis of these factors leads to an expression for the sedimentation coefficient of a macromolecule in terms of its physical properties (Bowen, 1970; Fish, 1975; Tanford, 1961), namely

$$S = \frac{M(1-\bar{v}p)}{Nf}$$

where S = sedimentation coefficient, M = molecular weight of the macromolecule, $\bar{v}$ = partial specific volume of the macromolecule, p = density of the solvent, N = Avogadro's number and f = the frictional coefficient of the macromolecule. Rearranging this equation yields

$$M = \frac{SNf}{(1-\bar{v}p)}$$

To calculate the molecular weight from the sedimentation coefficient, information about the shape of the macromolecule is required, i.e., the frictional coefficient needs to be known. This can be calculated from the Stoke's radius according to Stoke's equation,

$$f = 6\Pi\eta R \qquad \text{(Tanford, 1961)}$$

where R = the Stoke's radius and $\eta$ = solvent density. Substituting this expression into the above equation, we obtain

$$M = \frac{6\Pi\eta NSR}{(1-\bar{v}p)}$$

Since the sedimentation values are normalised relative to water at $20^{o}$, the values used for p and $\eta$ are those for water at $20^{o}$.

The results of molecular weight calculations for HA-1 and HA-2 using this approach are presented in Table 7.2. One source of uncertainty in these calculations is the value for the partial specific volume. Unfortunately, this could

TABLE 7.2

Molecular parameters of HA-1, HA-2 and LBP-3

| Protein | Stoke's radius (nm) | $S_{20,w}$* | | $\bar{v}$§ | Calculated mol. wt. | $f/f_o$ |
|---|---|---|---|---|---|---|
| HA-2 | 3.6 | 4.29 | 0.108 | 0.725¶ | 63,586 | 1.37 |
| | | | | 0.736# | 66,218 | 1.34 |
| | | | | 0.731£ | 64,995 | 1.35 |
| HA-1 | 6.5 | 5.68 | 0.217 | 0.725¶ £ | 152,006 | 1.84 |
| | | | | 0.736# | 158,298 | 1.81 |
| LBP-3 | 5.6 | - | | - | 140,000@ - 160,000 | - |

* Sedimentation coefficient in water at 20°.

§ Partial specific volume.

¶ Martin and Ames (1961).

# Smith (1970).

£ Calculated from amino acid composition.

@ Estimate based on gel filtration and SDS-PAGE data.

not be determined directly since the necessary equipment was not available, but this parameter varies only slightly between proteins. Smith (1970) quoted a mean of 0.736 ml/g, (S.D. = 0.02) while Martin and Ames (1961, citing Edsall, 1953) stated that the partial specific volumes of proteins lie between 0.70 and 0.75 ml/g and used a value of 0.725 ml/g in their calculations. Both these values, i.e. 0.736 ml/g and 0.725 ml/g were used in the present calculations. The partial specific volume of proteins is frequently estimated from amino acid compositions. These values are approximations but appear to be generally reliable for water soluble proteins (Fish, 1975). This has been done for HA-2 and HA-1 (Tables 7.5 and 7.7), the values obtained being included in the molecular weight calculations (Table 7.2). The largest variation in the molecular weights calculated using these values is only 4%. The mol. wts. for HA-1 and HA-2 are approximately 155,000 and 65,000 respectively.

An indication of the shape of a macromolecule can be obtained by calculating its frictional ratio. This is the ratio of the frictional coefficient of the macromolecule compared to that of a sphere with the same molecular weight and partial specific volume. Thus, if a protein is truly globular (i.e. spherical) its frictional ratio would be 1.00. Higher values indicate that a protein is non-globular. The frictional coefficient ($f_o$) of the hypothetical sphere is given by the expression:

$$f_o = 6\pi\eta(3M\bar{v}/4\pi N)^{1/3} \quad \text{(Tanford, 1961)}$$

As mentioned before, the frictional coefficient of a protein is related to its Stoke's radius by Stoke's equation,

$$f = 6\pi\eta R$$

Combining these equations we obtain:

$$\frac{f}{f_o} = \frac{R}{(3M\bar{v}/4\pi N)^{1/3}}$$ (Siegel and Monty, 1966)

Using this equation, the frictional ratio of HA-1 and HA-2 was calculated using the values for mol. wt. and Stoke's radius presented in Table 7.2.

There is some ambiguity as to what constitutes a globular protein. Tanford (1961) classifies proteins with frictional ratios of 1.1 - 1.3 as globular while Smith (1970) includes those with frictional ratios as high as 1.5. It is clear from the results of Table 7.2 that the HA-2 ($f/f_o$ = 1.34) is essentially globular while HA-1 ($f/f_o$ = 1.8) is decidedly non-globular.

The $S_{20,w}$ value for LBP-3 was not determined and as a result the mol. wt. and $f/f_o$ values for this protein could not be estimated using this method. However, in gel chromatography LBP-3 appeared to be smaller than HA-1, eluting in the same position as catalase (mol. wt. = 247,500) and IgG (mol. wt. = 150,000). SDS-PAGE analyses (results presented later) showed that unreduced LBP-3 is very similar in size to unreduced HA-1 and it therefore appears that LBP-3 is slightly aglobular, having a mol. wt. of between 140,000 and 160,000.

## 7.3 Determination of molecular weights under denaturing conditions

As the behaviour of proteins in transport systems under normal conditions is dependent not only on mass but also on shape and degree of solvation, there are no reliable empirical

relationships between the mobility of a protein, the single measurable parameter in these systems, and its molecular weight. If denaturing conditions are used, however, the parameters of shape and solvation can be expressed in terms of mass, since in these circumstances proteins adopt a gross conformation such that their size is a function of chain length, i.e. mass of the protein. Under these conditions the transport behaviour of the protein becomes directly related to its molecular weight. If electrophoretic rather than gel filtration techniques are used for such estimations, it is necessary that the denaturing solvent interacts with the protein in such a manner that not only the gross size but also the electrostatic charge of the product is proportional to the mass of the protein.

A denaturing agent known to fulfil these conditions is sodium dodecylsulphate (SDS). The use of SDS for the estimation of polypeptide mol. wts. by electrophoresis in polyacrylamide gels is well established (Polyacrylamide Gel Electrophoresis. laboratory techniques. Handbook, Pharmacia Fine Chemicals, 1981) and within certain constraints (Rodbard, 1976; Fish, 1975) is quite reliable.

### 7.3.1 Determination of the molecular weight of HA-1, HA-2 and LBP-3 by SDS-PAGE under non-reducing conditions

SDS-PAGE in non-reducing conditions was performed as described in Chapter 2, section 2.16. The behaviour of HA-1 and LBP-3 (7% gels) and HA-2 (11% gels) with a range of standard proteins is shown in Figure 7.4A. There are a number of empirical relationships between the relative mobility (Rf)

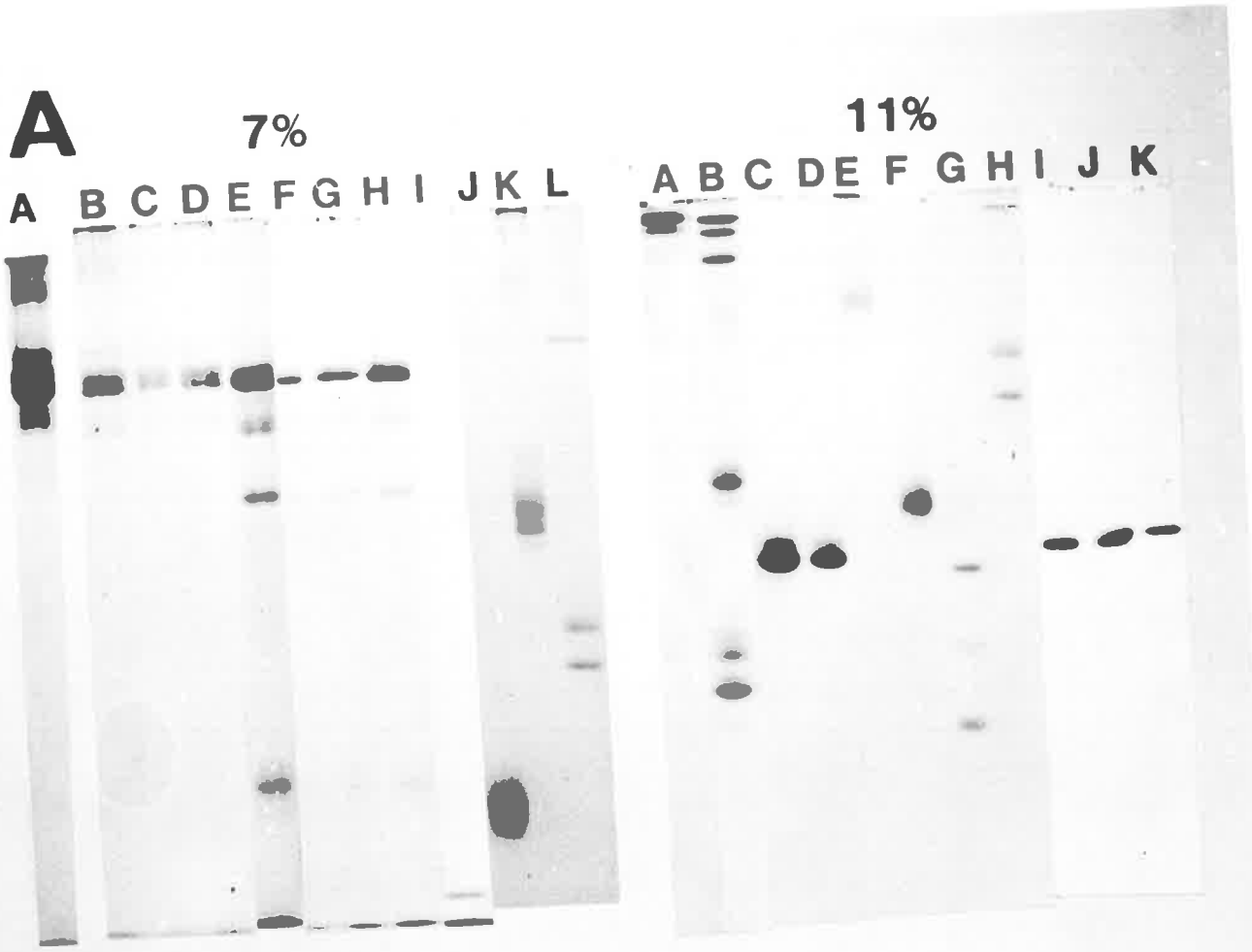
FIGURE 7.4

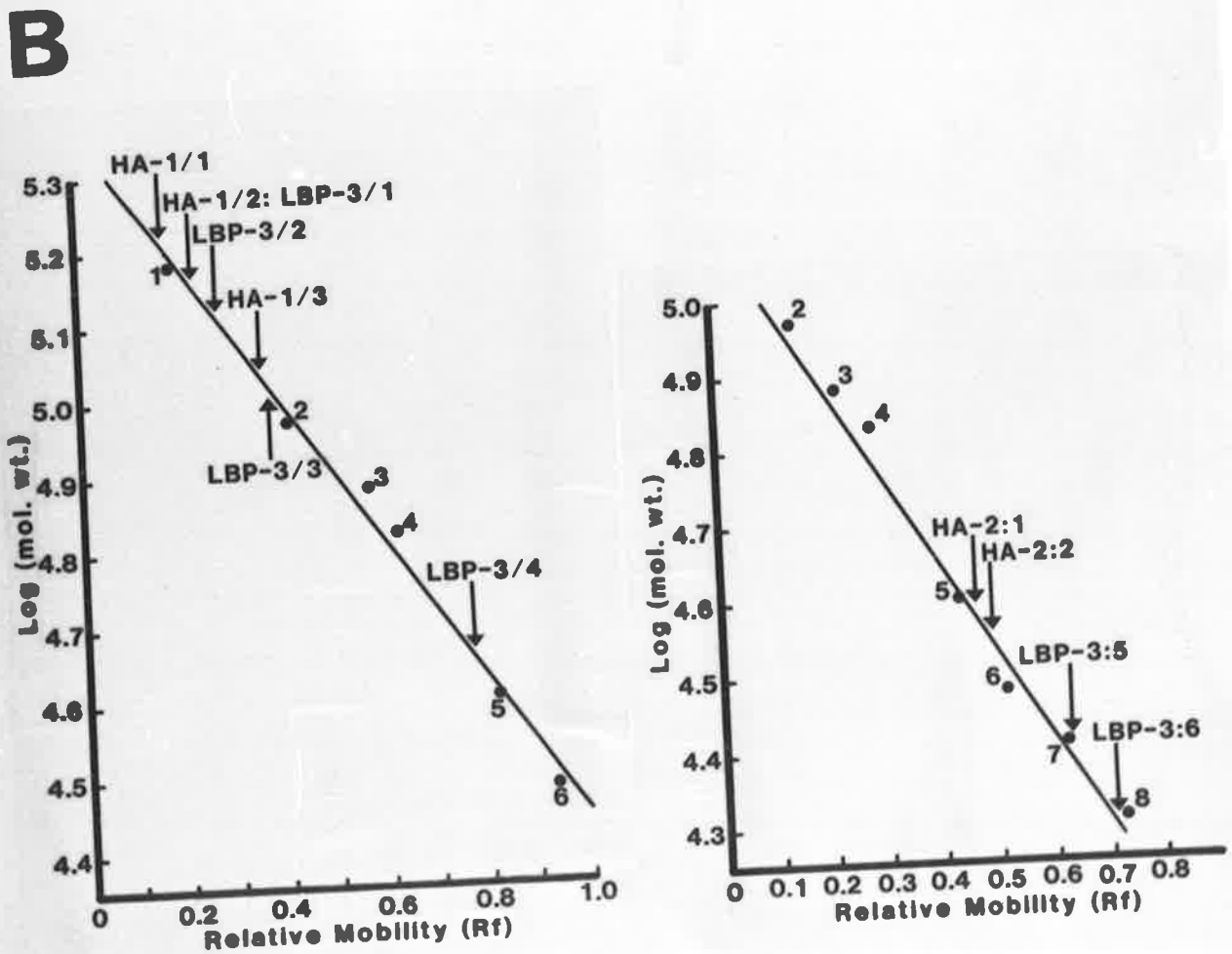
Determination of protein molecular weights by SDS-PAGE in non-reducing conditions

