RECOGNITION OF FOREIGN PARTICLES

BY HAEMOCYTES FROM THE

CRAYFISH, (Parachaeraps bicarinatus)

Christopher J. Tyson, B.Sc.Hons.(Adelaide)

A thesis submitted for the degree of Doctor of Philosophy

Department of Microbiology,
The University of Adelaide,
Adelaide,
South Australia.

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An investigation has been carried out on the factors involved in the recognition and uptake of bacteria by crayfish haemocytes.

Initial studies indicated that $^{32}$P-labelled bacteria were eliminated from the circulation of crayfish at different rates, depending upon the species of bacteria studied. Further investigations revealed that this removal could be depressed temporarily by a large blockading dose of the same organism. Such blockade could be reversed however by opsonisation of the challenge dose of bacteria with serum which suggested that blockade involved a depletion in serum opsonins.

Further confirmation of these findings came from studies which showed that the circulating haemocytes from the crayfish when cultured in vitro phagocytosed a number of different strains of bacteria. The ability of these cells to take bacteria could be depressed by treatment with trypsin. This state of depression could be reversed however by either incubating the trypsin-treated haemocytes in crayfish serum or opsonising the bacteria with the same. Data presented suggests that the recognition factors associated with the membrane of the phagocyte are similar to those circulating in the haemolymph.
Purification of the recognition factors for bacteria and erythrocytes by affinity chromatography and gel filtration revealed that they were an antigenically related group of molecules. Further studies demonstrated that the opsonins for bacteria and sheep erythrocytes had a molwt. of 81,000 daltons and consisted of 6 sub-units of 13,500 mol. wt.

Investigations were carried out also on the cytotoxic action of crayfish haemocytes for Ehrlich and Krebs ascites tumour cells. Such cytotoxicity was found to be dependent on cell associated recognition factors which were labile to trypsin.

The significance of these findings in relation to the recognition of foreignness by phagocytic cells from invertebrates is discussed.
This thesis contains no material previously submitted by me for a degree in any University, and to the best of my knowledge and belief it contains no material previously published or written by another person except where reference is made in the text.

CHRISTOPHER J. TYSON

June, 1974.
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INTRODUCTION

The recognition of foreign particles by phagocytic cells from the vertebrates has been shown to depend on the particles having first reacted with specific proteins known as the immunoglobulins. In the absence of these molecules recognition takes place slowly if at all.

In comparison little work has been carried out on the recognition of foreignness by phagocytic cells from the invertebrates. Research in this field has revealed however that phagocytic cells from a number of different invertebrates are capable of phagocytosing erythrocytes from various species of vertebrates provided they have been pretreated with haemolymph. It is apparent from these studies that there are recognition factors in the sera, which may be functionally equivalent to the immunoglobulins of vertebrates.

The aim of the present study was to investigate quantitatively the uptake of a number of different strains of bacteria by phagocytic cells from the crayfish, (Parachaeraps bicarinatus), and to purify these opsonins and characterise if possible these recognition factors.
CHAPTER I

PHAGOCYTOSIS IN THE VERTEBRATES

Considerable work over the last seventy years has established that the recognition of foreign material by phagocytic cells from vertebrates depends on the foreign material having first reacted with antibody. Such an understanding developed from the very early studies carried out near the end of the last century by Emil Metchnikoff, 1891 and his collaborators.

Metchnikoff was interested in the mobile phagocytic cells found in the tissues of many invertebrates. He found that rose slivers inserted into the body cavity of starfish were soon surrounded and encapsulated, by a mass of wandering cells. He observed also that the mobile cells of the water flea, (Daphnia magna) played an important role in host resistance to the fungus, (Monospora bicuspídata) by ingesting the fungal spores. In a few cases where the cells of the host failed to ingest these spores, they germinated, grew up and killed the host. Extending this work Metchnikoff and his collaborators found that certain species of insects rich in leucocytes were resistant to infection by various strains of bacteria even though large numbers of bacteria were injected into them. By contrast other species deficient in leucocytes were extremely susceptible to infection by these same bacteria.
From these studies Metchnikoff proposed that phagocytes were important in the elimination of foreign material and in defense against infection by various organisms.

However during the same period von Behring and Kitasato, 1890, showed that serum taken from animals which had received repeated injections of non-lethal doses of tetanus toxin, or of diphtheria toxin, had acquired the property of specifically neutralizing these toxins, and thus preventing their toxic effects. They regarded these anti-toxins in the blood of protected animals as being the major factors in defense against infection and they and others opposed strongly Metchnikoff's hypothesis.

These opposite points of view were resolved largely through the work of Wright and Douglas, 1903. They incubated white blood cells in thin capillary tubes with *Staphylococcus aureus* in the presence of serum or plasma. A cell smear was made after fifteen minutes and the number of bacteria ingested by the phagocytes was scored. Significant phagocytosis of *Staphylococcus aureus* by polymorphonuclear cells only occurred in the presence of serum or plasma. In the absence of serum or with heated serum (60°C for 15 minutes) little ingestion of the bacteria occurred. Wright spoke of this action of serum as an "opsonic" effect (opsono - I cater for; I prepare victuals for), and employed the term "opsonins" to designate these elements in serum or plasma which promoted phagocytosis. Sera from patients
immunized with sterilized cultures of various bacteria were found to have a greater opsonic effect against those strains than sera from normal patients. Both in vitro tests were carried out with polymorphonuclear cells from normal donors. The term "opsonic-index" referred to the phagocytic power of the blood from immunized patients as compared with normal patients and was determined by averaging the number of opsonized bacteria taken up by 20-30 polymorphonuclear cells. Wright proposed that this index was a useful means of follow-ing the course of an infective process in a truly quantitative way.

More recent research has established that these opsonins are in fact specific antibodies. Benacerraf, Sebestyen and Schlossman, 1959, found that the rate of phagocytosis of Escherichia coli and Staphylococcus aureus by the phagocytic cells of the liver and spleen correlated with the levels of specific antibody against these bacteria. Similar work in vivo by Whitby and Rowley, 1959; Jenkin and Rowley, 1961, has confirmed the importance of specific antibody in the phagocytosis of bacteria. Investigations carried out in vitro with macrophages and polymorphonuclear cells have established the importance of antibody in promoting the phagocytosis of bacteria. Thus Staphylococcus aureus was ingested by rabbit polymorphonuclear cells and macrophages only if it had been pretreated with specific antibody (Cohn and Morse, 1959;
Mackaness, 1960). Similarly, ingestion of *Escherichia coli* and *Salmonella typhimurium C5* by mouse peritoneal macrophages depended also upon the presence of specific antibody (Rowley and Whitby, 1959; Jenkin and Benacerraf, 1960).

While many workers have established that the phagocytic cells of vertebrates are capable of ingesting non-living particles such as carbon, carmine and quartz until recently little work had been done on the role of serum opsonins in this uptake. Benacerraf, Halpern, Bizzii and Benos', 1954, found that an intravenous injection of a large dose of carbon (16 mg. per 100 g. body weight) into rats could depress temporarily the uptake of a second dose of carbon by cells of the reticulo-endothelial system. They suggested that this state of "blockade" represented saturation of the phagocytic cells by the first dose of carbon and that the return of normal granulopoietic activity was dependent on the formation of new phagocytic cells. However other work to be discussed below suggested that serum opsonins played an important role in the phagocytosis of carbon and other colloids by these same cells. These observations stemmed from the work of Penn, 1921, who had found that polymorphonuclear cells would ingest carbon and quartz particles only in the presence of serum. Carbon was found to be phagocytosed more rapidly than quartz and this was related to the greater adsorptive properties of carbon particles for serum proteins.
Jenkin and Rowley, 1961, and Murray, 1963, found that the blockade of the reticulo-endothelial system of mice by carbon particles could be reversed if the challenge dose of carbon had been pretreated with serum. They suggested that blockade of the reticulo-endothelial system involved a temporary depression of serum opsonins rather than a saturation of the phagocytic cell with particles.

More recently Pisano, Patterson and Di Luzio, 1968, have found that the recovery from blockade by colloids in rats can be inhibited with puromycin. Puromycin did not inhibit the uptake of colloid pretreated with serum by isolated slices of liver from rats blockaded with colloid. Therefore it is possible that in vivo this drug depressed the synthesis of circulating opsonin resulting in a prolongation of blockade.

At this stage there is still considerable controversy whether blockade is due to the saturation of the phagocytic cells of the reticulo-endothelial system or a temporary depression in circulating opsonin level. Indeed Jeunet and Good, 1969, have suggested that blockade might be a combination of both these factors.

Whilst these experiments demonstrate that antibody plays an important role in the recognition of foreign material by phagocytic cells they offer no explanation as to the mechanism which allows phagocytic cells to recognize the antigen/antibody complexes.
Berken and Benacerraf, 1966, demonstrated that sheep erythrocytes pretreated with antibody adhered to the membrane of guinea pig macrophages resulting in the formation of rosettes. Furthermore they found that treatment of these antibodies with pepsin or with reducing agents such as β-mercapto-ethanol followed by alkylation with iodoacetamide diminished considerably their ability to promote rosette formation. Such reagents are known to destroy the Fc fragment of 7S immunoglobulin molecules. Pepsin treatment leaves a 5S bivalent antibody fragment which can still agglutinate erythrocytes but is unable to promote their adherence to guinea pig macrophages. It appears therefore that sheep erythrocytes pretreated with 7S immunoglobulin bind to receptors on the surface of guinea pig macrophages through a site on the Fc fragment.

The estimated amount of antibody required to promote recognition of one bacterium by a phagocytic cell appears to be extremely small (Rowley and Turner, 1966). These workers calculated that only 8 molecules of 19S antibody and 2,200 molecules of 7S antibody were required. Such a figure for 7S antibody agreed with the estimates made by Jenkin and Jackson, 1970, using Salmonella typhimurium C5 and mouse peritoneal macrophages. Thus the concept that opsonization involves a change in surface charge due to the coating of the bacteria with a layer of antibody would seem invalid from this data since one bacterium has been estimated to be able to accommo-
date 10,000 19S molecules in a close packed monolayer over its surface (Rowley and Turner, 1966).

CONCLUSION

In the vertebrates the phagocytosis of both living and possibly non-living particles (such as carbon and quartz) by macrophages and polymorphonuclear cells depends upon the presence of opsonins. These opsonins have been shown to be immunoglobulin molecules and only a small number of these molecules per particle appear to be necessary to promote recognition and phagocytosis. These immunoglobulin molecules mediate the recognition of foreign particles by attaching to receptors on the surface of phagocytic cells, though a site on the Fc fragment. Information regarding the recognition of foreign material by phagocytic cells from invertebrates is less comprehensive. Only recently have opsonins been described in the haemolymph of different species of invertebrates and their mechanism of action has not been investigated thoroughly.

One of the main problems in considering the mechanisms involved in the recognition of foreign material by invertebrates is the great diversity of these animals. In any discussion on this topic this diversity should be remembered and it is possible that observations and conclusions drawn about one group may not necessarily be applicable to others.
PHAGOCYTOSIS IN THE INVERTEBRATES

In vivo studies on Phagocytosis

Metchnikoff, 1891, studied the cellular reactions of many different species from the major invertebrate phyla to both living and inert materials. He found that lesions made by cauterizing the caudal appendages of insects led to an accumulation of blood cells around the injured site. Furthermore when foreign bodies such as wood splinters were introduced into insect larvae or starfish great numbers of haemocytes accumulated around them. From these types of observations done on a very extensive scale Metchnikoff proposed that phagocytic cells were of extreme importance in the elimination of foreign material and host defense against parasites.

Some forty years later a series of observations were published on the cellular reactions of the earthworm, (Lumbricus terrestris), to the injection of foreign material (Cameron, 1932). When carbon, carmine or mammalian erythrocytes were introduced into the coelomic cavity they were ingested by either fixed or circulating coelomic corpuscles. "Hanging drops" of coelomic fluid were used to study in vitro phagocytosis of bacteria, spermatozoa and red cells. Spermatozoa extracted from Allolobophora longa and Octolasium cyaneum were ingested by coelomic corpuscles from Lumbricus terrestris. However these same cells were unable to recognize as foreign, spermatozoa taken from different individuals within the species.
Cameron, 1934, extended this work with a study on inflammation in the caterpillar, (Galleria mellonella). He described the rapid phagocytosis of Indian ink, carmine or colloidal iron particles by cells of the blood, fat body and pericardium. However there was a difference in the ability of the larvae to recognize different species of bacteria. Bacteria such as pneumococci and Staphylococcus aureus were actively phagocytosed and completely destroyed by cells of the host. In contrast bacteria such as Proteus vulgaris and Bacillus mycoides although actively taken up by phagocytic cells of the host subsequently grew up and killed the larvae in less than 24 h. These results indicated therefore that in many but not all cases the phagocytic cells were effective in defense against infection.

Over the last ten years interest has again been revived in the cellular reactions of invertebrates to foreign material. However the research has been directed more towards establishing the mechanisms underlying these recognition processes than observing simply the uptake and removal of a number of different foreign particles by phagocytic cells.

Pauley, Krassner and Chapman, 1971, examined the removal of five different strains of bacteria from the circulation of the Californian sea hare, (Aplysia californica). While four of the five strains were taken up rapidly from the circulation over 24 hours it was found that Serratia marcescens was still
present in large numbers after 16 days. Examination of the
haemolymph showed that there was a correlation between the
absence of an agglutinin for *Serratia marcescens* and its slow
elimination from the circulation since there were agglutinins
present against the other four strains of bacteria which were
cleared rapidly. Furthermore concomitant with the removal of
these bacteria there was a temporary drop in the corresponding
agglutinin titres. This type of evidence has led the authors
to suggest that the agglutinins were also the opsonins for the
bacteria but such conclusions must await purification of the
agglutinins and a study of their biological function.

Other work has indicated the possible role of a humoral
factor in the response of insect haemocytes to the eggs of a
parasite (*Cardiochiles nigriceps*) (Lewis and Vinson, 1968). When eggs of *Cardiochiles nigriceps* were implanted into
*Heliothis* *zea* they were encapsulated whereas in *Heliothis
virescens* they survived and developed normally. Further
investigations showed that if the eggs were left in *Heliothis
zea* for various periods of time, then cleared of adhering cells
and transferred to *Heliothis virescens*, they were subsequently
encapsulated. These results indicated that a humoral factor
might be important for the encapsulation of the eggs in
*Heliothis* *zea* which was lacking in *Heliothis virescens*. It is
possible that the eggs avoid encapsulation in *Heliothis
virescens* because they possess antigens which share common
determinant groups with host antigens. The concept of shared antigens being an important factor in determining host susceptibility or resistance to infection had been suggested by Salt, 1961, from his studies on the cellular reactions of insects to various parasites.