A. SDS-PAGE in non-reducing conditions. The procedure is described in Chapter 2, section 2.16. The samples (20 ul), except where indicated, contained 2 ug of each protein.

| | 7% gel | | 11% gel |
|---|---|---|---|
| A | HA-1 (8 ug) | B | HA-1 (8 ug) |
| B | HA-1 (4 ug) | B | LBP-3 (8 ug) |
| C | HA-1 (1 ug) | C | HA-2 (4 ug) |
| D | HA-1 (2 ug) | D | HA-2 (2 ug) |
| E | LBP-3 (8 ug) | E | phosphorylase a |
| F | LBP-3 (1 ug) | F | aldolase |
| G | LBP-3 (2 ug) | G | carbonic anhydrase, α chymotrypsinogen, trypsin inhibitor |
| H | LBP-3 (4 ug) | H | mouse IgG, transferrin, BSA |
| I | carbonic anhydrase | I | HA-2, unreduced |
| J | aldolase | J | HA-2, unreduced |
| K | phosphorylase a | K | HA-2, reduced |
| L | mouse IgG, transferrin, BSA | | |

B. Relationship between the relative mobility ($R_f$ = distance migrated by protein ÷ distance migrated by dye front) of the marker proteins and their molecular weight The correlation coefficients were 0.993 and 0.994 for the 7% and 11% gels, respectively. The marker proteins were 1) IgG (150,000), 2) phosphorylase a (94,000), 3) transferrin (76,000) 4) BSA (67,000) 5) aldolase (40,000), 6) carbonic anhydrase (30,000), 7) α chymotrypsinogen (25,670), 8) trypsin inhibitor (20,500). The positions corresponding to the various HA-1, HA-2 and LBP-3 bands are indicated.


A
7%
A B C D E F G H I J K L
11%
A B C D E F G H I J K


B
HA-1/1
HA-1/2: LBP-3/1
LBP-3/2
HA-1/3
LBP-3/3
LBP-3/4
Log (mol. wt.)
Relative Mobility (Rf)
HA-2:1
HA-2:2
LBP-3:5
LBP-3:6

of a protein in SDS-PAGE and its molecular weight. These are outlined in detail by Rodbard (1976). Inspection of manual graphical plots of the data for the standard proteins indicated that the relationship log (mol. wt.) $\alpha$ Rf was approximately linear and a line was fitted using linear least-squares regression analysis. The results are shown in Figure 7.4B. The correlation coefficients were 0.993 (7% gel) and 0.994 (11% gel).

The HA-1 preparation yielded four protein bands, although three of these were only minor components as judged by staining intensities. One minor band only just entered the gel and the apparent mol. wts. of the other bands were 164,000, 107,600 and 147,200 for the major band. The LBP-3 preparation yielded a single major protein band (apparent mol. wt. = 147,200) and five quite minor bands having apparent mol. wts. of about 129,400, 104,200, 44,500, 24,500 and 19,900. The results indicated that the apparent mol. wt. of the major component of each preparation was similar. Both of these HA-1 and LBP-3 components had a diffuse appearance in the gels and it seems likely that each constitutes two overlapping molecular species, especially in regard to HA-1. Only one major polypeptide was revealed with HA-2 and this had an apparent mol. wt. of 35,500. However, a minor band was also present with an apparent mol. wt. of 38,900.

### 7.3.2 Determination of molecular weights by SDS-PAGE under reducing conditions

The SDS-PAGE analyses for HA-1, HA-2 and LBP-3 under reducing conditions are shown in Figure 7.5. The plot of relative mobility (Rf) versus log (mol. wt.) yielded a straight line with a correlation coefficient of 0.99. Only

FIGURE 7.5

Determination of polypeptide molecular weights by SDS-PAGE in reducing conditions

A. SDS-PAGE in reducing conditions. The procedure is described in Chapter 2, section 2.16. The gel was 13%. The samples (20 ul), except where indicated, contained 2 ug of each protein.

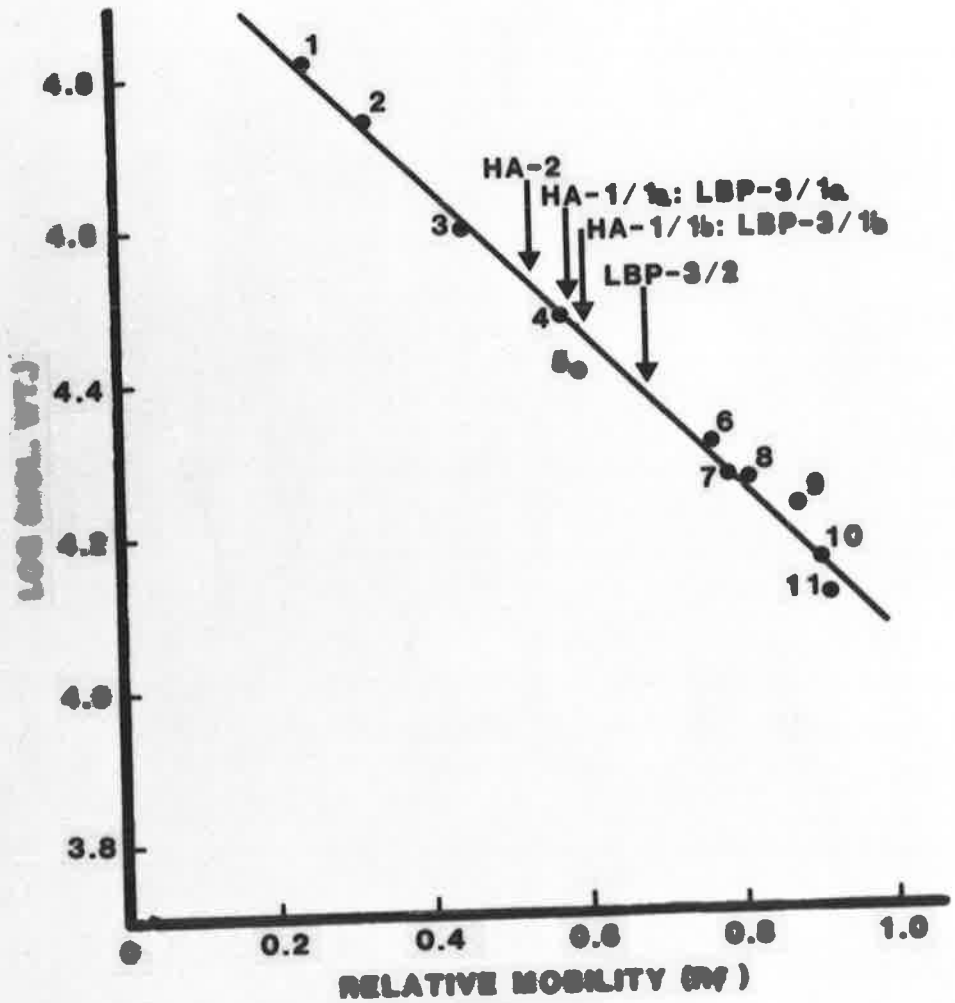
| | |
|---|---|
| A | lysozyme |
| B | myoglobin |
| C | ß-lactoglobulin |
| D | trypsin inhibitor |
| E | LBP-3 (3.5 ug) |
| F | bovine serum albumin, mouse IgG H chain, aldolase, carbonic anhydrase, IgG L chain, trypsin inhibitor, myoglobin, cytochrome c |
| G | HA-1 (3 ug) |
| H | α-chymotrypsinogen |
| I | HA-2 (3 ug) |
| J | mouse IgG H chain, L chain |
| K | ferritin |

B. Relationship between the relative mobility ($R_f$) of the marker proteins and their molecular weight. The correlation coefficient was 0.99. The marker proteins were: 1) bovine serum albumin (67,000), 2) IgG H chain (55,000), 3) aldolase (40,000), 4) carbonic anhydrase (30,000), 5) α-chymotrypsinogen (25,670), 6) trypsin inhibitor (20,500), 7) ferritin (18,500), 8) ß-lactoglobulin (18,365), 9) myoglobin (17,113), 10) lysozyme (14,316), 11) cytochrome c (13,000). The positions corresponding to HA-1, HA-2 and LBP-3 are indicated.

A


A B C D E F G H I J

B


4.8
4.6
4.4
4.2
4.0
3.8
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY (Rf)
1
2
3
4
5
6
7
8
9
10
11
HA-2
HA-1/1a: LBP-3/1a
HA-1/1b: LBP-3/1b
LBP-3/2

a single band was obtained with HA-2 and this had an apparent mol. wt. of 33,400, similar to that detected under non-denaturing conditions. In contrast, major differences were apparent upon reduction of both the HA-1 and the LBP-3. The HA-1 yielded two new bands (HA-1/1a and HA-1/1b) which were only marginally resolved and had apparent mol. wts. of approximately 29,600 and 28,200 respectively. The major 147,200 mol. wt. component seen in non-reduced gels was not evident. The LBP-3 appeared to be composed of three polypeptides. LBP-3/1a and LBP-3/1b were indistinguishable from HA-1/1a and HA-1/1b in terms of mobility, whilst the smaller third band (LBP-3/2), which stained more intensely than the other two, had an apparent mol. wt. of 23,500. No other bands were detected.

## 7.4 Two dimensional SDS-PAGE analysis of LBP-3 and HA-1

Although the analysis of unreduced LBP-3 and HA-1 showed that each was composed of only one major protein, several minor components were also present. It seemed unlikely that these were contaminating proteins since they would have needed to be lactose binding proteins with Stoke's radii very similar to those of HA-1 and LBP-3. Furthermore, on reduction with 2-mercaptoethanol only three or two bands appeared in SDS-PAGE suggesting that all the components were composed of the same subunits. To confirm this, the LBP-3 and HA-1 preparations were subjected to two dimensional SDS-PAGE using non-reducing conditions in the first dimension followed by reducing conditions in the second dimension.

The procedure used was an adaption of the method of O'Farrell (1974). Briefly, the normal first dimensional gel

was equilibrated with sample buffer containing 5% v/v 2-mercaptoethanol and then embedded into the stacking gel of the discontinuous SDS-PAGE system for the second dimension run. A full description of the procedure is in Chapter 2, section 2.17.

There were two conditions which had to be met before unequivocal results could be gained from this method. Firstly, the first dimensional gel needed to be incubated in the sample buffer long enough to ensure that the proteins were fully reduced, but short enough to ensure that there was no significant diffusion of proteins out of the gel. Secondly, the stacking gel of the second dimension run had to be long enough to ensure that all protein was eluted from the first dimensional gel and concentrated at the stacking gel front. It can be seen that artifacts would arise if some protein was behind the stacking gel front when this entered the separating gel. These points were investigated using iodine-125 labelled LBP-3. Firstly, the effect of incubating a first dimension gel strip in 15 ml of sample buffer containing 5% v/v 2-mercaptoethanol for 60 minutes at $37^{o}$ was investigated as outlined in Figure 7.6. These conditions were considered adequate to ensure complete reduction of all disulphide bonds. There appeared to be little loss of radioactive material from the gel by this procedure since similar radioactivity profiles were obtained for both the test and control gels (Fig. 7.6, A vs B) and the recovery of radioactivity was in each case greater than 90%. Consequently, this procedure was used to reduce the proteins in the first-dimension gels.

To investigate the length of stacking gel needed to stack the proteins in the second dimension, the first

FIGURE 7.6

Assessment of the 2-dimensional SDS-PAGE technique. Iodine-125 labelled LBP-3 (5 ug; 60,000 cpm) was subjected to 1st dimension SDS-PAGE under non-reducing conditions in 7% gels. Gel strips (1 x 8 cm) containing the individual sample running tracks were cut out. The radioactivity profile was determined by sectioning the gel strips into 2 mm slices before (A) and after (B) a 60 minute incubation in 15 ml of sample buffer containing 5% 2-mercaptoethanol. In (C), the length of stacking gel needed to stack the samples in the 2nd dimension was investigated. To allow the gel to be cut out and fractionated, the stacking gel was made using 1% agarose (Pharmacia, Type C). The radioactivity profile at a front which had moved 2 cm into the stacking gel is shown.

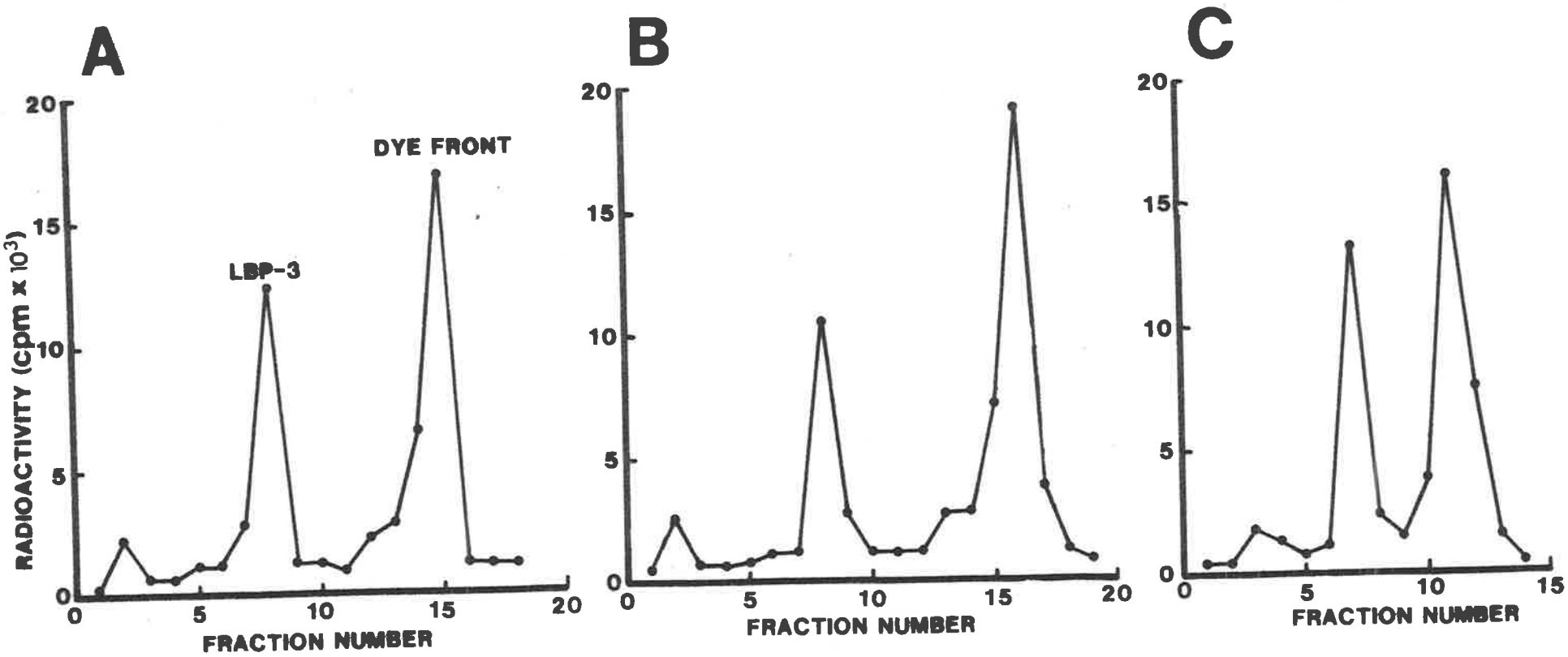

A
B
C
RADIOACTIVITY (cpm x $10^3$)
LBP-3
DYE FRONT
FRACTION NUMBER
FRACTION NUMBER
FRACTION NUMBER
0
5
10
15
20

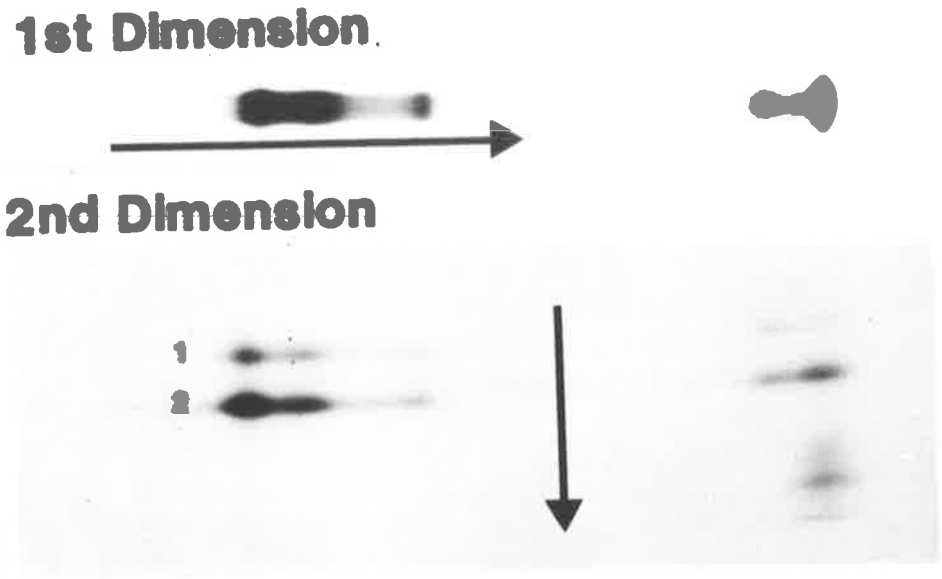
FIGURE 7.7

Two dimensional SDS-PAGE analyses of HA-1 and LBP-3. The 1st dimension was electrophoresed in 7% gels under non-reducing conditions and the 2nd dimension was electrophoresed in 11% gels under reducing conditions. The arrows indicate the direction of electrophoresis in each case. The gels were dried (Chapter 2, section 2.18) and autoradiographed (Chapter 2, section 2.19).

A. Analysis of LBP-3. The sample contained approximately 0.04 ug of radiolabelled LBP-3 (616,000 cpm) and 5 ug of unlabelled LBP-3.

b. Analysis of HA-1. The sample contained approximately 0.04 ug of radiolabelled HA-1 (400,000 cpm) and 5 ug of unlabelled HA-1.

A


1st Dimension
2nd Dimension
1
2

B


1st Dimension
2nd Dimension
1
2
3

dimension gel slices were embedded in stacking gels and the stacking gel fronts, as marked by bromophenol blue dye, were allowed to migrate into the stacking gel for various distances and the extent of stacking was evaluated by cutting out and fractionating a gel strip 5 mm either side of the bromophenol dye front. It was found that the maximum recovery of radioactivity (85-90%) was obtained when the dye front had moved at least 2 cm into the stacking gel, the radioactivity profile being very similar to that obtained for the first dimensional gels (Fig. 7.6, C vs B). Therefore, the stacking gel was made 3 cm long for the second dimension. As a final test of the complete two dimensional procedure, 10 µg of mouse IgG was used as the sample and this was visualised by Coomassie blue staining. As expected, only two bands (light chain and heavy chain) were seen (results not shown). There was no sign of higher molecular weight unreduced material.

Figure 7.7A shows the results obtained when labelled HA-1 and LBP-3 were analysed by this two dimensional SDS-PAGE procedure. The results were visualised by autoradiography. The patterns obtained with radiolabelled HA-1 and LBP-3 after electrophoresis in the 1st dimension were the same as those obtained with unlabelled protein (Fig. 7.4), indicating that the labelling procedure had not affected the integrity of the polypeptide chains. For the case of LBP-3 in the second dimension, all of the protein bands present in the first dimension appeared to be resolved into the same two polypeptide bands (bands 1 and 2, Fig. 7.7A) indicating that they were probably composed of the same subunits. Thus it appeared that the minor components were not contaminants. Additional low mol. wt. components

were detected in material which migrated with the dye front in the 1st dimension. This probably represented a small amount of degraded material.

In the case of HA-1 (Fig. 7.7B), all but one of the protein bands present in the 1st dimension gel appeared to be composed of the same major polypeptide component (band 2, Fig. 7.7B) and a very minor component (band 3, Fig. 7.7B). Thus it seemed that these minor components were not contaminants but were different forms of HA-1. In contrast to this result, the component (mol. wt. = 164,000) which migrated behind the major HA-1 band in the 1st dimension appeared to be composed of a different, much higher mol. wt. subunit (band 1, Fig. 7.7B). This suggested that this component is a contaminant.

## 7.5 Investigation of HA-1, HA-2 and LBP-3 by immunochemical analysis

Using the double immunodiffusion method of Ouchterlony, the three sugar binding proteins were tested for reactivity against anti HA-1 and anti HA-2 antisera. The results are shown in Figure 7.8. As judged by the formation of precipitin lines, anti HA-2 was reactive against HA-2 but not against HA-1 or LBP-3. Anti HA-1 was reactive against both HA-1 and LBP-3 but not against HA-2. Thus, there appeared to be no antigenic relationship between HA-2 and HA-1 or LBP-3.

The fusion of the HA-1 and LBP-3 precipitin lines (Fig. 7.8A) showed that these molecules were antigenically related. However, both the HA-1 and LBP-3 precipitin lines formed spurs indicating that the fusion was only partial.

FIGURE 7.8

Analyses of HA-1, HA-2 and LBP-3 by double immunodiffusion. Six point seven millilitres of 1% agarose dissolved in immunoelectrophoresis buffer containing 1 mM EGTA and 50 mM lactose was poured onto a 5 x 7.6 cm glass plate. Three millilitre diameter wells were cut at 8 mm spacing in the pattern shown and filled with the various antigen and antibody solutions (10 ul). The plates were incubated at 37° for 48 hr and then washed and stained as for rocket immunoelectrophoresis.

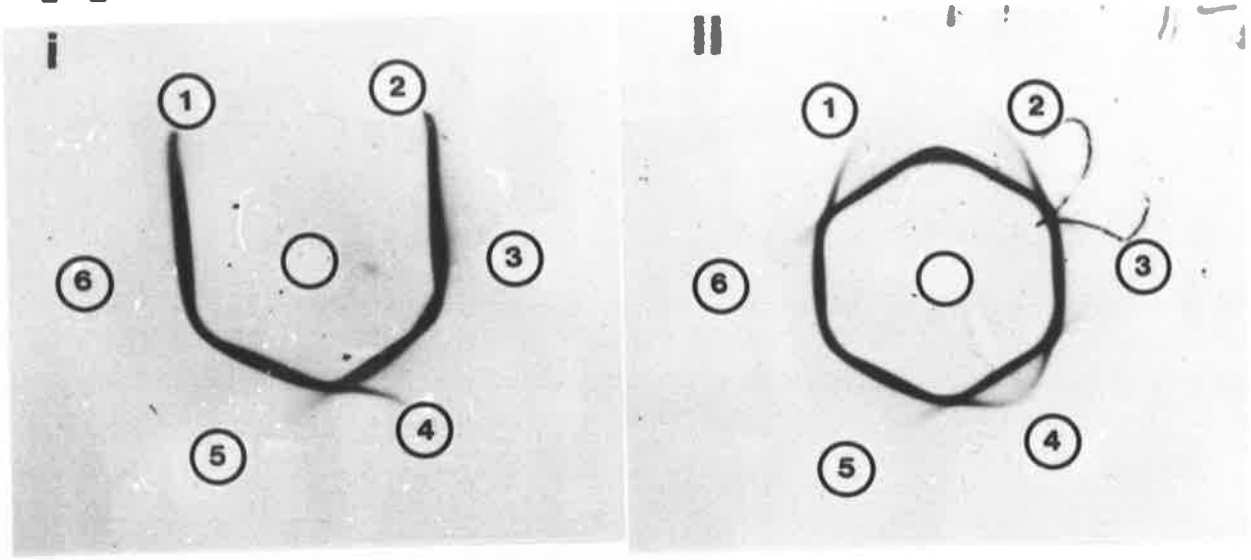
A. The centre well contained anti-(HA-1) rabbit IgG (12.26 mg/ml).

| i) well | sample | ii) well | sample |
|---|---|---|---|
| 1 | HA-2, 10.0 ug | 1 | HA-1, 1.5 ug |
| 2 | HA-2, 10.0 ug | 2 | HA-1, 1.5 ug |
| 3 | HA-1, 1.5 ug | 3 | LBP-3, 0.9 ug |
| 4 | HA-1, 1.5 ug | 4 | HA-1, 1.5 ug |
| 5 | LBP-3, 0.9 ug | 5 | LBP-3, 0.9 ug |
| 6 | LBP-3, 0.9 ug | 6 | LBP-3, 0.9 ug |

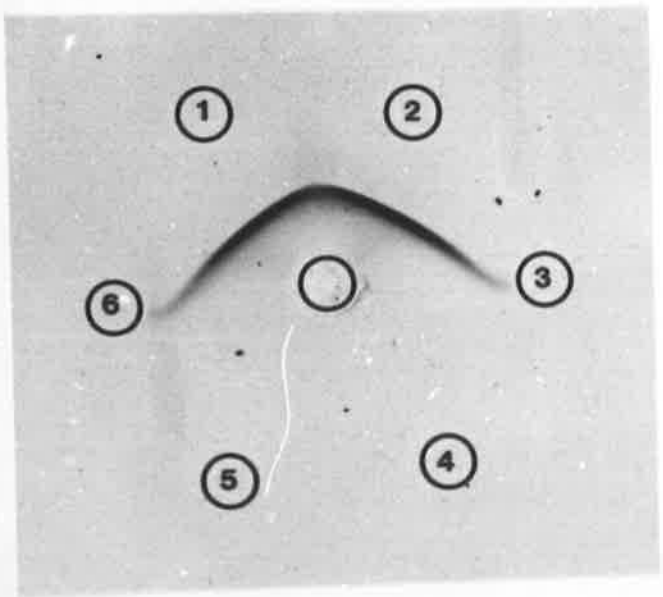
B. The centre well contained anti-(HA-2) rabbit IgG (9.9 mg/ml).

| well | sample |
|---|---|
| 1 | HA-2, 0.6 ug |
| 2 | HA-2, 0.6 ug |
| 3 | LBP-3, 15.0 ug |
| 4 | LBP-3, 15.0 ug |
| 5 | HA-1, 3.0 ug |
| 6 | HA-1, 3.0 ug |

**A**




**B**



The spurs formed by the LBP-3 precipitin line were very pronounced. By comparison, the HA-1 spurs were very weak. These preliminary results showed that HA-1 and LBP-3 shared many antigenic determinants but that both proteins possessed a number of unique determinants.