Harshbarger and Heimple, 1968, found that injection of zymosan into larvae of the wax moth, *Galleria mellonella* enhanced considerably their susceptibility to *Pseudomonas aeruginosa* and *Escherichia coli*. The zymosan caused the blood of the larvae to darken. It is possible that melanization of the blood precipitated proteins essential as recognition factors for the phagocytosis of these bacteria.

Using carbon, Reade, 1968, investigated the organs involved in removal of these particles from the circulations of the crayfish, *Parachaeraps bicarinatus* and the snail, *Helix pomatia*. He found that carbon introduced into the vascular system of either of these invertebrates was rapidly sequested into cells lining the blood vessels of the hepato-pancreas, an organ similar in function to the liver.

More recently Reade and Reade, 1972, have investigated the removal of carbon from the circulation of the clam, *Tridacna maxima*. In contrast to the crayfish, *Parachaeraps bicarinatus* and the snail, *Helix pomatia* circulating haemocytes appear to play the dominant role in the uptake of carbon. After ingestion they were observed to home to the
digestive organs, pass through the epithelium and be eliminated from the animal via the adductor valves. Thus in this animal there is a simple but very efficient way of eliminating unwanted material. Similarly in the cockroach, (*Periplaneta americana*) sheep red cells or carmine particles injected into the haemocoel were cleared rapidly by circulating haemocytes (Ryan and Nicholas, 1972). The sheep erythrocytes were subsequently digested by the haemocytes while carbon and carmine particles were deposited in cellular aggregates which later transformed into melanized nodules.

**In vitro studies on phagocytosis**

Until recently little research had been carried out in vitro on the phagocytosis of foreign particles by phagocytes from invertebrates. This was due in part to the difficulty of preparing suitable cell suspensions or monolayers of cells which would remain viable over extended periods of time. However with the improvement in tissue culture techniques a few papers in this area have appeared in recent years indicating the involvement of humoral factors in the recognition of foreign material by phagocytic cells from these animals.

Tripp, 1966, demonstrated that monolayers of amoebocytes from the oyster, (*Crassostrea virginica*) would phagocytose rabbit erythrocytes. The amoebocytes were cultured on coverslips and stained 30-60 min. after exposure to rabbit red blood cells. Pre-treatment of the erythrocytes with oyster
haemolymph enhanced ingestion but comparable levels of phagocytosis could be obtained if the amoebocytes were incubated with untreated cells for a much longer period of time.

Stuart, 1968, has carried out a series of investigations on the reticulo-endothelial system of the octopus, (Eledone cirrrosa). When suspensions of human erythrocytes and octopus macrophages were incubated together significant phagocytosis occurred only if the erythrocytes had been pretreated with Eledone serum. No ingestion occurred with unopsonized human erythrocytes or erythrocytes pretreated with specific antibody raised in rabbits. Thus there is an absolute requirement for specific opsonins in this system.

Amoebocytes from the snail, (Helix aspera) have been shown to phagocytose formalized yeast cells and formalized sheep erythrocytes (Prowse and Tait, 1969). Experiments were carried out with monolayers in small round bottom tubes. Phagocytosis was quantitated by counting the numbers of unphagocytosed foreign particles left in the supernatants of experimental tubes compared with control tubes. Monolayers were examined also under the microscope for visual evidence of phagocytosis. The particles had to be pretreated with snail haemolymph before significant phagocytosis occurred. Adsorption of haemolymph with yeast while abolishing its ability to promote the phagocytosis of yeast particles had no effect on the capacity of such haemolymph to promote phago-
cytosis of sheep erythrocytes. This last result is particularly interesting since it suggests that there are specific recognition factors (opsonins) for these particles in snail haemolymph.

The recognition of foreignness by cells of the crayfish, (Parachaeraps bicarinatus) has been examined extensively by McKay and Jenkin, 1970a. They maintained viable crayfish haemocytes in Leighton tubes over periods of up to 24 hours. Sheep or human red cells pretreated with crayfish serum were ingested by haemocytes. The requirement for serum was absolute and in its absence little phagocytosis occurred. At 0°C opsonized sheep red cells still adhered to the haemocyte membrane although ingestion was inhibited completely. Thus the initial step in phagocytosis seems to be adherence of the particle to the membrane of the cell which is independent of temperature. Sera from other invertebrates like the snail, (Helix aspera) or the Murray mussel, (Velesunio ambiguus) did not enhance the uptake of sheep erythrocytes by crayfish haemocytes. It appears therefore on this rather limited data that the opsonins which enhance the uptake of sheep erythrocytes by crayfish haemocytes are phylum specific. This is confirmed to some extent by the fact that serum from the reef crab, (Ozius truncatus) would also enhance the uptake of sheep erythrocytes by crayfish haemocytes. Both the crayfish, (Parachaeraps bicarinatus) and the reef crab, (Ozius truncatus)
are members of the Phylum Crustacea.

In contrast to these observations Scott, 1971, has demonstrated that the adherence of sheep and chicken erythrocytes to the surface of haemocytes of the cockroach, (Periplanata americana) is independent apparently of humoral factors. Monolayers were examined under the microscope and the haemocytes were scored for the adherence of sheep erythrocytes. The sheep red blood cells adhered to the surface of haemocytes but no phagocytosis occurred. Adherence was reduced considerably by treatment of the monolayers with 0.2% trypsin in contrast to lipase and lecithinase which had little effect. It is conceivable that trypsin destroyed a "receptor" for sheep erythrocytes which was on the surface of the haemocytes. Incubation of the trypsin treated haemocytes with cockroach haemolymph failed to restore the ability to these cells to take up sheep erythrocytes which suggested that the receptors if free and of a cytophilic nature were not present in the haemolymph to any significant level. The trypsin-treated haemocytes appeared quite viable as judged by trypan blue or neutral red uptake, and therefore it is possible that the receptor for sheep red blood cells is cell bound and never found free in the serum. Recently Anderson, Holmes and Good, 1973, have found that the haemocytes from the cockroach, (Blaberus craniifer) are capable of phagocytosing in vitro a number of different strains of bacteria. The uptake of
bacteria was independent of haemolymph which suggests that
cognition is mediated by receptors associated with the
membrane of the haemocyte.

CONCLUSION

The presence of serum is essential for phagocytosis of
erthrocytes by invertebrate phagocytes in most of the
examples described above. This is particularly interesting
since the sera from these invertebrates also show haemag-
glutinating activity for erythrocytes. The possibility that
the haemagglutinating activity and opsonic activity are
properties of the same molecule has not yet been resolved.
Adsorption of crayfish serum with sheep erythrocytes removed
both the haemagglutinating and opsonic activities (McKay,
1970). However this was carried out on only partially puri-
ified material and therefore does not answer the question.

Since it is possible that the haemagglutinins may
function as recognition units enabling invertebrate phagocytic
cells to recognize foreign erythrocytes it might be pertinent
to discuss what is known about the physico-chemical properties
of such molecules.

The physico-chemical properties of the haemagglutinins

Extensive studies by Cantacuzene, 1923, revealed the
presence of haemagglutinins in the sera of invertebrates from
various phyla such as Annelida, Arthropoda and Mollusca.
Furthermore his data indicated that in some cases these haemagglutinins were inducible by prior immunisation with sheep or rabbit erythrocytes (Cantacuzene, 1919). Such an observation has not been confirmed by recent research (Tripp, 1966; McKay, 1970; Cornick and Stewart, 1973).

The first attempts to purify haemagglutinins from the haemolymph of invertebrates was carried out by Tyler and Metz, 1945. They found that the haemolymph obtained from the spiny lobster, (Panulirus interruptus) agglutinated red cells from a number of different vertebrates and spermatozoa from some invertebrates. Based on extensive cross adsorption studies with erythrocytes and spermatozoa there appeared to be 8-10 different haemagglutinins. These haemagglutinins were purified by isoelectric precipitation at pH 4.8 - 5.0 which separated them from the haemocyanin (Tyler and Scheer, 1945). Electrophoresis of this material revealed two components of which the more slowly migrating one possessed the haemagglutinating activity. Antibodies raised against purified haemocyanin reacted with the purified haemagglutinin which led the authors to suggest that these proteins cross reacted. However it is possible that the haemocyanin preparation used for immunization was contaminated with the haemagglutinins and this resulted in antibodies to both materials.

More recently Marchalonis and Edelman, 1968, have purified and characterized the haemagglutinin for horse
erythrocytes from the horse shoe crab, (*Limulus polyphemus*).

Initially haemocyanin was separated from the serum by ultracentrifugation at 100,000 g. for 5 h. The clear supernatant was purified further by zone electrophoresis on starch gel and the eluted fraction containing the haemagglutinating activity was run on sephadex G 200. The active fraction was used for further analysis. The mol.wt. of the purified haemagglutinin was 400,000 and had a sedimentation coefficient of 13.4S. The molecule could be dissociated into sub units of 67,000 mol.wt. at pH 2.0 or pH 9.0. Further dissociation into units of 22,500 mol.wt. could be achieved with 20% acetic acid or 8 M urea. Thus the haemagglutinin appears to be made up of 6 units each composed of 3 sub units of 22,500 mol.wt.

Electron micrographs of the purified haemagglutinin showed molecules with a very uniform ring shape structure. Close examination of the single molecules have indicated that they have a hexagonal shape with a 6 fold symmetry which is consistent with the concept that the molecule is composed of 6 units. Although the haemagglutinin sub unit has a mol.wt. similar to the light chains of immunoglobulin and shows some similarity in amino acid composition (Table 1.1) it appears likely that the *Limulus* haemagglutinin and vertebrate antibodies are unrelated evolutionary developments. This is suggested from the fact that the sub units are all of the same mol.wt. and do not show the heterogeneity on starch gel electrophoresis.
characteristic of the polypeptide chains of antibody.

Acton, Bennett, Evans and Schroenloher, 1969, isolated and purified the haemagglutinin for sheep erythrocytes from the oyster, (Crassostrea virginica). The intact molecule had a sedimentation coefficient of 33.4 S but could be dissociated into sub units by acid or alkaline pH. Complete dissociation to a sub unit of 20,000 mol.wt. could be obtained by dialysis against 5 M-guanidine-HCl pH 7.5. Haemagglutinating activity was dependent on Ca^{++} ions and was abolished by dialysis against a chelating agent such as sodium citrate.

The haemagglutinin for human A erythrocytes from the snail, (Helix pomatia) has been extracted from the albumin gland in a highly purified form (Hammarstrom and Kabat, 1969). A single step purification was achieved by the specific adsorption of the snail haemagglutinin onto columns of poly-leucine chemically coupled to purified hog mucin containing A + H blood group activities. The haemagglutinin was eluted with N-acetyl-D-galactosamine (GNAC) in physiological saline. The material was free from contaminants as judged by gel filtration and ultracentrifugation. The mol.wt. of the haemagglutinin was estimated at 100,000 as determined by the analytical ultra-centrifuge. Amino acid analysis (Table 1.1) revealed that the molecule contained cysteine and was rich in aspartic acid, glutamic acid, serine, valine and arginine.

More recent work on this haemagglutinin (Hammarstrom,
1972) has established that the intact molecule has a mol.wt. of 79,000 as determined by further ultra-centrifugation studies. The molecule can be dissociated into 6 sub units of 13,000 mol.wt. by reduction with excess dithiothreitol in 6 M-guanidine-HCl and alkylation with iodoacetamide. The structural analysis done on this molecule however has not been related to biological function. Furthermore while the haemagglutinin is found in the albumin gland and eggs of snails it is absent from the haemolymph which suggests that it might have an important biological role in the reproductive processes of this animal, but not involved in the recognition of foreign material by the phagocytic cells.

The haemagglutinins to erythrocytes from various vertebrate species in the serum of the Murray mussel, (Velesunio ambiguus) have been purified and characterized (Jenkin and Rowley, 1970). Initially whole serum was precipitated with 50% (NH₄)₂SO₄ and the pellet containing haemagglutinating activity was purified further on a sucrose density gradient. This material gave a symmetrical peak in the ultra-centrifuge with a sedimentation coefficient of 28S. Further purification of the haemagglutinin specific for rabbit red cells was carried out on the material obtained from the sucrose density gradient. The haemagglutinin was adsorbed onto stroma prepared from rabbit erythrocytes and eluted with glycine/HCl buffer at pH 3.0. This purified haemagglutinin
was rich in aspartic acid, phenylalanine and glutamic acid (Table 1.1) and had only trace amounts of cysteine, methionine and tyrosine. The haemagglutinin from *Velesunio ambiguus* appears to have a sub unit structure similar to *Limulus polyphemus* (Marchalonis and Jenkin, 1970, unpublished observations).

Recently, Finstad, Litman, Finstad and Good, 1972, have described the purification of the haemagglutinins for horse erythrocytes from the horse shoe crab, (*Limulus polyphemus*) and the sea star, (*Asterias forbesi*). Initially, whole serum from *Limulus* or coelomic fluid from *Asterias* were spun at 100,000 G. in the ultracentrifuge to remove the haemocyanin. They were then subjected to zone electrophoresis on agar and finally, in the case of the agglutinin from *Asterias* run on a sucrose density gradient.

The activities of both agglutinins were dependent completely on Ca$^{++}$ ions and dialysis against ethylenediaminetetra-acetate (EDTA) caused irreversible inactivation. The peptide maps of the tryptic digests of the respective agglutinins were dissimilar in their patterns and also from the patterns of purified light and heavy chains of vertebrate immunoglobulins.

The *Limulus* agglutinin and *Asterias* agglutinin were of different molecular size having sedimentation coefficients of 12.6S and 6.5S respectively. Both consisted of sub units of 25-30,000 mol.wt. as defined by polyacrylamide-gel electro-
phoresis in sodium dodecyl sulphate. The proposed sub unit structure of the Limulus agglutinin (Marchalonis and Edelman, 1968) has therefore been confirmed by these studies. The amino acid analysis (Table 1.1) of these agglutinins revealed that they were rich in aspartic and glutamic acid and there were also detectable levels of cysteine.

CONCLUSION

There are considerable differences in the molecular size and weight of the haemagglutinins purified from various species of invertebrates. Thus the largest haemagglutinin from the oyster, (Crassostrea virginica) has a mol wt. greater than 1,000,000 while the haemagglutinin from the snail, (Helix aspera) has a mol wt. of 70,000. However there are certain similarities. Amino acid analyses (Table 1.1) indicate that all the haemagglutinins are rich in aspartic and glutamic acid and with the exception of the haemagglutinin from the Murray mussel, (Velesunio ambiguus) all the haemagglutinins contain demonstrable levels of cysteine.