The antigenic crossreactivity of HA-1 and LBP-3 found above was further investigated using crossed immunoelectrophoresis, a method which is capable of quite powerful resolution of antigen systems. When the HA-1 preparation was analysed by this method (Fig. 7.9B) one main peak, formed by a single, sharp precipitin line, was obtained. However, a number of very diffuse precipitin lines, which fused with the main peak, were also present in the cathodal descending region of the main peak. The diffuse appearance of these lines suggested that they were formed by weakly antigenic HA-1 material. After simple, one dimensional electrophoresis of HA-1 (Fig. 7.9C), the position of the haemagglutinating activity correlated only with the material which formed the main peak. No shoulder of activity correlating with the diffuse shoulder peaks was found. These results indicated that the HA-1 preparation was comprised mainly of a homogeneous population of HA-1 molecules but that a minor amount of inactive, antigenically-incomplete, possibly partially denatured HA-1 material was also present. The results obtained with the LBP-3 preparation (Fig. 7.8A) indicated that this too was composed mostly of a single homogeneous population of molecules. But the presence of two smaller peaks formed by well defined, less intense precipitin lines which fused with the main peak showed that minor quantities of different, electrophoretically slower moving

FIGURE 7.9

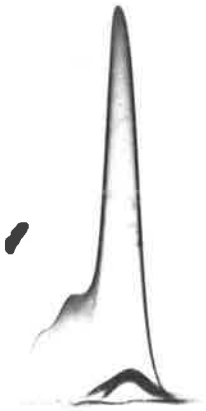
Crossed-immunoelectrophoresis of HA-1 and LBP-3. The procedure was as described in Chapter 2 section 2.20. The antibody gel contained anti-(keyhole limpet haemocyanin) (0.15 mg/cm$^2$) and anti-(HA-1) (0.2 mg/cm$^2$; raised in section 6.11) rabbit IgG. X = origin.

A. Analysis of LBP-3. Sample contained LBP-3 (16 ug) and included keyhole limpet haemocyanin (5 ug) as an internal marker.

B. Analysis of HA-1. Sample contained HA-1 (12 ug) and included keyhole limpet haemocyanin (5 ug) as an internal marker.

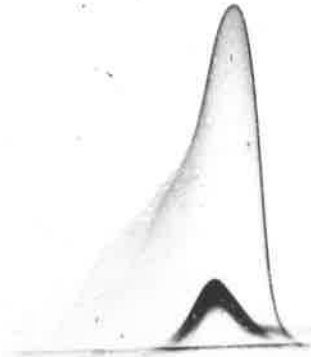
C. Electrophoresis in agarose of HA-1. A sample of HA-1 was electrophoresed in agarose as for the 1st dimension of crossed immunoelectrophoresis. The gel was sectioned into 0.2 mm slices and each of these were incubated overnight in 0.5 ml of TSA. The supernatants were then titrated against GPRBC.

A



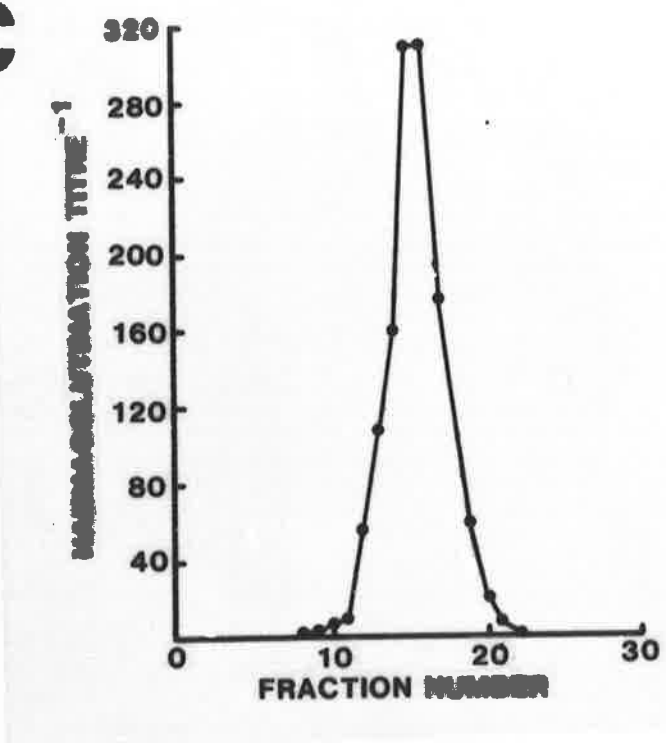
X LBP-3

B



X HA-1

C



LBP-3 molecules were also present. To investigate the antigenic cross reactivity of HA-1 and LBP-3 further, the effects of combining HA-1 and LBP-3 in the first dimension of (tandem) crossed immunoelectrophoresis was ascertained. Figure 7.10A shows the result when HA-1 and LBP-3 were placed in adjacent wells. No trace of the individual HA-1 and LBP-3 peaks can be seen. It appeared that the two overlapping peaks completely fused, resulting in a greatly enlarged peak which extended past the top of the gel plate. In Figure 7.10B the sample wells were staggered, HA-1 being placed ahead of LBP-3. This resulted in two partially overlapping peaks. The HA-1 peak could be identified since it ran ahead of the KLH peak. The HA-1 peak completely fused with the LBP-3 peak, there being no trace of a precipitin line extending past the LBP-3 peak. In contrast, the LBP-3 peak only partially fused with the HA-1 peak. The LBP-3 peak can clearly be seen extending past the fusion point with the HA-1 peak, though with less intensity. These results demonstrate that LBP-3 possesses antigenic determinants not shared with HA-1, while nearly all of the HA-1 antigenic determinants are common to LBP-3.

These conclusions were supported by the results of crossed line immunoelectrophoresis. In this technique an intermediate gel containing antigen is laid between the first dimension gel and the antibody containing gel, as shown in Figures 7.10C and D. When the first dimension gel contains no antigen, a precipitin line forms across the antibody-containing gel due to antigen moving from the intermediate gel. Figure 7.10D shows the results obtained when LBP-3 was electrophoresed in the first dimension and then run in the

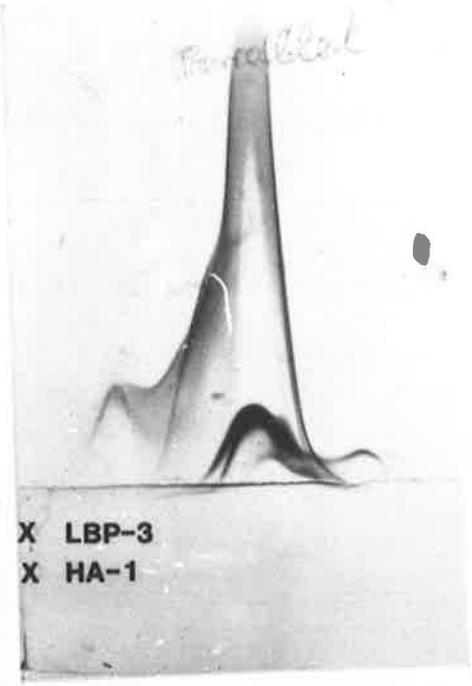
FIGURE 7.10

Immunochemical analyses of HA-1 and LBP-3. The procedures used were as described in Chapter 2, section 2.20 and Figure 7.8. X' = origin.

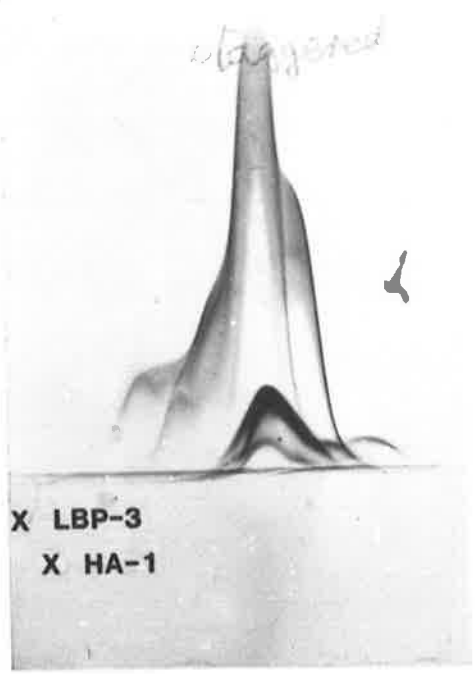
A plus B. Tandem crossed immunoelectrophoresis containing both HA-1 (plus keyhole limpet haemocyanin) and LBP-3 in the 1st dimension. In (A) the sample wells were adjacent and in (B) they were staggered.

C plus D. Crossed line immunoelectrophoresis. The intermediate gel contained 1.5 ug of LBP-3 per $cm^2$. The samples were (C) HA-1 and (D) LBP-3.

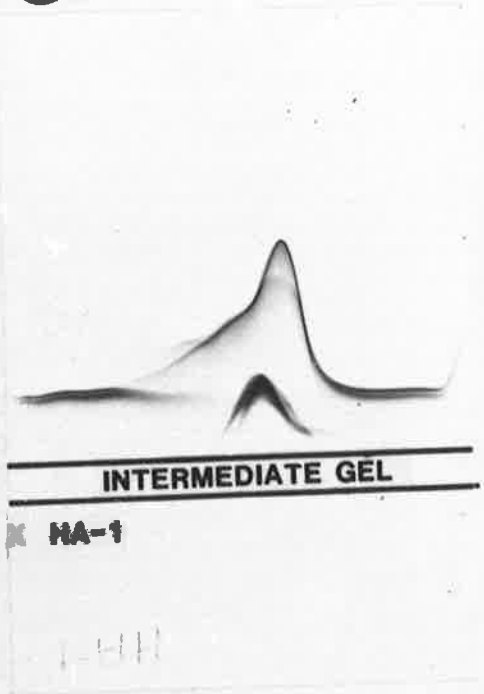
A


X LBP-3
X HA-1

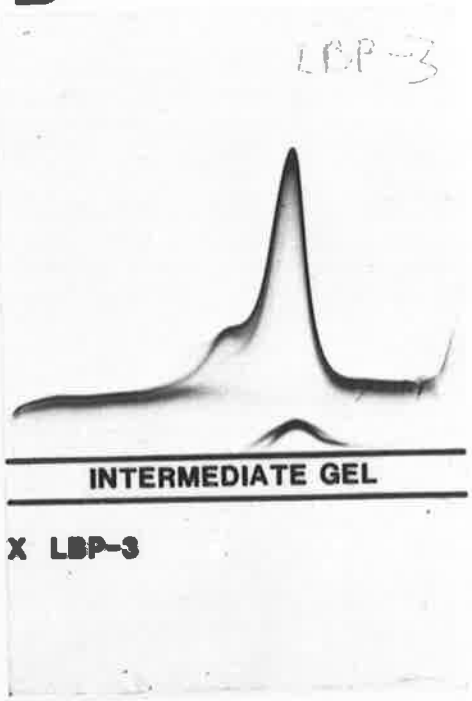
B


X LBP-3
X HA-1

C


INTERMEDIATE GEL
X HA-1

D


INTERMEDIATE GEL
X LBP-3

second dimension through an intermediate gel containing LBP-3. It can be seen that a normal precipitin peak formed (as in normal crossed immunoelectrophoresis; c.f. Fig. 7.9A) except that it was lifted in position and had fused completely with the base precipitin line. In contrast, the position of the antigenically unrelated KLH peak remained unchanged. Similarly, when HA-1 was electrophoresed in the first dimension gel and the intermediate gel contained LBP-3, the HA-1 peak was essentially normal (Fig. 7.10C) except that it too was lifted in position and had fused completely with the base LBP-3 precipitin line. These results indicated that most of the HA-1 antigenic determinants are shared with LBP-3.

## 7.6 Binding Specificity of LBP-3

Experience with the affinity column had suggested that the LBP-3 possessed a binding site similar to that of HA-1. For instance, both proteins bound galactose residues and required $Ca^{++}$ ions for this activity. However, unlike HA-1, LBP-3 did not agglutinate guinea pig erythrocytes. There were two possible explanations for this lack of agglutinating activity. Firstly it was possible that LBP-3 did not bind to guinea pig erythrocytes. If this was so it would mean that LBP-3 had a different binding site to that of HA-1. Or, secondly, LBP-3 may have bound to guinea pig erythrocytes but for some reason, probably due to its conformation, was unable to cross link the guinea pig erythrocytes.

With the availability of anti-LBP-3 antisera (Chapter 6) it became possible to quantitate the amount of LBP-3 in unknown samples by rocket immunoelectrophoresis (Chapter 2,

section 2.20.3) since the height of the rocket is normally proportional to the amount of antigen applied. Thus, the ability of LBP-3 to bind to guinea pig and human erythrocytes was assessed by determining by rocket immunoelectrophoresis the amount of LBP-3 remaining in the supernatants after incubation with each type of cell in the presence of either $Ca^{++}$, lactose or EGTA. The results are presented in Table 7.3. When LBP-3 was adsorbed with guinea pig erythrocytes in the presence of $Ca^{++}$, the height of the rocket compared to that of the control was considerably reduced, indicating that about 75-80% of the LBP-3 had bound to the guinea pig erythrocytes. The inability of this protein to agglutinate guinea pig erythrocytes must therefore lie its failure to adequately crosslink the cells. There was no adsorption to guinea pig erythrocytes in the presence of lactose or EGTA, nor to human erythrocytes in the presence of $Ca^{++}$. These findings demonstrate that the characteristics of the binding site of LBP-3 are indistinguishable from those of HA-1.

## 7.7 Amino acid analyses of HA-1, HA-2 and LBP-3

The amino acid analyses were performed as described in Chapter 2, section 2.25 on samples hydrolysed for 24 hr, 48 hr and 72 hr. Considerable losses of cysteine and half cystine occur during acid hydrolysis but cysteic acid, formed from these amino acids by performic acid treatment, is quite stable. Therefore, an analysis was also performed on samples which had been treated with performic acid (Glazer, De Lange and Sigman, 1975) and hydrolysed for 24 hr. Serine, threonine and tyrosine are partially destroyed in a time dependent manner by acid hydrolysis and these were determined by extra-

TABLE 7.3

Adsorption of LBP-3 with guinea pig and human erythrocytes*

| Incubation with cells in the presence of | Height of rocket (mm) after adsorption with§ | | |
|---|---|---|---|
| | Guinea pig erythrocytes | Human erythrocytes | Control |
| 5 mM $Ca^{++}$ | 13 (20%) | - | - |
| 54 mM lactose + 5 mM $Ca^{++}$ | 28 (100%) | - | - |
| 3.75 mM EGTA | 27 (100%) | - | - |
| 5 mM $Ca^{++}$ | - | 28 (100%) | - |
| No cells | - | - | 28 |

* 2.5% v/v suspensions of erythrocytes in TSA were incubated with 75 ug of LBP-3 in the presence of either $Ca^{++}$, lactose or EGTA as indicated. Total volume was 100 ul. Suspensions were incubated at room temperature for 45 minutes, centrifuged (900 g, 5 min.) and the amount of LBP-3 remaining in the supernatants determined by rocket immunoelectrophoresis (Chapter 2, section 2.20)

§ The values in brackets indicate the percent of LBP-3 remaining. This was determined from a plot of amount of LBP-3 versus height of rocket derived using the control supernatant.

polating to zero time the values for 24 hr, 48 hr and 74 hr. Asparagine and glutamine are quantitatively converted to aspartic acid and glutamic acid, respectively, by acid hydrolysis and so cannot be determined separately by this method. Analysis of the remaining amino acids is usually quite reliable.

The yield of each amino acid in micromoles is shown in Tables 7.4 and 7.6. Except where indicated, the average for the three hydrolysis time points was used to calculate the mole percent for each amino acid. The samples used for normal analysis and for performic acid treatment were different and the values for the yield of cysteic acid were accordingly standardised for each protein using a conversion factor calculated by averaging the ratios between the normal average value and the performic acid value for several stable amino acids, as indicated in Table 7.4.

The amino acid compositions shown here for HA-1 are an average for the 1a and 1b subunits, but would be largely biased towards those of the 1b subunit since the purified preparations used for the analyses contained mainly the 1b subunit (Fig. 7.4). The mole percent values for HA-1 were converted to residues per subunit (mol. wt. = 28,200) by assuming that proteins contain an average of 8.875 amino acid residues

TABLE 7.4

Amino acid composition of HA-2

| | 24 hr. | 48 hr. | 72 hr. | Performic acid treated* | Average | Mole% |
|---|---|---|---|---|---|---|
| | | | micro moles | | | |
| Asp | 0.091 | 0.090 | 0.081 | 0.05+ | 0.087 | 12.46 |
| Thr | 0.055 | 0.047 | 0.040 | 0.027 | 0.062# | 8.88 |
| Ser | 0.035 | 0.027 | 0.022 | 0.023 | 0.041# | 5.87 |
| Glu | 0.067 | 0.063 | 0.059 | 0.04+ | 0.063 | 9.03 |
| Pro | 0.039 | 0.035 | 0.035 | 0.021 | 0.036 | 5.16 |
| Gly | 0.081 | 0.065 | 0.062 | 0.44+ | 0.069 | 9.88 |
| Ala | 0.045 | 0.040 | 0.039 | 0.026+ | 0.041 | 5.87 |
| Val | 0.061 | 0.057 | 0.055 | 0.036 | 0.058 | 8.31 |
| Cys§ | - | - | - | 0.003 | 0.005 | 0.72 |
| Met | 0.015 | 0.010 | 0.009 | - | 0.011 | 1.58 |
| Ile | 0.061 | 0.055 | 0.054 | 0.029+ | 0.057 | 8.17 |
| Leu | 0.029 | 0.028 | 0.028 | 0.018+ | 0.028 | 4.01 |
| Tyr | 0.027 | 0.022 | 0.023 | - | 0.031# | 4.44 |
| Phe | 0.052 | 0.047 | 0.045 | 0.025 | 0.048 | 6.88 |
| Lys | 0.029 | 0.031 | 0.029 | 0.021 | 0.030 | 4.30 |
| His | 0.002 | 0.003 | 0.002 | - | 0.002 | 0.29 |
| Arg | 0.032 | 0.029 | 0.027 | 0.014 | 0.029 | 4.15 |
| Trp | - | - | - | - | - | - |

* Hydrolysed for 24 hr.

§ Determined as cysteic acid after performic acid treatment.

# Obtained by extrapolating to zero time.

+ These values were used to calculate the conversion factor for standardisation of cysteic acid (see text). The range was 1.55 to 1.96 and the average was 1.66.

TABLE 7.5

Determination of the molecular weight and partial specific volume of HA-2 from its amino acid composition

| Amino acid | Residues* per mole | Nearest integer | Mol. wt. of+ amino acid | ΣMol. wt. | Percent by wt. | $\bar{v}$ | $\Sigma \bar{v}$§ |
|---|---|---|---|---|---|---|---|
| Asp | 34.6 | 35 | 115.08 | 4027.8 | 13.1 | 0.6 | 7.86 |
| Thr | 24.7 | 25 | 101.10 | 2527.5 | 8.2 | 0.7 | 5.74 |
| Ser | 16.3 | 16 | 87.07 | 1393.12 | 4.67 | 0.63 | 2.94 |
| Glu | 25.1 | 25 | 129.11 | 3227.75 | 10.65 | 0.66 | 7.03 |
| Pro | 14.3 | 14 | 97.11 | 1359.54 | 4.58 | 0.76 | 3.48 |
| Gly | 27.4 | 27 | 57.05 | 1540.35 | 5.15 | 0.64 | 3.3 |
| Ala | 16.3 | 16 | 71.07 | 1137.12 | 3.81 | 0.74 | 2.82 |
| Val | 23.1 | 23 | 99.13 | 2279.99 | 7.52 | 0.86 | 6.47 |
| Cys¶ | 2.0 | 2 | 102.13 | 204.26 | 0.67 | 0.61 | 0.41 |
| Met | 4.4 | 4 | 131.19 | 524.76 | 1.89 | 0.75 | 1.42 |
| Ile | 22.7 | 23 | 113.15 | 2602.45 | 8.44 | 0.9 | 7.6 |
| Leu | 11.1 | 11 | 113.15 | 1244.65 | 4.14 | 0.9 | 3.73 |
| Tyr | 12.3 | 12 | 163.17 | 1958.04 | 6.62 | 0.71 | 4.7 |
| Phe | 19.1 | 19 | 147.17 | 2796.23 | 9.25 | 0.77 | 7.12 |
| Lys | 11.9 | 12 | 128.17 | 1538.04 | 5.03 | 0.82 | 4.12 |
| His | 0.8 | 1 | 137.14 | 137.14 | 0.36 | 0.67 | 0.24 |
| Arg | 11.5 | 12 | 156.19 | 1874.28 | 5.92 | 0.7 | 4.14 |
| Trp | - | - | 186.22 | - | - | - | - |
| | | | | 30,373 + 18# | | | 73.12 |
| | | | mol. wt. = 30,391 | | | $\bar{v}$ = 0.731 | |

* Calculated by multiplying mole% values (Table 7.4) by 2.777 = (no. of cys) ÷ (mole% of cys) = 2 ÷ 0.72.

\+ Mol. wt. = formular mol. wt. - mol.wt. of water.

§ $\Sigma \bar{v}$ = percent by wt. x $\bar{v}$.

¶ As half cystine.

\# Water of -COOH terminal amino acid.

TABLE 7.6

Amino acid composition of HA-1 and LBP-3

| | Micro moles* | | Mole percent | |
|---|---|---|---|---|
| | LBP-3 | HA-1 | LBP-3 | HA-1 |
| Asp | 0.0887 | 0.074 | 11.17 | 11.85 |
| Thr | 0.058 | 0.0384 | 7.3 | 6.15 |
| Ser | 0.064 | 0.049 | 8.06 | 7.85 |
| Glu | 0.102 | 0.0883 | 12.84 | 14.14 |
| Pro | 0.0207 | 0.0113 | 2.61 | 1.81 |
| Gly | 0.062 | 0.0427 | 7.81 | 6.84 |
| Ala | 0.0603 | 0.0417 | 7.59 | 6.68 |
| Val | 0.0263 | 0.022 | 3.31 | 3.52 |
| Cys§ | 0.0318 | 0.0242 | 4.00 | 3.87 |
| Met | 0.0153 | 0.012 | 1.93 | 1.92 |
| Ile | 0.0303 | 0.0293 | 3.82 | 4.69 |
| Leu | 0.0707 | 0.0603 | 8.90 | 9.66 |
| Try | 0.039 | 0.0276 | 4.91 | 4.42 |
| Phe | 0.0377 | 0.029 | 4.75 | 4.64 |
| Lys | 0.0403 | 0.044 | 5.07 | 7.05 |
| His | 0.0073 | 0.000 | 0.92 | 0.00 |
| Arg | 0.0397 | 0.0307 | 5.00 | 4.92 |
| Trp | - | - | - | - |

* Average for 24, 48 and 72 hour hydrolysis times. Thr, Ser, and Tyr were obtained by extrapolation to zero time.

§ Determined as cysteic acid after performic acid treatment. Value was standardised as in Table 7.4.

TABLE 7.7

Determination of the molecular weight and partial specific volume of the HA-1 subunit from its amino acid composition

| Amino acid | Residues* per subunit | ΣMol. wt.§ | $\Sigma\bar{v}$§ |
|---|---|---|---|
| Asp | 30 | 3452.4 | 7.32 |
| Thr | 15 | 1516.5 | 3.75 |
| Ser | 20 | 1741.4 | 3.88 |
| Glu | 35 | 4518.85 | 10.54 |
| Pro | 5 | 485.55 | 1.30 |
| Gly | 17 | 969.85 | 2.19 |
| Ala | 17 | 1208.19 | 3.16 |
| Val | 9 | 919.17 | 1.98 |
| Cys | 10 | 991.3 | 3.01 |
| Met | 5 | 655.95 | 1.74 |
| Ile | 12 | 1357.8 | 4.32 |
| Leu | 24 | 2715.6 | 8.64 |
| Try | 11 | 1794.87 | 4.5 |
| Phe | 12 | 1766.04 | 4.81 |
| Lys | 18 | 2307.06 | 6.69 |
| His | 0 | 0 | 0 |
| Arg | 12 | 1874.28 | 4.64 |
| Trp | - | - | - |
| | | 28,274.81 | 72.48 |
| | Mol. wt. = 28,293 | | $\bar{v}$ = 0.725 |

* Calculated by multipying mole% values by 2.5 and rounding to nearest integer. This conversion factor was derived by assuming that proteins contain an average of 8.875 amino acid residues per $10^3$ mol. wt. (see text).

§ Calculated as in Table 7.5.

per $10^3$ mol. wt. (calculated from compositions of cytochrome c, myoglobin, α chymotrypsinogen and lysozyme; Sober, 1970). This gave a conversion factor of 2.5 (Table 7.7). HA-2 appeared to contain at least one intrachain and no interchain disulphide bonds and a conversion factor based on a content of two half-cystines per subunit of HA-2 was calculated from the mole percent value for cysteic acid (Table 7.5).

These calculations are shown in Tables 7.5 and 7.7. Also shown is the calculation of subunit molecular weights from apparent amino acid composition. The molecular weights obtained for the subunits of HA-2 (30,391.02) and HA-1 (28,292) are very close to the values derived from SDS-PAGE.

These values are probably an underestimate since tryptophan was not included in the calculations. The partial specific volumes calculated by summing the contribution of each amino acid (Tables 7.5 and 7.7) are 0.731 ml/g for HA-2 and 0.725 ml/g for HA-1. Since the partial specific volumes of the amino acids are reasonably similar, these values should not be significantly prejudiced by not including the contribution of tryptophan in the calculations.