These proteins can be dissociated into sub units by a series of different chemical treatments which do not destroy covalent bonds. Therefore it is unlikely that interchain disulphide bridges between sub units are important in stabilizing the conformation of the intact molecule. It appears from the studies reported in this review that the sub
units of the agglutinins are dissimilar to the purified light and heavy chains of vertebrate immunoglobulins.
TABLE 1.1
The amino acid composition of the purified haemagglutinins from various invertebrate species.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>PHyla</th>
<th>Mollusca</th>
<th>Arthropoda</th>
<th>Echino-dermata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H.pomatia¹</td>
<td>C.virginica²</td>
<td>V.ambigus³</td>
<td>L.polyphemus⁴</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>11.4%</td>
<td>17.5%</td>
<td>15.3%</td>
<td>11.1%</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.9%</td>
<td>6.3%</td>
<td>6.4%</td>
<td>6.6%</td>
</tr>
<tr>
<td>Serine</td>
<td>10.9%</td>
<td>3.7%</td>
<td>15.3%</td>
<td>8.15%</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>7.6%</td>
<td>9.9%</td>
<td>8.9%</td>
<td>12.2%</td>
</tr>
<tr>
<td>Proline</td>
<td>6.5%</td>
<td>3.7%</td>
<td>2.2%</td>
<td>5.0%</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.7%</td>
<td>9.4%</td>
<td>10.1%</td>
<td>8.3%</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.4%</td>
<td>7.9%</td>
<td>6.4%</td>
<td>4.7%</td>
</tr>
<tr>
<td>Valine</td>
<td>7.3%</td>
<td>3.0%</td>
<td>6.4%</td>
<td>5.0%</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2.9%</td>
<td>5.1%</td>
<td>0%</td>
<td>3.0%</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.5%</td>
<td>1.2%</td>
<td>0%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.0%</td>
<td>4.1%</td>
<td>1.15%</td>
<td>4.5%</td>
</tr>
<tr>
<td>Leucine</td>
<td>6.0%</td>
<td>7.7%</td>
<td>5.1%</td>
<td>10.3%</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.6%</td>
<td>2.1%</td>
<td>0%</td>
<td>3.7%</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.1%</td>
<td>2.8%</td>
<td>12.7%</td>
<td>3.9%</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.2%</td>
<td>0%</td>
<td>3.8%</td>
<td>5.3%</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.3%</td>
<td>11.1%</td>
<td>2.55%</td>
<td>4.3%</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.9%</td>
<td>4.4%</td>
<td>3.8%</td>
<td>2.6%</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>4.5%</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
</tr>
</tbody>
</table>

* data expressed in amino acids, mole %.
1. Data obtained from Hammerstrom and Kabat, 1969, originally expressed as residues/100,000 g. protein.

2. Data obtained from Acton, Bennett, Evans and Schrohenloher, 1969, originally expressed as mole/moles of protein.

3. Data obtained from Jenkin and Rowley, 1970, originally expressed as g/100 g. protein.

4. Data obtained from Marchalonis and Edelman, 1968, originally expressed as g/100 g. of protein.

5. Data obtained from Finstad, Litman, Finstad and Good, 1972, originally expressed as u mole/mg. protein.

6. Data obtained from Finstad, Litman, Finstad and Good, 1972, originally expressed as u mole/mg. protein.
CHAPTER 2
MATERIALS AND METHODS

Animals used in this study
Crayfish, (Parachaeraps bicarinatus) were obtained from fresh water lakes at Wellington near the mouth of the River Murray, South Australia (Fig. 2.1). They were kept in shallow tanks with running water and continuous aeration and fed liver once a week. Animals for experimentation were held in shallow plastic tanks with continuous aeration in the laboratory at 19°C. These ranged in weight from 30 g. to 100 g. but for any particular experiment the variation in weight of the animals was kept minimal and did not exceed ±10 g.

Strains of bacteria
The following strains of bacteria were used, Escherichia coli 086, Salmonella abortus equi, Salmonella typhimurium C5, (Jenkin, 1963), Staphylococcus aureus and Pseudomonas CP (McKay and Jenkin, 1969).

All the above strains were obtained from stock cultures held in the Microbiology Department, University of Adelaide. Pseudomonas CP is pathogenic for the crayfish and was isolated from the blood of animals given lethal doses of endotoxin (McKay and Jenkin, 1969). It was maintained on agar slopes and subcultured every 2 weeks. Every 2 months it was tested
FIGURE 2.1

The Australian fresh water crayfish,
(Parachaeraps bicarinatus).
for virulence on groups of 10 crayfish held at 19°C. The LD$_{50}$ remained constant at about $5 \times 10^5$ organisms throughout this study.

The other strains of bacteria were maintained on agar slopes and sub-cultured every 2 months.

**Collection of Serum**

Animals were bled from the ventral haemal sinus with either a 5 ml or 10 ml syringe mounted with a 19 gauge needle. The haemolymph was allowed to clot at room temperature (19°C) for 10 to 15 min. and then spun at 2,000 g. for 20 min. The serum was drawn off with a Pasteur pipette and stored in 10 ml aliquots at -20°C. At this temperature haemagglutinating activity was completely stable and no loss in activity was recorded upon thawing.

**Collection of Plasma**

Animals were injected via the ventral haemal sinus with 2 ml of 0.25% cysteine hydrochloride in physiological saline containing 10 i.u/ml of preservative free heparin at pH 6.2. After 3 to 5 min. the animals were bled from the haemal sinus using a 10 ml syringe mounted with a 19 gauge needle. The syringe contained 1 ml of ice cold 0.25% cysteine hydrochloride and 10 i.u/ml of preservative free heparin. The haemocytes were spun down at 20 g. for 10 min. in a cold room at 4°C. The plasma was drawn off with a fine Pasteur pipette, pooled, and
finally dialysed overnight against van Harrevald's medium at pH 7.3 (van Harrevald, 1936).

**Preparation of $^{32}$P labelled bacteria**

These were prepared according to the method of Jenkin and Rowley, 1961.

An aliquot of an overnight broth culture of bacteria containing $5 \times 10^7$ organisms was added to a 500 ml sterile Erlenmeyer flask containing 100 ml of nutrient broth. One millicurie of $^{32}$P as orthophosphate was added to the broth and the culture incubated overnight at 37°C with shaking. Following incubation, the bacteria were recovered by centrifugation at 3,000 g. for 15 min. and washed 3 times by centrifugation with 10 ml of broth and finally resuspended in the above medium to give a concentration of $2 \times 10^8$ bacteria/ml. Bacterial suspensions were kept at 4°C and not used for longer than 5 days.

**Method for measuring the rate of elimination of $^{32}$P labelled bacteria from the circulation of the crayfish**

Various concentrations of bacteria were injected into the ventral haemal sinus. At known time intervals 0.1 ml samples of haemolymph were withdrawn using a 1 ml syringe mounted with a 22 gauge needle. Samples were spotted directly onto filter paper planchettes backed with plastic, and the radioactivity assayed using a thin mica end-window Geiger
counter installed in a Nuclear Chicago automatic sample change
C110A (Nuclear Chicago, Chicago).

The elimination of bacteria from the circulation
follows an exponential curve as in the vertebrates. The rate
of elimination or phagocytic index K was calculated from the
following equation:

\[ K = \frac{\log C_1 - \log C_2}{T_2 - T_1} \]

where \( C_1 \) and \( C_2 \) are the concentrations of bacteria at times
\( T_1 \) and \( T_2 \) (Biozzi, Benacerraf and Halpern, 1953).

Preparation of monolayers of haemocytes from the
crayfish.

This method was similar to that described by McKay and
Jenkin, 1970(a), with slight modifications. Crayfish were
injected via the ventral haemal sinus with 2 ml of 0.25%
cysteine hydrochloride in physiological saline containing 10
i.u/ml of preservative free heparin at pH 6.2. After 3-5 min.
the animals were bled from the ventral haemal sinus using a 5
or 10 ml syringe mounted with a 19 gauge needle. The syringe
contained 1 ml of ice-cold 0.25% cysteine hydrochloride and 10
i.u/ml of heparin. The cysteine hydrochloride prevents the
haemolymph from clotting without damaging the cells. In the
past some problems had been encountered with cells clumping
together either during bleeding or subsequent washing. Heparin
whilst not preventing clotting appears to prevent this clumping phenomenon. The contents of the syringe were squirted gently into an ice-cold tube after removing the needle and centrifuged at 20 g. for 10 min. Following centrifugation the cells were resuspended in ice-cold van Harrevald's medium at pH 7.2 containing 10 i.u./ml of preservative free heparin. Cells may be washed several times by the above method depending on the needs of the experiment without signs of clumping. Following washing the cells were added to Leighton tubes at 4°C such that each tube contained approximately 2 x 10^6 haemocytes. The haemocytes were allowed to settle onto glass over a period of 1 h. at 4°C. Following this settling period the tubes were allowed to stand at 19°C for a period of 40 min. before use. During this period the cells spread rapidly to form an even monolayer, and were finally washed in-situ with 2 separate changes of 1 ml of van Harrevald's medium without heparin.

**Method for Measuring Phagocytosis of Bacteria in-vitro**

Approximately 2 x 10^3 lag phase bacteria in 0.5 ml of van Harrevald's medium were added to each of 12 monolayers at 19°C. Such a volume just covered the monolayer of cells. At the same time a similar number of bacteria were added to 6 Leighton tubes without cells which served as controls. At the end of 60 min., 0.1 ml of the fluid from each tube was plated onto nutrient agar to determine the number of bacteria. Three
of the tubes containing cells (experimental tubes) were immediately put on ice and the supernatants of the other 9 removed. The monolayers of these 9 were washed with 3 separate changes of van Harrevald's medium. Following this washing procedure 0.5 ml of the medium was added to each tube and the tubes were incubated at 19°C. Previous studies had shown that 19°C was a suitable temperature to measure phagocytosis (McKay and Jenkin, 1970a). At known time intervals 3 tubes were removed, placed on ice, and together with the first 3 treated as follows. The supernatants were removed and 0.5 ml of ice cold van Harrevald's medium added to each monolayer. The cells were then scraped from the glass surface and following vigorous pipetting with a Pasteur pipette, 0.1 ml was plated onto nutrient agar. This enabled one to determine the number of viable bacteria associated with the haemocytes. The theoretical number of bacteria associated with these cells at 60 min. was calculated from the difference between the number of bacteria in supernatants of the control tubes compared to the number in the experimental. The difference between the theoretical number of bacteria ingested and that recovered from the monolayer of cells at different time intervals by plating reflected the number of bacteria killed over that time period (Jenkin and Benacerraf, 1960). These results were expressed as percentage survival.

Further experiments designed to study the rate of up-
take and survival of bacteria over a 60 min. period were essentially similar to the above except that 2 experimental tubes and 2 control tubes were removed at each time point.

**Treatment of monolayers with trypsin**

Monolayers of haemocytes were treated with 0.002% trypsin in van Harrevald's medium at pH 7.2 for 2 h. at room temperature. Following trypsin treatment the haemocytes were washed twice with 1 ml of the tissue culture medium. More than 90% of the cells treated in the above fashion were judged viable by trypan blue exclusion.

**Method of blockading crayfish**

Crayfish were injected with $3 \times 10^8$ unlabelled bacteria in 0.2 ml of broth and after 15 min. when more than 90% of the injected dose had been eliminated from the circulation, challenged with $5 \times 10^8$ isotopically $^{32}$P labelled bacteria. The rate of elimination of this second dose of bacteria was followed as indicated previously.

**Changes in the number of circulating cells following injection of particles**

Following the injection of various concentrations of bacteria or erythrocytes into the ventral haemal sinus, small samples (0.1 ml) of haemolymph were withdrawn and added to 0.2 ml of 5% cysteine hydrochloride in physiological saline
at pH 6.2 (McKay and Jenkin, 1970a). Cell numbers were determined using a haemocytometer.

Opsonisation of bacteria with crayfish serum

For opsonisation 1 ml of serum or a suitable dilution was added to 1 ml of a washed suspension of bacteria containing approximately $5 \times 10^9$ bacteria/ml. The mixture was incubated at 4°C for 60 min. After incubation the bacteria were washed twice by centrifugation at 2,000 g. with 20 ml of physiological saline. The bacteria were resuspended in physiological saline to give a final concentration of $5 \times 10^9$ bacteria/ml.

Maintenance of crayfish at different temperatures

Experiments were carried out on the effect of temperature on the elimination of bacteria from the circulation of the crayfish. Groups of these animals were maintained at 13°C, 19°C or 26°C for a week before the experiment. This allowed acclimatization of the animals to the required temperature. It was found that if the tanks were kept in an air-conditioned laboratory then the temperature in the water remained in the region of 18°C to 20°C. In order to maintain a temperature of 13°C, tanks were placed in a cold room with a thermomix and its thermostat set at 13°C. Under these conditions the temperature was maintained at 13°C with a maximum variation of ±1°C. A temperature of 26°C was achieved by placing the tanks in a
warm room at 30°C.

**Preparation of cell lysate**

Circulating cells were collected from the crayfish as described for the preparation of haemocyte monolayers. The cell count varied from 2 to 6 x 10⁶ haemocytes/ml. Following collection from 4 crayfish the haemolymph was centrifuged at 20 g. for 10 min at 4°C and the cells resuspended in 0.6 ml of the same medium. The cells were disrupted by ultra sonication for 1 min. in a H.S.E. ultrasonic power unit, the cellular debris was removed by centrifugation and the clear supernatant used in experiments to be described in Chapter 3.

**Maintenance of Ehrlich ascites tumour cells. (EAT)**

This tumour cell line was maintained in F1 mice (C₅7BL/6 x Balb/C₅7) by passive transfer every 2 weeks. Tumour cells were harvested from mice by collecting the ascitic fluid from the peritoneal cavity with a 10 ml syringe mounted with a 19 gauge needle. The fluid was squirted into 10 ml of ice-cold physiological saline containing 10 i.u./ml heparin. The tumour cells were washed 3 times with 12 ml of physiological saline by centrifugation at 270 g. for 2 min. respectively. They were finally resuspended in physiological saline to a concentration of 1 x 10⁷ tumour cells/ml and 0.2 ml injected i.p. into each of 5 mice.
Method for labelling EAT tumour cells with $^{51}\text{Cr}$

Tumour cells were prepared as above and made up to a final concentration of $5 \times 10^7$/ml in physiological saline. An aliquot (0.1 ml) of the tumour cell suspension was added to 1.9 ml of Hank's buffered salt soln. making a final concentration of $5 \times 10^6$ cells/ml. One hundred microcuries of $^{51}\text{Cr}$ (Na$_2$CrO$_4$, Radiochemical centre, Amersham, England) were added to the cells and the mixture incubated at 37°C for 1 h. with occasional shaking. The labelled cells were washed 5 times by centrifugation at 270 g. for 2 min. with 5 ml of Hank's buffered salt soln. They were finally resuspended in 4 ml of the same medium. Approximately 30-40% of the $^{51}\text{Cr}$ was associated with the tumour cells which were 99% viable as judged by trypan blue exclusion. Krebs II ascites tumour cells and Hela cells were labelled by the same technique.