## 7.8 Discussion

A major aim of the work presented in this chapter was to obtain estimates of the native mol. wts. of HA-1, HA-2 and

LBP-3. This was accomplished by calculating the mol. wts. from estimates of Stoke's radius and sedimentation coefficient. The reliability of the mol. wt. estimates depends on the accuracy and validity of the methods used to estimate these parameters. Although it is clear that the behaviour of macromolecules during gel chromatography is dependent upon their effective hydrodynamic size, it is by no means clear which theoretical model best describes the hydrodynamic particle. Early work suggested that the Stoke's radius best defined the radius of the hydrodynamic particle but more recent reports (Le Maire, Rivas and Moller, 1980; Nozaki, Schechter, Reynolds and Tanford, 1976) using Sepharose and Sephacryl indicate that this does not adequately describe the behaviour of macromolecules, especially non-globular proteins. However, the linear relationship between elution behaviour and Stoke's radius still appears to be valid for chromatography in Sephadex G-200. For example, Siegel and Monty (1966) tested a wide variety of proteins and found no deviations. Especially relevant in their work is the "correct" behaviour of the very aglobular protein fibrinogen ($f/f_o$ = 2.34). This is one of the molecules reported (Le Maire *et al.*, 1980; Nozaki *et al.*, 1976) to show large deviations from expected behaviour. Further support is provided by the behaviour of catalase and mouse IgG in the work reported here. Catalase is a globular protein ($f/f_o$ = 1.25) with a molecule weight of 247,500 and a Stoke's radius of 5.2 nm (Sober, 1970). The physical parameters of mouse IgG have not been reported but by comparison with human IgG ($f/f_o$ = 1.38; Stoke's radius = 5.4 nm, molecular weight = 160,000; Sobers, 1970) it can be expected

to be a slightly aglobular protein with a Stoke's radius of 5.0 - 5.4 nm. Catalase and IgG are quite dissimilar proteins, yet their behaviour during Sephadex G-200 chromatography was identical. This result appears to be only consistent with the hypothesis that hydrodynamic behaviour in Sephadex G-200 is dependent on Stoke's radius. These points lend confidence as to the validity of the Stoke's radii reported here.

The procedures used to construct isokinetic sucrose gradients were theoretically derived by assuming that all particles analysed on the gradients would be spherical and have the same densities. These conditions are approximately true for HA-2 and BSA but not so for HA-1, which is certainly not globular. However, in practice the results obtained appear to be quite insensitive to these parameters. For instance, mouse ribosomal RNA behaves correctly on isokinetic gradients (Noll, 1967) as does human IgG (Johns and Stanworth, 1976). Noll, (1967) has also shown that fairly large deviations in particle density from the value for which the gradient was constructed are needed in order to cause significant deviations from constant velocity behaviour. The values derived for the sedimentation constants in this work should therefore be quite accurate.

All the macromolecules, HA-1, HA-2 and LBP-3 are multimeric proteins and could be disassociated under denaturing conditions into smaller polypeptide components. HA-2 could be disassociated in non-reducing conditions but HA-1 and LBP-3 needed to be reduced. In non-reducing conditions the HA-2 disassociated into two polypeptide bands with similar mol. wts. of 38,900 and 35,500 respectively. In contrast, in

reducing conditions only a single polypeptide band could be seen and this had an apparent mol. wt. of 33,400. When reduced and unreduced samples were run in the same gel (Fig. 7.4), the single band from the reduced sample corresponded in position with the larger band from the unreduced sample. This result indicates that the HA-2 subunit contains an intrachain disulphide bond. Apparently, the presence of this bond restricts the extent of unfolding of the polypeptide during SDS-PAGE in non-reducing conditions (Fish, 1975) thereby causing the polypeptide to migrate faster. It seems that the unreduced sample was composed of a small proportion of subunits whose disulphide bonds had been broken. The data shows that HA-2 (apparent mol. wt. = 65,000) is a dimer composed of non-covalently linked identical subunits of mol. wt. 33,400 (total mol. wt. = 66,800). The results for HA-1 and LBP-3 are more complex. The data presented here suggest that the HA-1 is composed of two different subunits which differ only slightly in apparent mol. wt. However, recent work (P.L. Ey, personal communication) indicates that HA-1 can be separated by ion exchange chromatography into two groups of molecules which are composed of either one or the other of these subunits. This may explain why the major HA-1 component seemed to be composed of two overlapping bands in non-reduced SDS-PAGE gels (Fig. 7.4). It appears, then, that individual HA-1 molecules (apparent mol. wt. = 152,000) are composed of covalently linked subunits of one type only (apparent mol.wt. = 29,600 or 28,200) and are probably pentamers (total mol. wt. = 141,000-148,000), or possibly hexamers (total mol.wt. = 169,200-177,600). LBP-3 can similarly be separated by ion exchange chromatography (P.L. Ey, personal

communication) into two groups of molecules composed of subunit 2 and either one or the other of the 1a and 1b subunits. In SDS-PAGE gels, subunit 2 stains approximately 1.3 - 1.6 as intensely as the 1a plus 1b subunits (e.g. Fig. 7.11). Assuming that dye binding had not reached saturation (as seems to be the case, see Fig. 7.11) and was quantitatively the same for all subunits, then it appears that the mole ratio of the subunits in the native molecule is approximately 1.5-2 : 1. These results indicate that individual LBP-3 molecules (apparent mol. wt. = 140,000 - 160,000) are polymers of covalently linked subunits, comprising no more than two of either the 1a (apparent mol. wt. = 28,200) or 1b (apparent mol. wt. = 29,600) subunit and three, or probably four, of subunit 2 (apparent mol. wt. = 23,600) yielding an overall total mol. wt. of 127,200 - 130,000 or 150,800 - 153,600 respectively. These conclusions are summarised in Table 7.8.

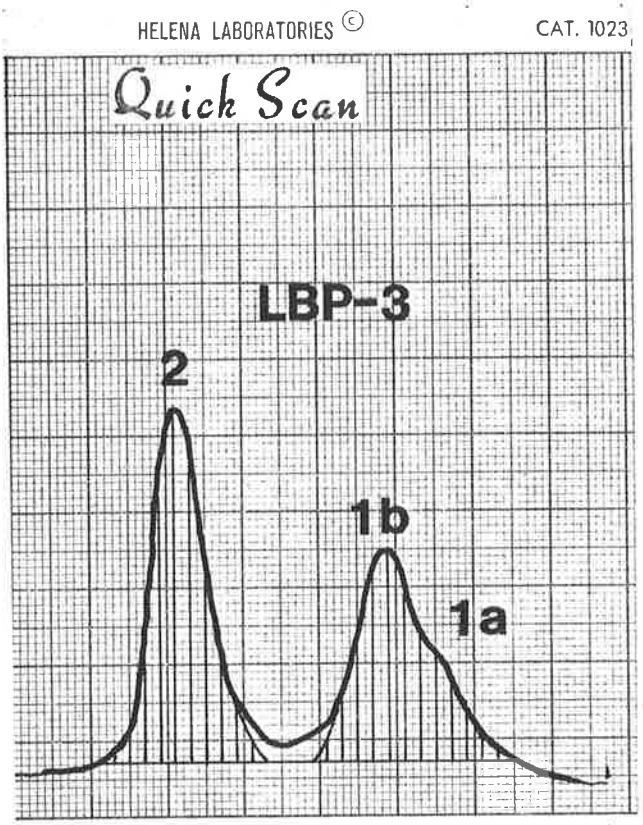
When analysed by SDS-PAGE in non-reducing conditions both the HA-1 and LBP-3 preparations appeared to contain minor amounts of proteins other than intact HA-1 or LBP-3. However, analysis by 2-dimensional SDS-PAGE showed that all these proteins were in each case composed of the same subunits as HA-1 or LBP-3. An exception was a minor component of the HA-1 preparation which appeared to be a contaminant. The SDS-PAGE results indicated a wide range of apparent mol. wts. for the unreduced proteins, yet all of these were derived from homogeneous preparations of HA-1 and LBP-3 purified by gel chromatography under native conditions. It would therefore appear that the variety of bands apparent in the SDS-PAGE gels represented incompletely disulphide-bonded forms of HA-1 and

FIGURE 7.11

Estimation of the subunit ratio within LBP-3. Various quantities (0.96, 1.35, 1.75, 2.62 ug) of purified LBP-3 were subjected to SDS-PAGE in reducing conditions (13% gel). The gel slab was stained in the normal manner and each sample track was then scanned twice in a Quick Scan Jr scanner (Helena Laboratories Corp., U.S.A.). The scan shown in (A) was obtained using 1.75 ug of LBP-3. To measure the amount of dye bound by the subunits, the peaks were cut out (as indicated by the shaded areas) and weighed. Since the 1a and 1b subunits were not resolved, these were treated as one peak. The total weight of the peaks from the two scans was plotted against amount of LBP-3 loaded onto the gel, as shown in (B) (▼——▼, LBP-3/2 ;●——●, LBP-3/1a + 1b). These plots are approximately linear, indicating that the amount of dye bound was proportional to the weight of protein present. Accordingly the weight ratio of the subunits was determined by calculating the ratio of the peak weight of the LBP-3/2 subunit compared to that of the LBP-3/1a + 1b subunits for each protein load (values shown in parentheses).

As the scans recorded no trough between the visually distinct, closely-spaced 1a and 1b bands (see Fig. 7.5) the estimates of the amount of dye bound by these subunits are probably in excess. Therefore, the calculated subunit ratios represent a minimum estimate of the actual value.

A


HELENA LABORATORIES ©
CAT. 1023
Quick Scan
LBP-3
2
1b
1a

B

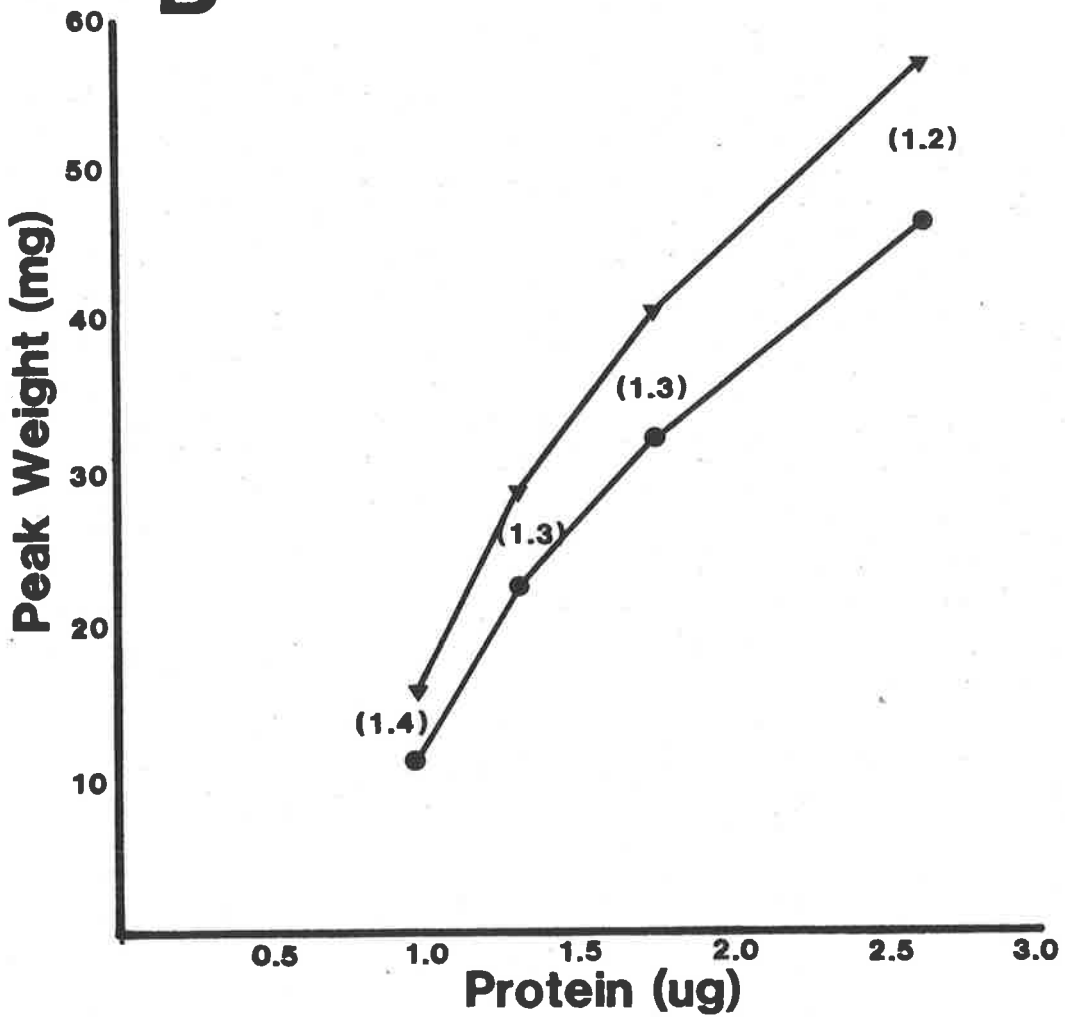

Peak Weight (mg)
60
50
40
30
20
10
0.5
1.0
1.5
2.0
2.5
3.0
Protein (ug)
(1.2)
(1.3)
(1.3)
(1.4)

TABLE 7.8

Summary of molecular weight data for HA-1, HA-2 and LBP-3

| Protein | Molecular weight | | | Probable structure |
|---|---|---|---|---|
| | Native | Denatured | | |
| | | Unreduced | Reduced | |
| HA-2 | 65,000* | 35,500 | 33,400 | DIMER |
| HA-1 | 152,000§ | 147,000 | 29,600 (1a)<br>28,200 (1b) | PENTAMER¶<br>$(1a)_5 + (1b)_5$ |
| LBP-3 | 140,000+<br>- 160,000 | 147,000 | 29,600 (1a)<br>28,200 (1b)<br>23,600 (2) | HEXAMER¶<br>$(1a)_2(2)_4 +$<br>$(1b)_2(2)_4$ |

* Based on $\bar{v}$ of 0.731 derived from amino acid composition (Table 7.5).

§ Based on $\bar{v}$ of 0.725 derived from amino acid composition (Table 7.7).

+ Approximation; see Table 7.2.

¶ Derived as explained in the text.

LBP-3 which became partially disassociated in the denaturing conditions of the gels.

The 2-dimensional SDS-PAGE analyses of HA-1 revealed the presence of an additional smaller polypeptide which had not been previously detected. This appeared not to be the result of the labelling procedure since the extra band could also be visualised by normal staining if a sufficient amount of protein was applied to the gels (results not shown). This polypeptide component was larger than subunit 2 of LBP-3 and consequently its presence could not be attributed to contamination with LBP-3. An attempt was made to estimate from its staining intensity (as in Fig. 7.11) the amount of this component present, but this was difficult since the amount of LBP-3 loaded in order to visualise the band resulted in the overloading of the 1a and 1b subunits. However, it appeared that this component represented about 5%-10% of the total protein. If one assumed that this component was in fact a subunit of HA-1 present as one unit per molecule (i.e., c.a. 25,000 per 152,000), then it would have to represent at least 15% of the total protein. Thus it seems that this component probably represents either a contaminant or a degradation product of the 1a or 1b subunit, although, given the uncertainty of the protein estimates, it is possible that it may be a subunit of the HA-1.

The apparent mol. wts. of the 1a and 1b subunits of HA-1 were identical to those of the 1a and 1b subunits of LBP-3. In addition, immunochemical analyses showed that most of the antigenic determinants of HA-1 were shared with LBP-3. Although both proteins possessed unique determinants, LBP-3 appeared to possess a much larger number than HA-1.

Considered together, these results lead to the conclusion that the 1a and 1b subunits of HA-1 and LBP-3 are equivalent, or at least very similar, molecules. It appears likely that the extra antigenicity of LBP-3 is due to its content of subunit 2. Since the binding site activity of HA-1 and LBP-3 appear to be identical, it is almost certain that this site is situated on the 1a/1b subunit(s). On this basis the LBP-3 estimated to contain two 1a/1b subunits, should be a divalent molecule. Recent work (P.L. Ey, personal communication) showing that the LBP-3 is in fact an extremely inefficient haemagglutinin for guinea pig erythrocytes supports this conclusion. There is no reason at the present time to suppose that subunit 2 has carbohydrate binding activity.

The abundance of hydroxyamino acids and acidic acids and the paucity of sulphur containing amino acids found in HA-2 is also characteristic of the amino acid composition of many other invertebrate (Ey and Jenkin, 1982) and plant lectins (Sharon and Lis, 1972; Lotan, Skutelsky, Danon and Sharon, 1975). In contrast, HA-1 and LBP-3 are rich in sulphur containing amino acids, especially cysteines, as are a number of other invertebrate haemagglutinins (Ey and Jenkin, 1982). The overall composition of HA-2 is quite dissimilar to that of LBP-3 and HA-1.

The LBP-3 appears to be somewhat unique in its subunit composition. A number of invertebrate (Ey and Jenkin, 1982; Bishayee and Dorai, 1980) and plant lectins have also been reported to be composed of non-identical subunits, but where

these have been fully characterised (Felsted, Egorin, Leavitt and Bachur, 1977; Leavitt, Felsted and Bachur, 1977; Murphy and Goldstein, 1979) all the subunits have been shown to possess carbohydrate binding sites. In addition, the arrangement of the subunits in these molecules appears to be random, resulting in the generation of a number of different species of the native protein. In contrast, LBP-3 appears to contain a subunit (LBP-3/2) lacking a binding site for lactose, and the association of the two would appear to be non-random since there is no detectable heterogeneity in the composition or size of the native molecule.

## 7.9 Summary

1) Using a technique which takes into account the possible non-globular nature of proteins, HA-1 was judged to be a non-globular protein ($f/f_o$ = 1.8) with a mol. wt. of 152,000 and HA-2 was judged to be a globular protein ($f/f_o$ = 1.34) with a mol. wt. of 65,000. LBP-3 was judged to be a slightly aglobular protein with a mol. wt. of approximately 140,000 - 160,000.

2) SDS-PAGE analyses indicated that HA-1 molecules were composed of two types of subunits (HA-1/1a, mol. wt. = 28,200; HA-1/1b, mol. wt. = 29,600), LBP-3 molecules of three types of subunits (LBP-3/1a, mol. wt. = 28,200; LBP-3/1b, mol. wt. = 29,600; LBP-3/2, mol. wt. = 23,600) and HA-2 molecules of one type of subunit (mol. wt. = 33,400). These results also showed that the subunits in native HA-1 and LBP-3 molecules were held together by covalent bonding whereas those in HA-2 molecules were held together solely by non-covalent bonding. It was estimated that native LBP-3 molecules

contained approximately twice as many LBP-3/2 subunits as LBP-3/1a plus LBP-3/1b subunits.

3) SDS-PAGE and immunochemical analyses of HA-1 and LBP-3 indicated that the HA-1/1a,1b subunits were identical to the LBP-3/1a,1b subunits. Also, even though LBP-3 did not appear to be a haemagglutinin, adsorption experiments showed that LBP-3, like HA-1, bound specifically to guinea pig erythrocytes and that this binding could be inhibited by lactose and EGTA. Therefore, it was concluded that the lactose binding sites of LBP-3 resided on the 1a,1b subunits.

4) The amino acid compositions of HA-1, HA-2 and LBP-3 were determined.

# CHAPTER 8

# GENERAL DISCUSSION

In this thesis the purification and physicochemical characterization of the haemagglutinins of *B. leachii* haemolymph is reported. The possibility that invertebrate haemagglutinins may function as opsonins has been discussed (Chapter 1, McKay and Jenkin, 1970; Tyson, 1974; Hall and Rowlands, 1974b). However, the evidence for this hypothesis is almost wholly circumstantial, the exception being the demonstration in the snail *H. pomatia* that the opsonic and haemagglutinating activities can be inhibited by the same sugar (Renwrantz, 1979; Harm and Renwrantz, 1980). The current work was undertaken as part of a study aimed at gaining definitive data on the structure and role of invertebrate haemagglutinins, including any possible involvement in nonself recognition. *Botrylloides leachii* was chosen for study since its haemolymph contained high levels of haemagglutinating activity for sheep and other erythrocytes and because preliminary experiments, in which sheep erythrocytes injected into the circulation of *B. leachii* were seen to be phagocytosed by circulating haemocytes, were consistent with the possibility that the haemagglutinins function as opsonins (D.R. Coombe, personal communication).

There is a lack of published information on protochordate immunity, particularly with regard to the means of self-nonself recognition. A number of haemagglutinins have been detected in the haemolymph of a variety of protochordates, but only one has been purified and partially characterized (Form *et al.*, 1977). It was not mentioned whether or not this molecule was tested for opsonic activity. Because the protochordates are considered

to be the survivors of the original stock of animals from which the chordates evolved (Berrill, 1955), there was a possibility, if protochordates utilise recognition molecules in their defense, that these molecules might bear some evolutionary relationship to vertebrate immunoglobulins. It was in this context that the work for this thesis was begun.

Two haemagglutinins, HA-1 and HA-2, were isolated from *B. leachii* haemolymph. A third protein, LBP-3, which exhibited the same specificity as HA-1 but lacked haemagglutinating activity, was also isolated. All these molecules were purified using an affinity column made from acid-treated Sepharose containing conjugated lactose, followed by gel filtration chromatography.

During initial investigations of the physicochemical properties of HA-1, it was found that this molecule eluted before IgG during gel chromatography but sedimented more slowly than IgG during velocity ultracentrifugation. The most likely explanation of this behaviour, considering the mechanisms involved in the separation of molecules by these methods, was that HA-1 was a non-globular protein. This necessitated finding a protocol whereby the non-globular nature of proteins could be taken into account when making estimates of mol. wts. This was accomplished by combining the results from both gel chromatography and sedimentation velocity experiments. Thus the mol. wt. of native HA-1 was estimated to be 152,000. The calculated frictional ratio ($f/f_o$ = 1.8) supported the idea that this molecule was non-globular. An alternative explanation for the behaviour of HA-1 was that the molecule had an

unusually low partial specific volume, but this did not appear to be true since the partial specific volume estimated from the amino acid composition of HA-1 (0.725 ml8g) was normal. Moreover, there was close agreement between the mol. wts. estimated using gel filtration and sedimentation velocity and those estimated using SDS-PAGE in non-reducing conditions. In contrast, HA-2 appeared to be a globular protein ($f/f_o$ = 1.34) with a native mol. wt. of 65,000. The mol. wt. of native LBP-3 could not be determined using the protocol described above, since its sedimentation coefficient was not determined. However, by comparison with HA-1 and other markers during gel chromatography and SDS-PAGE (non-reducing), LBP-3 appeared to be a slightly aglobular protein with a mol. wt. of 140,000 - 160,000.

The subunit composition of these molecules was investigated by SDS-PAGE. It appeared that LBP-3 was composed of three types of subunits, LBP-3/1a (apparent mol. wt. = 29,600), LBP-3/1b (apparent mol. wt. = 28,200) and LBP-3/2 (apparent mol. wt. = 23,600). HA-1 contained two types of subunits, HA-1/1a (apparent mol. wt. = 29,600) and HA-1/1b (apparent mol. wt. = 28,200) whilst HA-2 consisted of one type of subunit (apparent mol. wt. = 33,400). The staining intensities of the subunit bands indicated that the native LBP-3 molecules contained approximately twice as many LBP-3/2 subunits as LBP-3/(1a + 1b) subunits. Considering these results in conjunction with those of Ey (personal communication, see p. 153), which indicated that individual HA-1 and LBP-3 molecules are composed of either one or the other of the 1a or 1b sub-

units, it was concluded that HA-1 molecules are pentamers composed of covalently linked identical subunits and LBP-3 molecules are covalently linked hexamers comprising four LBP-3/2 subunits and no more than two LBP-3/1a or 1b subunits (see Figure 8.1). HA-2 molecules appeared to be dimers composed of non-covalently linked identical subunits.

It is possible that the 1a and 1b subunits of HA-1 and LBP-3 are in fact identical polypeptide chains differing in their degree of glycosylation. The presence of carbohydrates on proteins is known to affect the binding of sodium dodecylsulphate and the consequent abnormal behaviour of glycoproteins during SDS-PAGE can lead to large errors in the estimates of polypeptide mol. wts. (Fish, 1975). Alternatively, the two subunits may be different polypeptides with the 1a subunit containing about 10 more amino acids than the 1b subunit. Initial investigations of the carbohydrate content of the subunits by periodic-acid-Schiff staining of SDS-PAGE gels (results not known) were inconclusive since non-glycoproteins (e.g., BSA) gave significant positive responses. This is an important point concerning the structure of HA-1 and LBP-3 molecules. It would be best resolved by separately isolating sufficient quantities of the subunits and determining the amino acid and carbohydrate compositions of each. As the subunits are similar in size, their separation may be best achieved using methods based on native change, e.g., PAGE, ion exchange or isoelectric focussing (in the presence of urea if denaturing conditions are necessary). The degree to which denaturing conditions would need to be used will

FIGURE 8.1

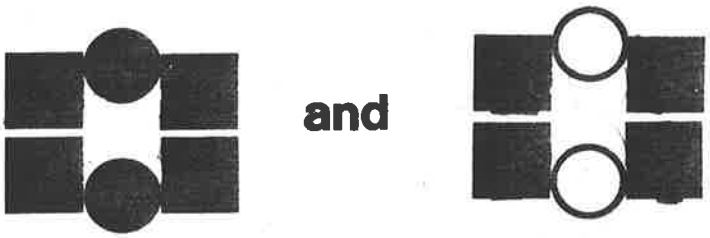
Diagram showing the proposed subunit structures of HA-1 and LBP-3.

● HA-1/1a: LBP-3/1a

○ HA-1/1b: LBP-3/1b

■ LBP-3/2


**HA-1**




**LBP-3**

depend on the lability of the interchain disulphide bonds in the native molecules and the strength of non-covalent bonding between the subunits. Obviously, if one was interested in maintaining the antigenicity of the subunits it would be desirable to keep the use of denaturing conditions to a minimum during purification.

LBP-3 shares several features with HA-1. The binding sites of the molecules appear to be identical, being specific for guinea pig erythrocytes and inhibited by lactose and requiring the presence of $Ca^{++}$ ions for activity. In addition, the results of SDS-PAGE and immunochemical analyses of these proteins indicate that the HA-1/1a and 1b subunits may be identical to the LBP-3/1a and 1b subunits. Since the HA-1 molecule lacks any additional subunits it seems reasonable to conclude that the carbohydrate binding site of LBP-3 is situated on the 1a/1b subunits. There are no data to suggest that the LBP-3/2 subunit possesses a carbohydrate binding site.