Collection and maintenance of Kreb's II ascites tumour cells

These tumour cells were maintained in F1 mice ($C_5\times BL_6 \times Balb/C_0$) by passage every 10 days. The collection of these cells was similar to that described for the EAT cells with a slight modification. It was found that this tumour caused haemorrhages in mice and therefore the tumour cell suspension was washed 3 times by centrifugation as above and then resuspended in 0.144 NH$_4$Cl in 0.017M tris (tris (hydroxy-
methyl) amino methane) pH 7.4 for 10 min. at 4°C. This treatment lyses selectively the erythrocytes but the tumour cells remain viable as judged by trypan blue exclusion. Following this treatment the tumour cells were washed 3 times by centrifugation as above and finally resuspended in physiological saline to a concentration of $1 \times 10^7$ cells/ml. Each of 5 mice were injected i.p. with 0.2 ml of the tumour cell suspension.

**Collection and maintenance of Hela cells**

This cell line was obtained from the Institute of Medical and Veterinary Science (I.M.V.S.) South Australia when required.

The cells were harvested from the culture bottles by incubating them for 15 min. at 37°C with 0.25% trypsin in Hank's buffered salt soln. at pH 7.1. Following incubation the bottles were shaken (manually) from side to side until the Hela cells were dislodged. The cells were washed 3 times with 10 ml of Hank's buffered salt soln. by centrifugation at 270 g. for 2 min. and resuspended finally in the same medium to give a concentration of $5 \times 10^6$ cells/ml.

**Preparation of Lipopolysaccharide from gram negative bacteria**

Lipopolysaccharide from either *Salmonella abortus equi* or *Salmonella typhimurium C5* was prepared by the phenol-water
Method of Westphal, Lüderitz and Bister, 1952.

Method for sensitizing Hela cells with lipopolysaccharide

Two ml of lipopolysaccharide from Salmonella abortus equi or Salmonella typhimurium C5 at a concentration of 2 mg/ml was treated with 0.5 ml of 0.1N NaOH for 4 h. at 37°C. It was subsequently neutralized to pH 7.4 by adding dropwise 0.02N NaOH with a Pasteur pipette. The alkali treated lipopolysaccharide was stored in 0.2 ml aliquots at -4°C.

Hela cells (5 x 10⁶ cells/ml) were labelled with C5 as described above. Following washing, 2 ml of the labelled suspension were incubated with 100 ug/ml of alkali treated lipopolysaccharide for 1 h. at 37°C with occasional shaking. The cells were then washed 3 times with 5 ml of Hank's buffered salt soln. by centrifugation at 270 g for 2 min. and finally resuspended in 2 ml of the same medium. Treatment of the labelled Hela cells with alkali treated lipopolysaccharide did not effect the viability of the cells as judged by trypan blue exclusion.

Method for assaying the cytotoxicity of haemocytes against ⁵¹Cr-labelled tumour cells

Monolayers of crayfish haemocytes were set up in Leighton tubes as previously described. The ⁵¹Cr-labelled tumour cells were adjusted to 5 x 10⁶ cells/ml in van
Harrevald's buffered salt soln. and 0.5 ml of this suspension was added to each Leighton tube and the tubes incubated at 19°C. At regular time intervals over 4 h, 2 Leighton tubes were removed and the supernatants withdrawn with a 1 ml pipette and centrifuged at 270 g. for 2 min. to remove free tumour cells. An aliquot (0.2 ml) was taken from each supernatant with a 1 ml pipette, and counted in a glass tube in a Packard Auto Gamma Spectrometer.

**Preparation of antibodies against the haemagglutinins in crayfish serum**

It had been shown previously by McKay and Jenkin, 1970a that serum from the crayfish possesses haemagglutinins which appear to function as opsonins in so far as they enhance the uptake of different mammalian erythrocytes by cultures of haemocytes. Experimental results to be given in the text indicated that the various haemagglutinins were antigenically related. Thus in order to raise an antiserum in rabbits against these molecules, rabbit erythrocytes were sensitized with normal crayfish serum and re-injected into the same animal.

Two rabbits were used in the immunization procedure. Ten millilitres of blood was collected from the ear vein of each of the rabbits into 2 bottles each containing 10 ml of 3.8% sodium citrate. The cells were washed 3 times by centrifugation at 1,000 g. for 10 min. with 30 ml of physio-
logical saline. Following washing they were incubated with 6 ml of normal crayfish serum for 2 h. at 19°C with occasional shaking, and finally washed 8 times with 10 ml of physiological saline as above. One millilitre of packed cells was injected I.V. into the ear vein of each of the respective rabbits and the sera tested at a later stage for antibody activity using the haemolytic assay described below.

Preparation of a rabbit antiserum against opsonins for various strains of bacteria in crayfish serum

Antibodies were raised against Salmonella abortus equi, Staphylococcus aureus, Escherichia coli 086, Salmonella typhimurium C5 and Pseudomonas CP, that had been opsonised previously with normal crayfish serum as above.

Two rabbits were used for each strain of bacteria. Ten millilitres of an overnight broth culture of bacteria (5 x 10⁸/ml) were washed 3 times by centrifugation at 2,000 g. for 10 min. and finally resuspended in 10 ml of normal crayfish serum. The suspension was incubated at 19°C for 1 h. and finally washed 3 times by centrifugation as above. The bacteria were resuspended in physiological saline to a concentration of 5 x 10⁸/ml and 0.2 ml were injected into the ear vein of each rabbit. Rabbits were immunized over 7 weeks and test bled on the 8th week. Antibody activity was assayed by a haemolytic assay described below. In the case of Salmonella typhimurium C5, Staphylococcus aureus and Pseudomonas CP
immunization was carried out using heat killed \(56^\circ\text{C}/60\text{ min.}\) organisms treated with crayfish serum.

Two rabbits were immunized also with crayfish haemocytes in an attempt to raise antibodies against cell bound opsonins. Four crayfish were bled (as has been described on p.27) and their haemocytes washed 3 times and then pooled in 2 ml of pyrogen free saline. This suspension containing 2 to \(4 \times 10^8\) haemocytes was slowly injected into the ear vein of a rabbit in a cold room (6\(^\circ\text{C}\)) since suspensions of haemocytes were found to clump within 2 min. at room temperature. Both rabbits were injected every week over a period of 5 weeks and bled at the end of the 6th week. Their sera was assayed for antibody activity using the haemolytic assay described below.

**Use of the modified Coomb's technique for detecting rabbit antibody against the opsonins in crayfish serum**

In order to avoid confusion at this stage, experiments to be described in the text showed that the opsonins for bacteria appeared to be antigenically related to the haemagglutinins, and so antibody raised in rabbits against opsonins to bacteria could be assayed conveniently by the following method.

A modification of the Coomb's technique was used (Coombs, Mourant and Race, 1945). An aliquot (0.2 ml) of a
\[1/10\] dilution of specific antisera in physiological saline (for details of antisera see p.37-38) was titrated serially in 0.2 ml of saline in small glass tubes. To each of these tubes was added 0.4 ml of 1% (V/V) washed sheep erythrocytes which had been pre-opsonised for 60 min. at 19°C with a \[1/10\] dilution of normal crayfish serum. An aliquot (0.2 ml) of a \[1/10\] dilution of fresh guinea pig serum was used as a source of complement. The rabbit antiserum had been adsorbed with an equal vol. of sheep red blood cells at 4°C overnight to remove natural antibody to sheep erythrocytes. The tubes were incubated at 37°C for 30 min. The end point of haemolysis was taken as the tube in which approximately 50% of the sheep erythrocytes had lysed as judged by the optical density at 541 mp using a Schimadzu Spectrophotometer.

**Use of the modified Coomb's technique for detecting the haemagglutinin for sheep erythrocytes in crayfish serum**

The modified Coomb's technique was used as a more sensitive assay for the haemagglutinin against sheep red blood cells in crayfish serum (for details of the other assay see p.40).

An aliquot (0.2 ml) of normal crayfish serum was diluted serially in 0.2 ml of physiological saline in small glass tubes. To each of these tubes was added 0.2 ml of 1% (V/V) washed sheep erythrocytes and the mixture incubated for 1 h. at 19°C with occasional shaking. The cells were then
washed 3 times with 3 ml of physiological saline by centrifugation at 1,000 g. for 5 min. respectively and resuspended in 0.4 ml of the same medium. An aliquot (0.2 ml) of a $1/30$ dilution in saline of an antiserum raised in rabbits against the haemagglutinins (for details see p.36) was added to each tube. Finally 0.2 ml of a $1/10$ dilution of fresh guinea pig serum in physiological saline was used as a source of complement. The rabbit antiserum had been adsorbed with an equal volume of sheep red blood cells overnight to remove natural antibody to sheep erythrocytes. The tubes were incubated at 37°C for 30 min. and the end-point of haemolysis was determined as above.

**Titration of haemagglutinating activity in the blood of the crayfish**

The haemagglutinins in the serum of the crayfish agglutinate erythrocytes from a number of different species of vertebrates (McKay, Jenkin and Rowley, 1969). Normal crayfish serum (0.2 ml) was diluted serially in 0.2 ml of physiological saline. Aliquots of 0.2 ml of a 1% (V/V) washed erythrocyte suspension was added to each well. The trays were incubated at room temperature for 2 h. and the agglutination titre expressed as the last well showing visible agglutination.
Techniques used for the purification of the 'opsonins' for erythrocytes and bacteria

Affinity Chromatography

This technique was used as the first step in the purification of the opsonins from normal crayfish serum (Axen, Porath and Ernback, 1967: Cuatrecasas and Anfinson, 1971).

Preparation of the column

Biogel A 50M (agarose bead) was used as the supporting structure in the column. Twenty five millilitres of Biogel A 50M in deionised water was mixed mechanically with 1 g. of cyanogen bromide. The reaction was carried out in a fume hood and maintained at pH 10.5 - 11.5 by adding dropwise 2N NaOH for a period of 15-20 min. The pH reading was recorded on a Beckman Zeromatic pH Meter. The "activated" beads were washed with 200 ml of cold deionised distilled water on a Buchner filter funnel using a vacuum pump. Finally they were washed with 200 ml of 0.1M NaHCO₃ and care was taken not to allow the beads to dry out. This slurry was added to 25 ml of an immunoglobulin fraction containing specific antibody to the crayfish opsonins (for details of antisera see pp.36-38).

The immunoglobulin fraction had been prepared previously by the following method. Fifteen millilitres of the antisera (for details pp.36-38) were mixed mechanically with 15 ml of 0.05M tris buffer at pH 8.0 in a cold room (6°C). A total
volume of 22.5 ml of saturated (NH₄)₂SO₄ was added slowly to the antiserum while mixing to give a final concentration of 40%(NH₄)₂SO₄. The mixture was allowed to stand with constant stirring at 6°C for 30 min. The precipitate containing the immunoglobulin fraction was centrifuged at 10,000 g. for 20 min. in a Servall refrigerated centrifuge (Ivan Sorvall, Connecticut, USA). The deposit was resuspended in distilled water and dialysed overnight at 6°C against the same.

Following this a further dialysis was carried out at 6°C against 0.1M NaHCO₃ in order to redissolve the precipitate within the bag. The immunoglobulin preparation was finally dialysed overnight at 6°C against 0.05M phosphate buffered saline pH 7.1 and passed through a haemocyanin column (for details see below). This was done in order to remove antibodies raised against the haemocyanin of normal crayfish serum which appeared to bind in low levels to the rabbit erythrocytes even after repeated washings with physiological saline.

The immunoglobulin preparation and the slurry of beads were stirred mechanically for 3 h. at 19°C. After this treatment the beads were washed with 200 ml of 0.1 M NaHCO₃ followed by 400 ml of deionised distilled water. Eighty percent of the protein and antibody activity was found to be adsorbed onto the surface of the beads. The washed slurry of beads were stirred with 25 ml of 1 M ethanolamine in boric acid pH 8.5 for 2 h. at 19°C. This reaction blocks any remaining active
groups on the Biogel agarose which might bind other proteins in crayfish serum. Finally the beads were washed with 400 ml of deionised distilled water and resuspended in 25 ml of 0.1 M tris buffered saline at pH 7.1. The column 30 cm x 1.1 cm was packed with the slurry of beads and equilibrated with 0.1 M tris buffered saline at pH 7.1. Samples of crayfish serum were loaded onto the column and run through at a flow rate of 66 ml/h.

**Preparation of Haemocyanin column**

This column was prepared to remove antibodies against haemocyanin in the immunoglobulin fraction. Whole crayfish serum was run through an affinity column (prepared as above). The excluded material was found to have no haemagglutinating, haemolytic or opsonic activity for sheep red blood cells or bacteria. Thirty millilitres of this material containing 20 mg/ml protein were dialysed overnight against 0.1 M NaHCO₃ at 6°C and coupled to cyanogen bromide activated beads as described above.

**Column Chromatography of the active fraction obtained by Affinity Chromatography**

Column chromatography was used as the second step in the purification of the opsonins from crayfish serum.

Ten grams of Biogel P-150 (100-200 mesh) polyacrylamide beads (BIO-RAD, California, USA) were added to 500 ml of 0.1 M
tris buffered saline at pH 8.0 and allowed to swell overnight. The excess fluid was drawn off with a vacuum pump and the beads were washed 5 times by decantation with 0.1 M tris buffered saline at pH 8.0. The slurry of beads was poured into a column 100 cm x 1.2 cm under gravitational flow. The column was equilibrated for 48 h. at 6°C with 0.1 M buffered saline pH 8.0 at a flow rate of 3.2 ml/h. A sample (2 ml) was loaded on the column and fractions of 1.6 ml were collected every 30 min. with an ISCO automatic fraction collector (Instrument Specialities Co., Nebraska, USA).

Essentially similar procedures were carried out in packing and equilibrating a Biogel agarose column. Biogel A 0.5 M (200-400 mesh) agarose beads (BIO-RAD, California, USA) were used in 0.1 M tris buffered saline pH 8.0 with 0.01 M ethylenediaminetetra-acetic acid (EDTA). The presence of EDTA blocked the binding of the haemagglutinins to the agarose.

A sephadex G 100 column was poured also and equilibrated as above. Sephadex G 100 (40-120 u) beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were used in 0.01 M tris buffered saline pH 8.0.

In all three types of column chromatography described above the fractions were assayed for activity by the modified Coombs's test (see p.39)

Polyacrylamide gel electrophoresis
Polyacrylamide gel electrophoresis was carried out
following the procedure described by Neville, 1971.

<table>
<thead>
<tr>
<th>Lower gel acrylamide stock soln.</th>
<th>Catalyst soln.A</th>
</tr>
</thead>
<tbody>
<tr>
<td>N, N'-methylene bisacrylamide</td>
<td>N,N,N',N'-tetramethylethyl-</td>
</tr>
<tr>
<td></td>
<td>enediamine (temet)</td>
</tr>
<tr>
<td>0.2g</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>acrylamide</td>
<td>distilled water</td>
</tr>
<tr>
<td>22g</td>
<td>9.5 ml</td>
</tr>
<tr>
<td>distilled water to 1,000 ml</td>
<td>(store in the dark)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Upper gel acrylamide stock soln.</th>
<th>Catalyst soln.B</th>
</tr>
</thead>
<tbody>
<tr>
<td>N, N'-methylene bisacrylamide</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>0.6g</td>
<td>200 mg</td>
</tr>
<tr>
<td>acrylamide</td>
<td>distilled water</td>
</tr>
<tr>
<td>9.6g</td>
<td>10 ml</td>
</tr>
<tr>
<td>distilled water to 1,000 ml</td>
<td>(store in the dark)</td>
</tr>
</tbody>
</table>

Buffer stock soln.

**Lower running buffer pH 9.18**

- tris 257g
- concentrated HCl 15.5 ml
- distilled water to 1,000 ml

The stock soln. was diluted 5 times with distilled water before use.

**Upper running buffer pH 8.64**

- sodium dodecyl sulphate (SDS) 10 g
- tris 49.7 g
- boric acid 24.7 g
- distilled water to 1,000 ml
The stock soln. was diluted 10 times with distilled water before use.

**Upper gel buffer pH 6.1**

\[
\begin{align*}
\text{H}_2\text{SO}_4 & \quad 267 \text{ ml of a 1M stock soln.} \\
\text{tris} & \quad 65.4 \\
\text{distilled water} & \quad \text{to } 1,000 \text{ ml}
\end{align*}
\]

The stock soln. was diluted 10 times with distilled water before use.

The gels consisted of a lower gel and an upper stacking gel. The lower gel was made by adding 12.5 ml of lower gel acrylamide stock soln. to 5.0 ml of a 5 fold concentrate lower running buffer together with 6.2 ml of distilled water in a 40 ml glass tube. The air was drawn off with a vacuum pump and the mixture was placed in a 15°C water bath. Aliquots (0.7 ml) of catalyst soln.A and (0.6 ml) of catalyst soln.B were added and the gel solution was poured carefully in 3 ml volumes into glass tubes (13 cm x 0.6 cm) in a 15°C water bath. The tubes were sealed at the bottom with 2 layers of plastic strips (1 cm x 1 cm). The lower gels were overlayed with 0.2 ml of isobutyl alcohol which was removed after 20 min. when the gels had set. The upper gel was made by adding 3.3 ml of upper gel acrylamide stock soln. to 5.0 ml of distilled water and 1.0 ml of 10 fold concentrate upper gel buffer in a 20 ml glass tube. The air was drawn off with a vacuum pump and the soln. was placed in a 15°C water bath. Catalyst soln.A (0.3 ml) and catalyst soln.B (0.25 ml) was
added to the upper gel and 0.3 ml of this mixture was poured
into each of the tubes containing the lower gel followed by
0.2 ml of isobutyl alcohol. After 20 min. the isobutyl
alcohol layer was removed and the tubes placed in a cylindri-
cal container filled with lower running buffer which was
immersed in a 25°C water bath (Fig. 2.2). Upper running buffer
was put into the upper compartment of the polyacrylamide
apparatus and the samples were loaded onto the gels (as
described below).

The fractions to be tested were concentrated in 2% SDS by freeze drying in an Edwards' Centrifugal freeze dryer
(Edwards High Vacuum Ltd., England). This was due to the low
levels of protein in the fractions from the columns showing
activity as measured by the Coomb's enhancement technique.
The freeze dried material was resuspended in 20 μl of 0.05M
Na₂CO₃ and 2 μl of B mercapto-ethanol added. The fractions
were incubated for 1 h. at 19°C and then diluted with 100 μl
of upper gel buffer containing 2% sucrose and 0.02% bromo
phenol blue. One hundred micro litres of the above were loaded
onto the polyacrylamide gels with a 100 μl syringe and gels
were run for 4 h. at 1.5 ma/tube at 25°C. After the run was
completed the gels were removed from the glass tubes and
stained overnight in 0.02% Coomassie blue in 50% methanol and
7.5% acetic acid. Following this procedure they were washed
for 48 h. in 20% methanol and 7.5% acetic acid with gentle
FIGURE 2.2

The electrophoresis apparatus used for running polyacrylamide gels.
shaking. The following protein markers were used; lysozyme (13,000 mol.wt.), carbonic anhydrase (29,000 mol.wt.), ovalbumin (43,000 mol.wt.), catalase (60,000 mol.wt.), bovine serum albumin (68,000 mol.wt.) and transferrin (77,000 mol.wt.)

**The Double-Diffusion test:**

The double diffusion technique essentially followed the procedure outlined by Ouchterlony, 1962. One gram of agarose (BDH laboratory reagent) was melted in 50 ml of distilled water in a beaker in 100°C water bath. The suspension was dispensed into 7.5 ml aliquots and stored at 6°C with 0.02% sodium azide. Before use, an aliquot was remelted in a 100°C water bath and 7.5 ml of 0.05 M phosphate buffered saline pH 7.1 and 0.15 ml of SP54 (Bene Chemie, West Germany) added. This suspension was poured carefully into the space formed by placing a glass slide on four glass supports (about 1 mm thick) resting on a glass plate. The glass slides had been boiled for 1-2 h. in a 2% solution of RBS-25 detergent (R Borghgraef, Belgium) and washed by boiling in distilled water for 2 h. and then coated with a thin layer of agar (0.1%). The glass plate had been washed with distilled water then alcohol and finally covered with a thin layer of silicone in chloroform before each preparation. The complete assembly was prewarmed to 37°C before pouring the agarose. The agarose was allowed to set for 30 min. at 4°C and then the glass slide was removed carefully by sliding it across the glass plate.
The wells were cut in the agarose using a template containing cylindrical openings and a close fitting metal cutter which fitted through the cylindrical holes. The agar plug was sucked out through a glass tube connected to a vacuum pump.

Samples were placed in the wells using a 5 μl syringe and the plates incubated at 19°C for 48 h. in a humid chamber. Following incubation they were washed in physiological saline for 36 h. at 19°C and finally in distilled water for 12 h. The plates were dried at 100°C for 1 h. and then immersed for 5 min. in a saturated solution of xylene brilliant cyanogen G in 45% methanol and 10% acetic acid. After this they were washed for 1 min. in 45% methanol and 10% acetic acid, and dried at 19°C.

Buffers used in tissue culture

Hank's buffered salt soln.

The Hank's medium was prepared according to the method described by Weller, Enders, Robbins and Stoddard, 1952, with the exception that NaHCO₃ was used as the buffer in this system. Two stock soln. designated A and B were prepared.

Soln. A contained:-

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>160 g</td>
</tr>
<tr>
<td>KCl</td>
<td>8 g</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>4 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.8 g</td>
</tr>
<tr>
<td>deionised distilled water.</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>
Soln. B contained:

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} & \quad 3.04\text{ g} \\
\text{KH}_2\text{PO}_4 & \quad 1.2\text{ g} \\
\text{glucose} & \quad 20\text{ g} \\
deionised\text{ distilled water} & \quad 900\text{ ml} \\
0.4\%\text{ phenol red soln.} & \quad 100\text{ ml}
\end{align*}
\]

The Hank's buffered salt soln. was prepared by adding 5 ml of soln. A to 5 ml of soln. B in a 100 ml bottle containing 90 ml of distilled water. An aliquot (2.5 ml) of 4.6% NaHCO\textsubscript{3} was added to the bottle and the soln. was gassed with 5% CO\textsubscript{2} to pH 7.1.

van Harrevald's buffered salt soln. pH 7.5

This balanced salt soln. was devised for studies of nerve conduction in fresh water crustaceans (van Harrevald, 1936).

It consists of:

\[
\begin{align*}
\text{NaCl} & \quad 60\text{ g} \\
\text{KCl} & \quad 2\text{ g} \\
\text{CaCl}_2 & \quad 7.5\text{ g} \\
\text{MgCl}_2 & \quad 2.6\text{ g}
\end{align*}
\]

This mixture was made up to 500 ml with distilled deionised water and autoclaved in 20 ml aliquots. Before use, 5 ml of the stock soln. was added to 45 ml of distilled deionised water containing 0.25 ml of 4.6% NaHCO\textsubscript{3}. 
Method for cleaning Leighton tubes used for haemocyte culture work

Leighton tubes were boiled for 1-2 h. in 2% RBS-25 detergent (R. Borghgraef, Belgium). Following this treatment they were washed and boiled in distilled water for 2 h., dried at 100°C, and finally autoclaved before use.

Buffers used in the purification of the opsonins for sheep erythrocytes and bacteria

Phosphate buffered saline pH 7.1

This buffer was made up from the following stock soln.

It consists of:–

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 106.5 \text{ g} \\
\text{Na H}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} & \quad 39 \text{ g} \\
\text{Na N}_3 & \quad 20 \text{ g} \\
\text{Na Cl} & \quad 175 \text{ g} \\
\text{distilled water} & \quad 2,000 \text{ ml}
\end{align*}
\]

The stock soln. was diluted 10 times before use with distilled water.

Tris buffered saline pH 7.1

This buffer was used for affinity chromatography and was prepared from the following stock soln.

\[
\begin{align*}
\text{tris} & \quad 60.6 \text{ g} \\
\text{Na N}_3 & \quad 5 \text{ g} \\
\text{Na Cl} & \quad 58 \text{ g} \\
\text{Concentrated HCl} & \quad 41.4 \text{ ml}
\end{align*}
\]

This was made up to 1,000 ml with distilled water.
The soln. was diluted 10 times with distilled water before use.

Tris buffered saline pH 8.0

This buffer used for column chromatography was made up from the following stock soln.

It consists of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>tris</td>
<td>121 g</td>
</tr>
<tr>
<td>NaN</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>87 g</td>
</tr>
</tbody>
</table>

This mixture was dissolved in 700 ml of distilled water and adjusted to pH 8.0 with concentrated HCl using a Beckman Zeromatic pH Meter. The volume was made up to 1,000 ml with distilled water. The stock soln. was diluted 10 times with distilled water before use.
CHAPTER 3

The importance of opsonins in the removal of bacteria from the circulation of the cray-fish (Parachaeraps bicarinatus).

In the vertebrates the rate of elimination of bacteria and foreign erythrocytes from the circulation is related to the concentration of specific antibody. The rate of elimination (K) bears a linear relationship to the concentration of antibody until K approaches a maximum. The rate of elimination (K) or, as it is sometimes called, the phagocytic index (K), measures in effect the reaction (recognition) between such foreign particles and the macrophages of the liver and spleen. Invertebrates are able also to remove foreign particles from their circulation but the mechanism which enables their phagocytic cells to recognise self from non-self is poorly understood. In vitro studies with erythrocytes and phagocytic cells from several different species of invertebrates suggest that the naturally occurring haemagglutinins in the haemolymph of these animals may function as opsonins and are therefore in this sense analogous in function to the immunoglobulins of vertebrates (Tripp, 1966; Stuart, 1968; Prowse and Tait, 1969; McKay and Jenkin, 1970(a)).

In this chapter is described the kinetics of the removal of bacteria from the circulation of the fresh-water
crayfish and the dependence of this uptake on opsonins in the haemolymph.

Rate of elimination of bacteria from the circulation of the cray-fish

The 5 strains of bacteria used in this study were labelled with $^{32}$P and their rates of removal from the circulation of the crayfish determined in groups of 5 crayfish. Various concentrations of bacteria were injected ranging from $5 \times 10^7$ to $1 \times 10^9$ organisms. The weights of the crayfish used in these studies varied from 45-60 g.

The data in Table 3.1 show that the different strains of bacteria are eliminated from the circulation at different rates. Thus *Salmonella typhimurium* C5 is removed very slowly with a $K$ value of 0.03 whereas *Escherichia coli* 086 is removed rapidly with a $K$ value of 0.19. Furthermore it would appear that with the exception of *Salmonella abortus equi* the rate is independent of the concentration of bacteria injected. Similar results have been obtained in vertebrates (Biozzi, Howard, Halpern, Stiffel and Mouten, 1960). At this stage there is no adequate explanation as to the reason why the rate of elimination of *Salmonella abortus equi* increases with the concentration of the bacteria injected into the crayfish.
TABLE 3.1 Rate of elimination (K) of various strains of bacteria from the circulation of the crayfish (*Parachaeraps bicarinatus*).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of bacteria injected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 x 10⁷</td>
</tr>
<tr>
<td>Salmonella typhimurium C₅</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.14±0.02</td>
</tr>
<tr>
<td>Escherichia coli 086</td>
<td>0.22±0.04</td>
</tr>
<tr>
<td>Pseudomonas strain C.P.</td>
<td>0.09±0.02</td>
</tr>
</tbody>
</table>

* Each group consists of 5 crayfish. The figures represent the mean K value of the group ± standard deviation.
Effect of Temperature and Weight of the crayfish on the elimination of \(^{32}\text{P}\)-labelled Salmonella abortus equi from the circulation

Groups of 5 crayfish were acclimatised for one week at 13°C, 19°C and 26°C and the rate of elimination of \(5 \times 10^8\) \(^{32}\text{P}\)-labelled Salmonella abortus equi from the circulation determined at the temperatures at which the animals had been held (Table 3.2). The data presented show that the rate of removal of these bacteria from the circulation of crayfish was independent of temperature. This will be commented on later.

It was important also to determine whether the rate of removal of \(^{32}\text{P}\)-labelled bacteria from the circulation of crayfish was influenced by the weight of the animals since crayfish of different body weights were used in the experiments described in the text. The rates of removal of \(5 \times 10^8\) \(^{32}\text{P}\)-labelled Salmonella abortus equi from the circulation of crayfish covering the range 45-175 g body weight was determined (Table 3.3). It can be seen from the data that the rate of elimination of Salmonella abortus equi is independent of body weight.