As discussed in Chapter 3, a comparison of ascidian haemagglutinins has not been possible due to a lack of physicochemical data. Apart from the *B. leachii* haemagglutinins, the only ascidian haemagglutinin to have been purified is that from *H. pyriformis*. Although the *H. pyriformis* haemagglutinin requires $Ca^{++}$ ions for activity (Anderson and Good, 1975), it appears to be unrelated to the *B. leachii* haemagglutinins since it has a mol. wt. of 800,000, is composed of 20,000 mol. wt. subunits (Form *et al.*, 1977) and is specific for sialic acid (Anderson and Good, 1975). The haemagglutinins from *B. schlosseri*, an ascidian closely related to *B. leachii*,

were also partially characterized in this thesis. A haemagglutinin was identified that appeared to be very similar to HA-1, since it only agglutinated guinea pig erythrocytes, was inhibited by the same sugars and it required $Ca^{++}$ ions for activity. Also, the results of gel filtration, sedimentation velocity ultracentrifugation and SDS-PAGE indicated that the size of this haemagglutinin was very similar if not identical to that of the HA-1.

A major aim of the study of the *B. leachii* haemagglutinins is to determine whether they play a role in non-self recognition. In this regard, results of some preliminary experiments have suggested that HA-2 may be opsonic (D.R. Coombe, personal communication). When washed *B. leachii* haemocytes and sheep erythrocytes were incubated together in the absence of haemolymph, a significant degree of rosette formation followed by phagocytosis of the bound erythrocytes was seen. If lactose was included in the incubation medium the formation of rosettes was prevented. In addition to lactose, a polysaccharide fraction, obtained by phenol-water extraction of the Cohn fraction V of bovine serum (bovine serum albumin; Commonwealth Serum Laboratories, Melbourne, Australia), was also capable of inhibiting the binding of erythrocytes to haemocytes. This extract was prepared after the "albumin" (included in haemagglutination assay diluents as carrier protein) was found in my early experiments to inhibit the agglutination of several types of erythrocytes by *B. leachii* haemolymph (data not included in this thesis). Significantly, the extract was found to be a potent inhibitor of the HA-2 agglutinin but proved ineffective against the HA-1 agglutinin. Washed haemocytes

incubated with the rabbit anti-(HA-2) serum (Chapter 6, section 6.11) were strongly agglutinated (D.R. Coombe, personal communication), indicating that HA-2 molecules were present on the surface of many haemocytes. These results suggested indirectly that the cell-bound HA-2 molecules were involved in the attachment of erythrocytes to the haemocyte surface. Interestingly, prewashing the haemocytes with lactose (0.5 M) had no effect on the level of rosette formation subsequently obtained. This may mean that the lactose binding site plays no role in the attachment of HA-2 molecules to the haemocyte membrane. If this was so, binding might be mediated by another site similar, for instance, to the Fc site of cytophilic antibody, or the HA-2 molecules might be integral components of the haemocyte membrane. Alternatively, the HA-2 molecules may attach to the membrane through the carbohydrate binding site but with a very much greater binding affinity than that of lactose/HA-2 interactions. Thus, lactose would be very inefficient at eluting haemocyte bound HA-2 molecules.

When considering non-self recognition systems, it seems reasonable to propose *a priori* that in order to deal with the variety of foreign organisms an animal may encounter, a large number of recognition molecules (opsonins) with different specificities would be needed, as is the case with antibodies in the vertebrates. In the invertebrates, adsorption studies have shown that both the snail *H. aspersa* and the freshwater crayfish *C. destructor* possess a number of opsonins which have different specificities (Prowse and Tait, 1969; Tyson and Jenkin, 1974). Two models have been proposed to explain how opsonins exhibiting a range of specificities may be generated

in invertebrates. The model of Jenkin and Hardy (1975) is based on the apparent hexameric structure of the opsonins from *C. destructor*. These authors envisaged the random combination of six different subunits to form a variety of hexameric molecules in which the combination of e.g. subunits A and B generated a specificity different from that generated by the combination of e.g. subunits A and C. It was estimated that this type of system could yield several thousand specificities. There is some experimental evidence to support this hypothesis (Jenkin and Hardy, 1975).

In an alternative model, Parish (1977) suggested that nonself discrimination could be effected by a recognition system based on carbohydrate complementarity. Parish proposed that the recognition factors are formed by glycosyl transferase enzymes which the animal otherwise used to synthesize its own carbohydrate molecules. Recognition factors comprising different specificities could be formed by the random association of different enzymes into a polymer which included an additional protein recognized by an acceptor site on phagocyte surfaces. The incorporation of this protein would allow the recognition molecules to function as opsonins. Both these models propose that recognition molecules are formed by random association of different subunits. A precedence for such a system is provided by a number of examples of proteins which while not known to be involved in recognition phenomena, have been shown to be formed in such a manner (Felsted, Egorin, Leavitt and Bachur, 1977; Leavitt, Felsted and Bachur, 1977; Murphy and Goldstein, 1979; Murray and Jeffrey, 1974; Jeffrey, Shaw and Treacy, 1978).

None of the hypotheses outlined above appears to be

applicable to the *B. leachii* lactose binding proteins. Firstly, it is unlikely that subunits 1 and 2 of HA-1 and LBP-3 randomly associate since only two forms of the native molecules, i.e., HA-1 and LBP-3, are found in haemolymph. Furthermore, the results of Ey (see p. 153) suggest that not even the HA-1 or LBP-3 1a and 1b subunits will associate with each other although the existence of molecules containing either 1a or 1b subunits might be explained if the differences between these molecules arise by post-assembly and/or age-dependent modification. The major finding, however, which can be used to argue against the applicability of these models in this animal is that the HA-1, HA-2 and LBP-3 proteins do not appear to be comprised of populations of molecules having different specificities. On the contrary, each type of protein seems to be composed of molecules having only one type of binding site. Thus, even though HA-2 agglutinated all five types of erythrocytes tested, extensive cross-adsorption experiments and sugar inhibition experiments showed clearly that this activity was due to a single monospecific haemagglutinin. Furthermore, the lactose affinity column could adsorb all HA-2 molecules from haemolymph. Similarly, HA-1 and LBP-3 were specific for guinea pig erythrocytes and the affinity column adsorbed all of these molecules from haemolymph.

Given that each of the *B. leachii* lactose binding proteins are homogeneous in their binding specificities, we are faced with the question that if any of these molecules are opsonins, as may be the case for HA-2 at least, how might they be used in host defense against a wide diversity

of pathogenic organisms. One possibility is that they recognise carbohydrate moieties common to most potential pathogens. For instance, in the case of HA-2 the binding site may be relatively small and capable of recognising only the Gal-β1→4-Glu section of a polysaccharide. Accordingly, the type and configuration of other sugars which may be connected to this dissaccharide may have little influence on HA-2 binding. If this were the case and if the dissaccharide moiety Gal-β1→4-Glu is present in the carbohydrate of many marine micro-organisms, then HA-2 would bind to most of these organisms. Alternatively, if such opsonins were only required to recognise a limited number of different pathogens, extra protection could be supplied by other as yet undetected proteins having other specificities, or by a different type of recognition system not based on carbohydrate complimentarity. For instance, the amoeba *Dictyostelium discoideum* possesses two types of cell associated recognition sites mediating phagocytosis, one a lectin-like receptor which can be blocked by glucose and the other a "non-specific" receptor relying on hydrophobic interactions (Vogel, Thilo, Schwarz and Steinhart, 1980).

In the final part of this discussion I would like to consider whether the *B. leachii* lactose binding proteins might be related to vertebrate immunoglobulin. Vertebrate immunoglobulin chains can be divided into sections or domains which have a high degree of sequence homology with each other (Putnam, 1977a). This observation has led to the proposal that immunoglobulins may have evolved from a single gene by a series of gene duplications (Putnam,

1977b; Nisonoff, Hopper and Spring, 1975a). Studies of cloned immunoglobulin genes which show that the regions of DNA coding for each domain are separated from each other by non-coding intervening regions support this hypothesis (Molgaard, 1980). An early form of the immunoglobulin "chain", expressed prior to the acquisition of a V-gene pool, may have been structurally similar to present day light chains, being the result of a single gene duplication, but possessing an invariant "variable" region. Studies of vertebrate immunoglobulins indicate that the non-covalent bonding holding the chains together is strong and the secondary structure of immunoglobulin molecules remains essentially unchanged when the inter-chain disulphide bonds are broken (Nisonoff *et al.*, 1975b). Purified light and heavy chains of rabbit IgG have a strong tendency to self-associate non-covalently upon renaturation, 35-45% of the light chains forming dimers ($f/f_o = 1.35$) and the rest remaining in monomeric form (Björk and Tanford, 1971). Furthermore, the immunoglobulin from the lamprey lacks interchain disulphide bonds (Marchalonis and Edelman, 1967) and the earliest forms of immuno-globulin may also have lacked interchain disulphide bonds. In view of these findings one could argue that any "early" immunoglobulins would probably have occurred as dimers whose chains were held together by strong non-covalent forces. Clearly, the dimeric HA-2 molecule, consisting of two polypeptide chains with mol. wts. slightly larger than an immunoglobulin light chain, is very similar to this postulated precursor. However, a characteristic invariant feature of vertebrate immunoglobulin molecules

is that the core of each domain is formed by some 60 amino acid residues enclosed within an intrachain disulphide loop (Putnam, 1977a). It seems highly likely that any primoidal immunoglobulin must have possessed this feature. The HA-2 molecule has only one intrachain disulphide loop per subunit and on this basis it would appear that this molecule is probably unrelated to immunoglobulin. The non-globular nature of LBP-3 and especially HA-1 and the requirement of $Ca^{++}$ ions for their binding activity makes it highly unlikely that either of these proteins has any relationship to vertebrate immunoglobulins.

The degree of similarity between the *B. leachii* lactose binding proteins and immunoglobulin has also been investigated by examination of their amino acid compositions. A number of methods of assessing the relatedness of proteins from amino acid compositions have been published (Metzger, Shapiro, Mosimann and Vinton, 1968; Marchalonis and Weltman, 1971; Shapiro, 1971) and it appears that they all give essentially the same results (Osmand, 1972). The method used here is the one described by Marchalonis and Weltman (1971). In this method the parameter

$$S\Delta Q = \sum_{i=1}^{20} (x_{ij} - x_{ik})^2,$$

where $x_i$ is the mole percent of amino acid i in the two proteins j and k being compared, is used as an estimate of compositional relatedness. Marchalonis and Weltman (1971) found a good correlation between $S\Delta Q$ values and comparisons based on amino acid sequences, and in a survey of a large number of proteins regarded as unrelated showed that only

2% of comparisons differed by less than 100 SΔQ units. Table 8.1 shows the results of comparisons of HA-1, HA-2, LBP-3 and the heavy and light chains of human IgM and lamprey immunoglobulin.

A number of points emerge from inspection of this table. The SΔQ for HA-1 and LBP-3 is very low. One would expect to find some degree of compositional similarity between these proteins since subunit one comprises about 37% by weight of LBP-3 and this subunit is thought to be identical for both proteins (see earlier). The low SΔQ value found, however, indicates that the LBP-3/1a,1b and LBP-3/2 subunits must also have similar amino acid compositions. LBP-3 and HA-1 are clearly unrelated to HA-2 and none of the *B. leachii* proteins are related to human IgM. In contrast, the SΔQ values for comparisons of HA-1, HA-2 and LBP-3 with lamprey L chain and, in particular, lamprey heavy chain are less than 100 and of the same order of magnitude as the difference between human μ chain and lamprey L chain or human μ and human α or γ chain (Marchalonis and Weltman, 1971), relatively conserved polypeptides which would be expected to be distantly related. The lamprey is a very primitive vertebrate and these results could be taken to indicate a relationship between the *B. leachii* proteins and phylogenetically early forms of immunoglobulin. However, the *B. leachii* and lamprey proteins are "serum" components and in order to comply with certain limitations imposed by solubility in that medium may have relatively high proportions of hydrophylic amino acids which could bias the SΔQ values. Based on this premise, Osmand (1972) compared unrelated human plasma

TABLE 8.1

Values of SΔQ for comparisons of HA-1, HA-2, LBP-3 and immunoglobulins

| | HA-1 | LBP-3 | HA-2 | Human IgM* | | Lamprey§ | |
|---|---|---|---|---|---|---|---|
| | | | | L | H | L | H |
| HA-1 | 0 | | | | | | |
| LBP-3 | 12.3 | 0 | | | | | |
| HA-2 | 151.3 | 123.2 | 0 | | | | |
| IgM H | 131.9 | 102.8 | 171.5 | 0 | | | |
| IgM L | 124.8 | 91.2 | 104.6 | 26.6 | 0 | | |
| Lamprey H | 63.6 | 50.1 | 112.1 | 91.2 | 45.8 | 0 | |
| Lamprey L | 64.7 | 50.1 | 71.1 | 74.4 | 31 | 8.3 | 0 |

* Composition from Cohen and Porter (1964).

§ Composition from Marchalonis and Edelman (1968a).

proteins and found that 18% of comparisons had SΔQ values of 60 or less. Thus the significance of the low SΔQ values is questionable.

It is not possible to state conclusively from the data presently available whether or not the *B. leachii* lactose binding proteins are related to vertebrate immunoglobulins, though on a structural comparison it seems more likely that they are not. However, an unequivocal assessment of the relatedness of proteins can only be gained from analyses based on amino acid sequence data.

The results of this thesis provide a basis for further more detailed studies on the structure of the *B. leachii* lactose binding proteins and also on the role(s) which they may play in the animal.

# APPENDIX

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

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