Since the rate of removal of different strains of bacteria from the circulation varied, it suggested by analogy with the vertebrates, that factors in the haemolymph may be necessary for the recognition and phagocytosis of bacteria by phagocytic cells. This was investigated further as follows.
TABLE 3.2 The effect of temperature on the rate of elimination of *Salmonella abortus equi* from the circulation of the crayfish, *(Parachaeraps bicarinatus)*.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Phagocytic Index K</th>
<th>Mean phagocytic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>13°C Group 1</td>
<td>0.13, 0.16, 0.12, 0.12, 0.10</td>
<td>0.13</td>
</tr>
<tr>
<td>19°C Group 2</td>
<td>0.14, 0.16, 0.12, 0.09, 0.16</td>
<td>0.13</td>
</tr>
<tr>
<td>26°C Group 3</td>
<td>0.16, 0.18, 0.12, 0.12, 0.15</td>
<td>0.15</td>
</tr>
</tbody>
</table>
TABLE 3.3 The effect of body weight on the elimination of *Salmonella abortus equi* from the circulation of the crayfish, *Paracharaps bicarinatus*.

<table>
<thead>
<tr>
<th>Weight/g</th>
<th>Phagocytic Index K</th>
<th>Mean phagocytic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>45-55g</td>
<td>0.12, 0.16, 0.12, 0.09</td>
<td>0.12</td>
</tr>
<tr>
<td>75-85g</td>
<td>0.1, 0.11, 0.13, 0.10</td>
<td>0.11</td>
</tr>
<tr>
<td>155-175g</td>
<td>0.12, 0.12, 0.10, 0.20</td>
<td>0.14</td>
</tr>
</tbody>
</table>
Factors involved in the removal of bacteria from the circulation of the crayfish

If, in vertebrates that have just eliminated a primary dose of bacteria from the circulation, a second similar dose of bacteria is given, the latter is removed much more slowly. However, if the second dose is pretreated (opsonised) with specific antibody the rate of elimination may be returned to normal. This type of experiment is a very sensitive assay for antibody.

It was necessary before carrying out such experiments in crayfish to establish the numbers of Salmonella abortus equi required to establish a state of blockade to challenge with the same organism. Groups of 4 crayfish were injected with different numbers of Salmonella abortus equi and after 15 min challenged with $5 \times 10^8$ $^{32}$P-labelled Salmonella abortus equi. It is apparent from Table 3.4 that large numbers of organisms i.e., $3 \times 10^9$ organisms produced complete blockade in all the animals while lower doses produced only a partial blockade.

Since a good blockade could be established it was of interest to see if this could be reversed by pretreating the challenge dose with crayfish serum. Groups of 5 crayfish were blockaded with $3 \times 10^9$ Salmonella abortus equi and were challenged 15 min later (at which time more than 90% of the initial dose had been eliminated) with either $5 \times 10^8$ $^{32}$P-labelled unopsonised Salmonella abortus equi or $5 \times 10^8$ $^{32}$P-labelled
TABLE 3.4 Dose of *Salmonella abortus equi* required to establish a state of blockade in the crayfish (*Parachaeraps bicarinatus*).

<table>
<thead>
<tr>
<th>Dose of blockading organism</th>
<th>Challenge organism</th>
<th>Mean phagocytic Index K</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$5 \times 10^8$ <em>Salmonella abortus equi</em></td>
<td>0.15</td>
</tr>
<tr>
<td>$4 \times 10^8$ <em>Salmonella abortus equi</em></td>
<td>$5 \times 10^8$ <em>Salmonella abortus equi</em></td>
<td>0.14</td>
</tr>
<tr>
<td>$1 \times 10^9$ <em>Salmonella abortus equi</em></td>
<td>$5 \times 10^8$ <em>Salmonella abortus equi</em></td>
<td>0.06</td>
</tr>
<tr>
<td>$3 \times 10^9$ <em>Salmonella abortus equi</em></td>
<td>$5 \times 10^8$ <em>Salmonella abortus equi</em></td>
<td>0.025</td>
</tr>
</tbody>
</table>
Salmonella abortus equi pretreated with crayfish serum (Table 3.5). It is clear from the data that the reversal of blockade may be effected by pretreating the second dose of bacteria with crayfish serum. Prior adsorption of serum used for opsonisation of the second dose with the specific strain of bacteria prevented the reversal of blockade. Furthermore, if the primary dose of bacteria was pretreated with crayfish serum prior to injection, thus preventing removal of recognition factors when injected, the animal was not blockaded and the second dose was eliminated at normal rates. It appears from this data that blockade in the crayfish involves a depletion in serum opsonins rather than a saturation of the phagocytic cells of the animal.

**Titration of the opsonins in crayfish serum for Salmonella abortus equi**

The system of blockade described above suggested a useful means of titrating the opsonic activity of the serum against any particular strain of bacteria. Groups of 5 crayfish were blockaded with $3 \times 10^9$ Salmonella abortus equi and challenged 15 min later with $5 \times 10^8$ $^{32}$P-labelled Salmonella abortus equi pretreated with various dilutions of serum. Results presented in Table 3.6 show that a $\frac{1}{15}$ dilution of the serum completely reversed the blockade whereas a $\frac{1}{25}$ dilution did not. Further experiments on preparations of sera pooled from different groups of animals indicated that the opsonic
The elimination of *Salmonella abortus equi* from the circulation of normal and blockaded crayfish.

<table>
<thead>
<tr>
<th>Blockading organism</th>
<th>Challenge organism</th>
<th>Mean phagocytic Index K</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td><em>Salmonella abortus equi</em></td>
<td>0.15</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em></td>
<td>0.025</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em> pretreated with normal crayfish serum</td>
<td>0.15</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em> pretreated with serum adsorbed with <em>Salmonella abortus equi</em></td>
<td>0.025</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em> pretreated with normal crayfish serum</td>
<td><em>Salmonella abortus equi</em></td>
<td>0.11</td>
</tr>
<tr>
<td>Blockading organism</td>
<td>Challenge organism</td>
<td>Mean phagocytic Index K</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Salmonella abortus equi</td>
<td>0.03</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Salmonella abortus equi pretreated with 1/5 dilution of normal crayfish serum</td>
<td>0.17</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Salmonella abortus equi pretreated with 1/15 dilution of normal crayfish serum</td>
<td>0.15</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Salmonella abortus equi pretreated with 1/25 dilution of normal crayfish serum</td>
<td>0.047</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Salmonella abortus equi pretreated with 1/50 dilution of normal crayfish serum</td>
<td>0.045</td>
</tr>
</tbody>
</table>
titre could range from $1/5$ in one batch of serum to $1/50$ in another preparation.

Specificity of blockade and the opsonins in the serum of the crayfish

The specificity of blockade was investigated using groups of 5 crayfish and the following strains of bacteria; Salmonella abortus equi, Salmonella typhimurium C5, Escherichia coli 086 and Staphylococcus aureus. The data shown in Table 3.7 indicate that the elimination of Salmonella abortus equi was depressed significantly by prior treatment of the animal with Salmonella typhimurium C5, Escherichia coli 086 and Staphylococcus aureus. Furthermore the uptake of Escherichia coli 086 was depressed by the prior injection of Salmonella abortus equi and so was the elimination of Staphylococcus aureus.

It would appear from these results that the establishment of the state of blockade is non specific for the 4 strains of bacteria tested. Since blockade has been shown from data given above to involve a depletion of serum opsonins it would appear that there may be one species of opsonin for the 4 strains of bacteria.

This conclusion was supported by the following experiment. Forty millilitres of crayfish serum was prepared from 10 crayfish and 2 ml aliquots were adsorbed with $1 \times 10^9$/ml and then $1 \times 10^{10}$/ml of each of the bacterial strains for 30 min at
### TABLE 3.7 Specificity of blockade in the crayfish.

<table>
<thead>
<tr>
<th>Challenge organism</th>
<th>Blockading organism</th>
<th>Mean phagocytic Index K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella abortus equi</td>
<td>None</td>
<td>0.15 ± 0.01*</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Salmonella abortus equi</td>
<td>0.025 ± 0.01</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Salmonella typhimurium C5</td>
<td>0.06 ± 0.025</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Escherichia coli 086</td>
<td>0.026 ± 0.007</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Staphylococcus aureus</td>
<td>0.02 ± 0.004</td>
</tr>
<tr>
<td>Escherichia coli 086</td>
<td>None</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Escherichia coli 086</td>
<td>Escherichia coli 086</td>
<td>0.055 ± 0.015</td>
</tr>
<tr>
<td>Escherichia coli 086</td>
<td>Salmonella abortus equi</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>None</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Staphylococcus aureus</td>
<td>0.04 ± 0.015</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Salmonella abortus equi</td>
<td>0.085 ± 0.015</td>
</tr>
</tbody>
</table>

* Each group consists of 5 crayfish. The figures represent the mean K value of the group ± standard deviation.
4°C. The rate of elimination of $^{32}$P-labelled Salmonella abortus equi opsonised with the adsorbed serum was determined in groups of 4 crayfish blockaded with the same organism. The data given in Table 3.8 show that the opsonins for Salmonella abortus equi could be adsorbed out by Salmonella typhimurium C5, Escherichia coli O86 and sheep erythrocytes. Adsorption with Staphylococcus aureus reduced significantly the level of opsonins for Salmonella abortus equi although there was some residual activity.

Association of opsonic factors with circulating haemocytes

In view of the fact that the crayfish has, apart from the genital organs, only two other glandular organs with specialised functions of digestion and excretion, it was not unreasonable to suppose that within the population of circulating haemocytes were cells capable of synthesising these opsonins. Haemocytes were collected and lysed as described in Chapter 2, p.32. The lysate diluted with an equal volume of physiological saline was used to pretreat Salmonella abortus equi prior to injection into crayfish which had been blockaded with Salmonella abortus equi. The data given in Fig. 3.1 show that the lysate contains opsonins that can reverse the blockade. The haemocytes had been washed 3 times before pooling for sonication and therefore it is unlikely that contamination with serum is responsible for this effect.
TABLE 3.8 Specificity of the opsonins in normal crayfish serum for *Salmonella abortus equi*.

<table>
<thead>
<tr>
<th>Blockading organism</th>
<th>Challenge organism</th>
<th>Mean phagocytic Index K</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em></td>
<td>0.025 ± 0.01*</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em> pretreated with normal crayfish serum</td>
<td>0.16 ± 0.012</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em> pretreated with crayfish serum adsorbed with <em>Salmonella abortus equi</em></td>
<td>0.028 ± 0.01</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em> pretreated with crayfish serum adsorbed with <em>Escherichia coli</em> 086</td>
<td>0.052 ± 0.014</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em> pretreated with crayfish serum adsorbed with <em>Staphylococcus aureus</em></td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td><em>Salmonella abortus equi</em> pretreated with crayfish serum adsorbed with <em>Salmonella typhimurium</em> C5</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

(Cont'd...)
<table>
<thead>
<tr>
<th>Blockading organism</th>
<th>Challenge organism</th>
<th>Mean phagocytic Index K</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella abortus</em></td>
<td><em>Salmonella abortus</em></td>
<td>0.029 ± 0.013</td>
</tr>
<tr>
<td><em>equi</em></td>
<td><em>equi</em></td>
<td>pretreated with cray-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fish serum adsorbed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with sheep erythro-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cytes (one adsorption</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with 5 x 10^9 cell/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>for 2h/19°C)</td>
</tr>
</tbody>
</table>

* Each group consists of 4 crayfish. The figures represent mean K value of the group ± standard deviation.
FIGURE 3.1

Rate of elimination of *Salmonella abortus equi* from the circulation of the crayfish before and after opsonisation with a lysate prepared from the circulating haemocytes.

- 0—0 Unopsonised bacteria
- ●●● Opsonised bacteria
It has been established earlier in this Chapter that the rate of elimination of *Escherichia coli* 086 could be depressed significantly in crayfish blockaded with *Salmonella abortus equi*. This state of blockade could be reversed if the *Escherichia coli* 086 was pretreated with lysate. The data as shown in Table 3.9 demonstrate that the titre of opsonic activity for *Escherichia coli* 086 in the lysate from crayfish haemocytes is of the order of $\frac{1}{5} - \frac{1}{10}$. It has been shown previously in the Chapter that there may be only one species of opsonin in crayfish serum for *Salmonella abortus equi* and *Escherichia coli* 086. It appears that a similar situation may be applicable for the opsonins associated with the circulating haemocytes.

**The change in the number of circulating haemocytes following the injection of sheep erythrocytes and bacteria**

Mckay, Jenkin and Rowley, 1969, found that the population of the circulating haemocytes in the crayfish was depressed following the injection of $2 \times 10^9$ sheep erythrocytes into the ventral haemal sinus. Haemocytes surrounded by rosettes of sheep erythrocytes were found on the gill filaments of animals 10 min after injection. It was suggested that the erythrocytes attached to the circulating haemocytes following injection and caused them to settle out temporarily onto the membranes of cell lining the gill filaments. They implied also from data obtained on the distribution of radioactivity following injection of
TABLE 3.9 Titration of the opsonic activity of the lysate from crayfish haemocytes against *Escherichia coli 086.*

<table>
<thead>
<tr>
<th>Blockading organism</th>
<th>Challenge organism</th>
<th>Mean phagocytic Index K</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td><em>Escherichia coli 086</em></td>
<td>0.19 ± 0.01*</td>
</tr>
<tr>
<td><em>Salmonella abortus</em></td>
<td><em>Escherichia coli 086</em></td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td><em>equi</em></td>
<td><em>Escherichia coli 086</em></td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Pretreated with a 1/5 dilution of lysate.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella abortus</em></td>
<td><em>Escherichia coli 086</em></td>
<td>0.1 ± 0.07</td>
</tr>
<tr>
<td><em>equi</em></td>
<td>Pretreated with a 1/10 dilution of lysate.</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella abortus</em></td>
<td><em>Escherichia coli 086</em></td>
<td>0.05 ± 0.014</td>
</tr>
<tr>
<td><em>equi</em></td>
<td>Pretreated with a 1/20 dilution of lysate.</td>
<td></td>
</tr>
</tbody>
</table>

* Each value represents the mean of 4 crayfish ± standard deviation.
32P-labelled bacteria, that the circulating haemocytes represented the most important population of phagocytic cells involved in the elimination of foreign material (McKay and Jenkin, 1970(b)).

Since previous data with various concentrations of bacteria indicated that in general the rate of elimination was independent of concentration, it seemed pertinent to study changes in the haemocyte population following the injection of either erythrocytes or bacteria.

Volumes (0.2 ml) of each of the respective concentrations of sheep erythrocytes, 5 x 10⁷/ml, 5 x 10⁸/ml, 5 x 10⁹/ml, were injected into groups of 4 crayfish via the ventral haemal sinus. Samples of haemolymph were taken from the crayfish at regular intervals over 90 min and cell numbers were determined using a haemocytometer. The data in Fig. 3.2 show that the degree of depression in the number of circulating haemocytes over the first 5 min is directly related to the number of erythrocytes injected. This supported the above conclusion of McKay, Jenkin and Rowley, 1969, that the circulating haemocytes were very important in the uptake of sheep erythrocytes. However a group of 4 crayfish which had been injected with 2 x 10⁹ sheep erythrocytes still eliminated a dose of 5 x 10⁸ 32P-labelled Salmonella abortus equi as rapidly as control untreated animals, at a time when 95% of the circulating haemocytes had settled out. The blood volume of the crayfish used in the
Percentage fall in the number of circulating haemocytes in the crayfish following injection of various concentrations of sheep erythrocytes.

0---0  $1 \times 10^7$ sheep erythrocytes

$\Delta$---$\Delta$  $1 \times 10^8$ sheep erythrocytes

$\bullet$---$\bullet$  $1 \times 10^9$ sheep erythrocytes
studies had a mean value of 10 ml which meant that the effective concentration of sheep erythrocytes was $2 \times 10^8$ cells/ml in haemolymph. Such levels have been found insufficient to effect a removal of opsonic activity for *Salmonella abortus equi* from crayfish serum (see Table 3.8). It would appear therefore that the circulating haemocytes do not play a major role in the elimination of bacteria although it is possible that the haemocytes having settled out could still phagocytose a second dose of particles.

In order to resolve this dilemma the above experiments were repeated using bacteria instead of sheep erythrocytes. Volumes (0.2 ml) of *Salmonella typhimurium C5* containing $5 \times 10^7$, $5 \times 10^8$ or $3 \times 10^9$ organisms were injected into the ventral haemal sinus and the change in the number of circulating haemocytes was recorded over 120 min. The data given in Fig. 3.3 show that the fall in the number of circulating haemocytes was dependent on the concentration of bacteria injected and unlike the situation with erythrocytes, they did not return into the circulation. This data taken together with the observations that the elimination of most of the strains of bacteria tested was independent of concentration suggest that the circulating haemocytes are not the major cells responsible for the uptake of bacteria. It was found also that the rate of elimination of *Salmonella abortus equi* in animals that had been blockaded with *Salmonella typhimurium C5* had returned to normal 45 min
FIGURE 3.3

Percentage fall in the number of circulating haemocytes in the crayfish following injection of various concentrations of *Salmonella typhimurium* C5.

- □——□ Control injected with saline
- ⊗——⊗ 5 x 10⁷ bacteria
- ▲——▲ 5 x 10⁸ bacteria
- ●——● 2 x 10⁹ bacteria
PERCENT CELLS CIRCULATING

TIME (MINUTES)
following the blockading dose (Table 3.10). At this stage only 10% of the haemocytes were left in the circulation. This suggests that by 45 min there was a return of sufficient levels of opsonins into the circulation of the crayfish to enable the elimination of Salmonella abortus equi to occur at rates comparable with normal control crayfish. Comparable data has been obtained in the vertebrates by Jenkin and Rowley, 1961. They found that the rate of removal of $^{32}$P-labelled bacteria from the circulation of mice returned to normal 60 min following a blockading dose of carbon.

**DISCUSSION**

In this chapter it has been established that the rate of elimination of $^{32}$P-labelled bacteria from the circulation of the crayfish followed an exponential curve as has been found in vertebrates (Biozzi, Howard, Halpern, Stiffel and Mouten, 1960). Furthermore the rate of elimination of Salmonella abortus equi from the circulation of the crayfish was found to be independent of temperature. It has been shown by McKay and Jenkin, 1970(a), that the recognition of sheep erythrocytes by crayfish haemocytes involves the adherence of sheep red blood cells to the membrane of the haemocyte followed by phagocytosis. They have found further that the adherence phase is independent of temperature over the range 0°C to 22°C while phagocytosis is inhibited at lower temperatures. In the
TABLE 3.10  The period of time over which blockade of the elimination of *Salmonella abortus equi* from the crayfish is effective.

<table>
<thead>
<tr>
<th>Blockading organism</th>
<th>Challenge organism</th>
<th>Time of challenge</th>
<th>Mean phagocytic Index K</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhimurium</em> C5</td>
<td><em>Salmonella abortus equi</em></td>
<td>15'</td>
<td>0.06</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> C5</td>
<td><em>Salmonella abortus equi</em></td>
<td>45'</td>
<td>0.13</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> C5</td>
<td><em>Salmonella abortus equi</em></td>
<td>120'</td>
<td>0.12</td>
</tr>
</tbody>
</table>
light of these observations it appears likely that measurement of the rate of removal of bacteria from the circulation of the crayfish involves the measurement of the adherence of these organisms to the phagocytic cells of the animal.

The rate of elimination of $^{32}\text{P}$-labelled bacteria could be depressed significantly if a large dose of unlabelled bacteria ($3 \times 10^9$ organisms/animal) was injected into the crayfish before challenge. This state of blockade was temporary however and could be reversed by opsonisation of the challenge innoculum with normal crayfish serum. This reversal of blockade therefore revealed the presence of opsonins for bacteria in crayfish serum.

Further investigations revealed that this state of blockade lacked specificity for Salmonella abortus equi, Escherichia coli 086, Salmonella typhimurium C5 and Staphylococcus aureus. These studies suggested that there might be only one species of opsonin in crayfish haemolymph for all these strains of bacteria. Such conclusions received further support from data which showed that the opsonic activity in crayfish serum for Salmonella abortus equi could be removed by adsorption with Escherichia coli 086 and Salmonella typhimurium C5. Adsorption with comparable levels of Staphylococcus aureus resulted in only a partial removal of opsonic activity for Salmonella abortus equi. This may be due to the opsonins in crayfish serum possessing a lower affinity for the antigenic
determinants on this strain of bacteria.

A series of experiments reported in the text showed that the rate of elimination of bacteria from the circulation of the crayfish was independent of the dose of bacteria injected, in contrast to the fall in the number of circulating haemocytes which was related closely to the concentration. It appears likely from these results that the cells lining the haemal sinus are of importance in removing foreign material from the haemolymph although the cells in the circulation are actively phagocytic. An analogy may be drawn here with the vertebrates where injected foreign particles are removed by the fixed phagocytic cells of the liver and spleen despite the fact that quite a large population of phagocytic cells are present in the circulation.

Opsonic activity could be found also in the cell lysate of well washed crayfish haemocytes. Whether these opsonins are produced by these cells or whether they are produced from other sites in the crayfish and bind to receptors on the membrane of the haemocyte is not known at this stage. However in further work to be reported in the following chapter evidence is presented to show that opsonins for *Salmonella abortus equi* and other bacteria may be found either on the surface of the haemocytes or free in the circulation.
CHAPTER 4

Phagocytosis of bacteria by crayfish haemocytes

In-vitro

In vitro studies on the recognition of foreign particles by phagocytic cells from several different invertebrates indicate that opsonic factors in the haemolymph may function in a similar fashion to the immunoglobulins of the vertebrates (Tripp, 1966; Stuart, 1968; Prowse and Tait, 1969; McKay and Jenkin, 1970(a); Pauley, Krassner and Chapman, 1971.

However the work by Scott, 1971, has indicated that the adherence of sheep erythrocytes to the haemocytes of the cockroach, (Periplaneta americana) occurs in the absence of serum and depends on a trypsin labile receptor on the membrane of the haemocyte. Recently Anderson, Holmes and Good, 1973, have shown that haemocytes from the cockroach, (Blaberus craniifer) are capable of phagocytosing and destroying a number of strains of bacteria and that this uptake is independent of haemolymph.

In view of the apparent conflict between data presented by various workers it appeared desirable to study in detail in vitro the factors involved in the recognition of bacteria by crayfish haemocytes.
Phagocytosis of bacteria by crayfish haemocytes

In separate experiments the 5 strains of bacteria Salmonella typhimurium C5, Salmonella abortus equi, Escherichia coli 086, Staphylococcus aureus and Pseudomonas CP were added to monolayers of haemocytes before and after the bacteria had been treated with crayfish serum. The protocol for these experiments are described in Chapter 2, p.28.

The data in Table 4.1 show that the phagocytosis of the 5 different strains of bacteria by crayfish haemocytes appears to be independent of serum. Indeed the uptake of Salmonella abortus equi and Pseudomonas CP was inhibited when the bacteria were pretreated with crayfish serum. The significance of this finding will be discussed later in the chapter. It seems unlikely that the uptake of bacteria is due to small amounts of haemolymph carried over during preparation of the monolayers since haemocytes washed 4 times with 20 ml of van Harrevald's buffered salt solution ingested Salmonella abortus equi as efficiently as unwashed cells.

Studies were carried out also on the intracellular fate of bacteria following uptake by crayfish haemocytes. The experimental protocol for these experiments has been outlined in Chapter 2, p.29. The data in Fig. 4.1 shows the intracellular survival of Salmonella abortus equi, Salmonella
### TABLE 4.1 Phagocytosis in-vitro of various strains of bacteria by crayfish haemocytes.

<table>
<thead>
<tr>
<th>STRAIN OF BACTERIA</th>
<th>TREATMENT OF BACTERIA</th>
<th>NO SERUM</th>
<th>%P</th>
<th>SERUM</th>
<th>%P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EXPERIMENTAL</td>
<td>CONTROL</td>
<td></td>
<td>EXPERTIMENTAL</td>
<td>CONTROL</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>Experiment 1</td>
<td>103±22*</td>
<td>75</td>
<td>179±34</td>
<td>346±21</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>160±23</td>
<td>62</td>
<td>171±26</td>
<td>380±37</td>
</tr>
<tr>
<td></td>
<td>Experiment 3</td>
<td>193±58</td>
<td>55</td>
<td>149±19</td>
<td>217±11</td>
</tr>
<tr>
<td>Salmonella typhimurium C5</td>
<td>Experiment 1</td>
<td>127±51</td>
<td>53</td>
<td>214±26</td>
<td>347±35</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>298±43</td>
<td>50</td>
<td>376±43</td>
<td>711±82</td>
</tr>
<tr>
<td></td>
<td>Experiment 3</td>
<td>181±28</td>
<td>69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli 086</td>
<td>Experiment 1</td>
<td>279±22</td>
<td>25</td>
<td>63±16</td>
<td>128±20</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>173±16</td>
<td>44</td>
<td>44± 7</td>
<td>73± 7</td>
</tr>
<tr>
<td></td>
<td>Experiment 3</td>
<td>208±16</td>
<td>44</td>
<td>56± 8</td>
<td>169±21</td>
</tr>
<tr>
<td>Pseudomonas CP</td>
<td>Experiment 1</td>
<td>172±10</td>
<td>60</td>
<td>590±61</td>
<td>772±48</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>106±44</td>
<td>79</td>
<td>211±17</td>
<td>304±17</td>
</tr>
<tr>
<td></td>
<td>Experiment 3</td>
<td>117±10</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Experiment 1</td>
<td>30± 8</td>
<td>25</td>
<td>183±20</td>
<td>292±57</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>147±34</td>
<td>33</td>
<td>147±25</td>
<td>232±23</td>
</tr>
</tbody>
</table>

* Number of bacteria in 0.1 ml ± standard deviation.
FIGURE 4.1

The survival of bacteria following uptake by haemocytes of the crayfish, (Parachaeraps bicarinatus).

- Staphylococcus aureus
- Salmonella typhimurium C5
- Pseudomonas CP
- Escherichia coli 086
- Salmonella abortus equi
typhimurium C5, Pseudomonas CP, Staphylococcus aureus and
Escherichia coli 086 in crayfish haemocytes. It is interest-
ing to note that as with phagocytic cells from the vertebrates,
there was a rapid phase of killing over the first 60 min.
Bacteria surviving after this time were killed much more
slowly over the period studied as has been found in the
vertebrates. (Cohn and Morse, 1959; Jenkin and Benacerraf
1960).

In view of the above a more detailed investigation was
carried out on the interaction between bacteria and crayfish
haemocytes over the first 60 min in order to establish the
rate of phagocytosis and intracellular death. The method used
in these experiments followed the protocol outlined in Chapter
2, p.29. Ten Leighton tubes containing monolayers of crayfish
haemocytes were prepared for each experiment together with 10
Leighton tubes containing no haemocytes. The percentage phago-
cytosis and survival of bacteria in crayfish haemocytes were
determined from duplicate tubes sampled at 0 min, 10 min, 20
min 40 min and 60 min. The data are shown for Salmonella
abortus equi, Escherichia coli 086 and Staphylococcus aureus
(Figs. 4.2, 4.3, 4.4). In all three cases at a time when 30% of
the bacteria had been taken up by crayfish haemocytes greater
then 80% had been killed by these phagocytic cells. This indicat-
a very short half life for the engulfed bacteria.
FIGURE 4.2

Rate of phagocytosis of *Salmonella abortus equi* by haemocytes from the crayfish, (*Parachaeraps bicarinatus*) and their subsequent fate within the phagocytic cells.

- Percent phagocytosis
- Percent survival within haemocytes
PERCENT PHAGOCYTOSIS

PERCENT SURVIVAL

TIME (MINUTES)
FIGURE 4.3

Rate of phagocytosis of *Escherichia coli* 086 by haemocytes from the crayfish, *(Parachaeraps bicarinatus)* and their subsequent fate within the phagocytic cells.

- Percent phagocytosis

- Percent survival within haemocytes.
FIGURE 4.4

Rate of phagocytosis of *Staphylococcus aureus* by haemocytes from the crayfish, (*Parachaeraps bicarinatus*) and their subsequent fate within the phagocytic cells.

--- Percent phagocytosis

△△△ Percent survival within haemocytes.
The effect of supernatants from haemocyte monolayers on the viability of Salmonella abortus equi

It was conceivable that the apparent killing of bacteria by crayfish haemocytes was due to the release of bactericidal principles from these phagocytic cells into the culture medium either before or after they had contacted the bacteria. These possibilities were investigated in the following way. Twelve haemocyte monolayers prepared as above were incubated at 19°C for 1h in either van Harrevald's buffered salt solution alone or in the presence of $1 \times 10^3$ Salmonella abortus equi. The supernatants were drawn off from the haemocyte monolayers and pooled to give two samples. The first consisted of supernatants from haemocyte monolayers incubated in tissue culture medium alone while the second consisted of supernatants from haemocyte monolayers incubated in the presence of $1 \times 10^3$ Salmonella abortus equi. The supernatants pooled from the haemocyte monolayers that had been in contact with Salmonella abortus equi were centrifuged at 3,000 g for 20 min to remove the bacteria. Salmonella abortus equi at a concentration of $2 \times 10^3$ organisms/ml was incubated then for 1h at 19°C in each sample of the pooled supernatants. As shown in Fig. 4.5 in neither case was there any killing of Salmonella abortus equi by the culture supernatants. It appears therefore that the uptake and killing of bacteria by haemocytes are entirely a function of these cells and is not due to the culture medium or cell products.
The effect of the supernatants from the cultures of haemocytes on the viability of *Salmonella abortus equi*.

- Supernatants pooled from normal haemocyte monolayers.
- Supernatants pooled from haemocyte monolayers preincubated with *Salmonella abortus equi* for 60 min at 19°C.
- van Harrevald's buffered salt solution.
elaborated during incubation.

Effect of treating haemocytes with trypsin on their ability to phagocytose bacteria

The previous experiments have shown that haemocytes from the crayfish are capable of ingesting *Salmonella abortus equi*, *Escherichia coli* 086, *Staphylococcus aureus*, *Salmonella typhimurium C5* and *Pseudomonas CP* irrespective of whether they had been pretreated with serum. These results suggest that the opsonins or recognition factors responsible for the uptake of these bacteria could either form an integral part of the cell membrane or else be removed passively from the haemolymph of the crayfish in a manner similar to the adsorption of cytophilic antibody onto the surface of macrophages in the vertebrates.

In order to decide between these possibilities monolayers of haemocytes were pretreated with the proteolytic enzyme trypsin (Difco, USA) before the addition of bacteria. It has been found in the vertebrates that trypsin treatment of guinea pig and mouse macrophages removed their cytophilic antibody and resulted in the exposure of receptors for these antibodies on the surface of the cells (Uhr, 1965; Nelson and Boyden, 1967). Furthermore incubation of trypsin treated macrophages with cytophilic antibody against sheep erythrocytes or bacteria restored to these cells the ability to take up such particles.
It was found that treatment of haemocytes with 0.002% trypsin for 2h at 19°C caused a significant reduction in the uptake of *Salmonella abortus equi* and other bacteria. Treatment with lower concentrations of trypsin, 0.0002%, 0.0005% was less effective while higher levels of trypsin i.e. 0.2% caused cell death after 60 min as judged by trypan blue exclusion. Attempts to pretreat the haemocytes in suspension with 0.002% trypsin was unsuccessful due to rapidity with which they aggregate at 19°C.

Haemocyte monolayers were prepared as described in Chapter 2, p.27 and treated with aliquots (0.5 ml) of 0.002% trypsin in van Harrevald's buffered salt solution for 2h at 19°C. After the first hour of incubation the medium was removed and replaced with fresh aliquots (0.5 ml) of 0.002% trypsin in van Harrevald's buffered salt solution. The data given in Table 4.2 indicate that the trypsin treatment of haemocytes significantly lowered their capacity to take up *Salmonella abortus equi*, *Salmonella typhimurium C5*, *Escherichia coli 086* and *Pseudomonas CP*. However, recognition appears to be unimpaired if the bacteria have been pretreated with crayfish serum. These findings indicate the presence of trypsin labile recognition factors associated with the cell membrane of the haemocytes but give no indication as to whether they are similar to those present in the haemolymph. In order to explore this possible relationship, trypsin treated haemocytes
TABLE 4.2  The phagocytosis of opsonised and unopsonised bacteria by haemocytes from the crayfish, *(Parachaeraps bicarinatus)* before and after trypsin treatment.

<table>
<thead>
<tr>
<th>Strain of Bacteria</th>
<th>Trypsin treated cells</th>
<th>Normal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% phagocytosis of bacteria in 60 min</td>
<td>Opsonised</td>
</tr>
<tr>
<td>Salmonella abortus equi</td>
<td>52</td>
<td>11</td>
</tr>
<tr>
<td>Escherichia coli 086</td>
<td>43</td>
<td>16</td>
</tr>
<tr>
<td>Salmonella typhimurium C5</td>
<td>53</td>
<td>35</td>
</tr>
<tr>
<td>Pseudomonas species CP</td>
<td>43</td>
<td>17</td>
</tr>
</tbody>
</table>

* These values are the mean of three separate experiments. The variation between experiments was not greater than 8%.
were incubated with crayfish serum using the following procedure.

Monolayers of trypsin treated haemocytes were each incubated for 60 min with 0.5 ml of crayfish serum which had been dialysed overnight at 6°C against van Harssevald's buffered salt solution. The dialysis was found necessary in order to remove cytotoxic substances for crayfish haemocytes produced as a result of clotting which is mediated by the lysis of certain cells. All the monolayers were washed three times with van Harssevald's buffered salt solution in situ and then incubated at 19°C with unopsonised bacteria and the percentage phagocytosis determined at the end of 60 min.

The data in Table 4.3 demonstrate that the uptake of Salmonella abortus equi by trypsin treated haemocytes was restored by preincubating these cells with crayfish serum. One could argue that because the cells were in a more nutritional medium they could synthesize these recognition factors. However, this seems unlikely since the activity was not restored to trypsin treated cells incubated with serum which had been adsorbed previously with Salmonella abortus equi. Thus it appears that the opsonins for Salmonella abortus equi can exist free in the haemolymph or associated with receptors on the membrane of the circulating haemocytes. Therefore they may be similar functionally to cytophilic antibody described in the vertebrates. The capacity of trypsin treated haemocytes to ingest Staphylococcus aureus was restored also by
TABLE 4.3  Phagocytosis of unopsonised *Salmonella abortus equi* by trypsin treated haemocytes before and after exposure to serum.

<table>
<thead>
<tr>
<th></th>
<th>Percent phagocytosis in 60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>No crayfish serum</td>
<td>19</td>
</tr>
<tr>
<td>Incubated with crayfish</td>
<td>50</td>
</tr>
<tr>
<td>serum previously adsorbed</td>
<td></td>
</tr>
<tr>
<td>with *Salmonella abortus</td>
<td>18</td>
</tr>
<tr>
<td>equi</td>
<td></td>
</tr>
</tbody>
</table>

* These values are the mean of 3 separate experiments. The variation between experiments was not greater than 8%.
pre-incubation with crayfish serum (Table 4.4). The experimental procedure was similar to that described above. This is a particularly interesting result since trypsin treated haemocytes would not ingest Staphylococcus aureus preopsonised with crayfish serum in contrast to the other strains. It might be that the opsonins free in the haemolymph have a low affinity for Staphylococcus aureus but function more efficiently if they are associated with the membrane of the haemocyte.

Phagocytosis of bacteria by trypsin treated haemocytes maintained in tissue culture for varying periods of time

It has been shown above that the recognition factors (opsonins) for bacteria were associated either with the cell membrane or free in the haemolymph. It was of interest therefore to see whether trypsin treated haemocytes maintained in tissue culture over 24h could synthesize these recognition factors. Thirty haemocyte monolayers were treated with trypsin as described earlier and maintained for varying periods of time at 19°C. Six trypsin treated haemocyte monolayers were removed at 2h, 4h, 8h and 24h respectively and tested for their ability to phagocytose unopsonised Salmonella abortus equi following the procedure outlined in Chapter 2, p.28. Thirty untreated haemocyte monolayers were set up as controls. Six of these haemocyte monolayers were removed at 2h, 4h, 8h and 24h respectively and assayed for their ability to take up unopsonised Salmonella abortus equi as above. The data presented in
## TABLE 4.4

Phagocytosis of unopsonised *Staphylococcus aureus* by trypsin treated haemocytes before and after exposure to serum.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percent phagocytosis in 60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>No haemolymph</td>
<td>6</td>
</tr>
<tr>
<td>Incubated with haemolymph</td>
<td>28</td>
</tr>
<tr>
<td>Normal haemocytes (CONTROL)</td>
<td>30</td>
</tr>
</tbody>
</table>

* Mean of 2 separate experiments.
Fig. 4.6 show that the ability to take up unopsonised Salmonella abortus equi by normal haemocytes was maintained over the 24h period although there was some reduction in this ability during the first 4h. However haemocytes treated with trypsin did not recover their ability to ingest unopsonised Salmonella abortus equi which suggests that these cells are unable to synthesise the recognition factors (opsonins) associated with the cell membrane.

**Survival of bacteria within trypsin treated haemocytes**

It has been shown above that trypsin treatment of haemocyte monolayers impaired their ability to take up Salmonella abortus equi and other strains of bacteria. It was of interest therefore to follow the fate of unopsonised bacteria associated with trypsin treated haemocytes to determine whether they were killed by these phagocytic cells.

The survival of unopsonised and opsonised bacteria in crayfish haemocytes pretreated with trypsin was determined by cell counts done on triplicate monolayers at regular time intervals over 3h (for further details of protocol see Chapter 2, p.28). The data for Salmonella abortus equi and Escherichia coli 086 (Figs. 4.7; 4.8) show that trypsin treatment impaired the ability of haemocytes to kill the small numbers of unopsonised bacteria associated with these cells. This could however be reversed by pretreatment of the bacteria with
FIGURE 4.6

The uptake of Salmonella abortus equi by normal and trypsin treated haemocytes maintained in tissue culture over 24h.

○○ Phagocytosis of Salmonella abortus equi by normal haemocytes.

▲▲ Phagocytosis of Salmonella abortus equi by trypsin treated haemocytes.
FIGURE 4.7

The survival of *Salmonella abortus equi* following uptake by trypsin treated haemocytes.

- Unopsonised *Salmonella abortus equi*
- Opsonised *Salmonella abortus equi*

FIGURE 4.8

The survival of *Escherichia coli 086* following uptake by trypsin treated haemocytes.

- Unopsonised *Escherichia coli 086*
- Opsonised *Escherichia coli 086*.
crayfish serum.

In view of the above observations it was necessary to determine whether the small numbers of unopsonised bacteria associated with the trypsin treated haemocytes were adhering non specifically to the membrane of these cells or were surviving inside the phagocytic cells. This aspect was investigated in the following way. Twelve monolayers of trypsin treated haemocytes were incubated with $1 \times 10^3$ unopsonised *Salmonella abortus equi* for 60 min at 19°C and then washed three times with 3 ml of van Harrevald's buffered salt solution. Cell counts were done on 2 of the monolayers and 5 of the remaining monolayers were incubated with van Harrevald's buffered salt solution containing 12.5 ug/mL Streptomycin while the other 5 monolayers served as controls. This concentration of Streptomycin was found to be the minimum required to kill 90% of *Escherichia coli* 086 over 150 min. The monolayers containing Streptomycin were washed twice with 2 ml of van Harrevald's buffered salt solution to remove residual levels of Streptomycin before sampling. The procedure for determining the number of surviving bacteria associated with the trypsin treated haemocytes followed the protocol outlined in Chapter 2, p.29. The data in Fig. 4.9 show that unopsonised *Escherichia coli* 086 taken up by trypsin treated haemocytes
FIGURE 4.9

The effect of 12.5 μg/ml Streptomycin on the population of unopsonised *Escherichia coli 086* associated with trypsin treated haemocytes.

- The fate of *Escherichia coli 086* in the absence of Streptomycin

- The fate of *Escherichia coli 086* in the presence of Streptomycin.
were killed when the monolayers were exposed to 12.5 μg/ml Streptomycin. This suggests that the bacteria were present on the surface of the trypsin treated haemocytes rather than inside the cells. It appears likely therefore that the small numbers of unopsonised bacteria taken up by trypsin treated haemocytes were caused by non-specific attachment of the bacteria to the membranes.

**Titration of opsonic activity in the serum and plasma of the crayfish**

The titre of opsonic activity for *Salmonella abortus equi* in the serum and plasma of the crayfish was determined in order to see whether the recognition factors in the serum could have been derived from the cells during the process of clotting. Twenty millilitres of serum were prepared from 5 crayfish and its titre of opsonic activity for *Salmonella abortus equi* was determined using groups of 6 trypsin-treated haemocyte monolayers for each dilution of serum. Thirty millilitres of crayfish plasma was prepared also from 5 crayfish and the opsonic titre was determined in a similar way. The crayfish plasma was dialysed overnight at 4°C against van Harrevald's buffered salt solution before opsonisation of the bacteria.

The data for the uptake of *Salmonella abortus equi* pretreated with various dilutions of serum or plasma by trypsin treated haemocytes is shown in Table 4.5. It is apparent from the data that there is appreciable opsonic activity for
TABLE 4.5 Titration of the opsonic activity in crayfish serum and plasma for *Salmonella abortus equi*.

<table>
<thead>
<tr>
<th>Dilution of serum or plasma</th>
<th>Bacteria opsonised with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td>1/2</td>
<td>32</td>
</tr>
<tr>
<td>1/5</td>
<td>35</td>
</tr>
<tr>
<td>1/10</td>
<td>37</td>
</tr>
<tr>
<td>1/20</td>
<td>5</td>
</tr>
</tbody>
</table>
Salmonella abortus equi in crayfish plasma as well as crayfish serum. This result tends to discount the possibility that a large portion of the opsonins for bacteria in crayfish serum are derived from damaged or lysed haemocytes during clotting.

**Specificity of the opsonins in normal crayfish serum**

A series of cross-adsorptions of crayfish serum with the various bacteria used in these studies was carried out in order to investigate the specificity of the opsonins. Sixty millilitres of serum were prepared from 8 crayfish and 10 ml aliquots were adsorbed for 90 min at 4° with 2 x 10^{10}/ml alcohol killed Salmonella abortus equi, Escherichia coli 086, and Salmonella typhimurium C5 respectively. The remaining 20 ml of normal serum were used to opsonise the bacteria in the control tubes for each experiment. Ten monolayers of trypsin treated haemocytes were used for each strain of bacteria, 5 monolayers for the bacteria pretreated with adsorbed serum and 5 monolayers for the bacteria pretreated with normal serum.

The results in Table 4.6 indicate that crayfish serum after adsorption with either Salmonella abortus equi or Salmonella typhimurium C5 had opsonic activity removed not only against that particular strain but also the other strain. However the opsonic activity for Escherichia coli 086 in neat crayfish serum adsorbed with the above strains of bacteria was reduced only slightly by such adsorptions.
TABLE 4.6  Investigations on the specificity of
the opsonins for bacteria in crayfish serum.

<table>
<thead>
<tr>
<th>Serum adsorbed with</th>
<th>S. abortus equi</th>
<th>E. coli 086</th>
<th>S. typhimurium C5</th>
<th>Pseudomonas CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. abortus equi</td>
<td>0 (34)*</td>
<td>36 (41)*</td>
<td>3 (31)*</td>
<td>28 (29)*</td>
</tr>
<tr>
<td>E. coli 086</td>
<td>50 (52)</td>
<td>18 (36)</td>
<td>32 (30)</td>
<td>44 (46)</td>
</tr>
<tr>
<td>S. typhimurium C5</td>
<td>11 (41)</td>
<td>32 (34)</td>
<td>10 (35)</td>
<td>17 (33)</td>
</tr>
</tbody>
</table>

* The value in brackets indicates the degree of uptake of bacteria opsonised with normal crayfish serum by trypsin treated haemocytes.
At first these data may appear to contradict results obtained in Chapter 3 on the specificity of the opsonins for Salmonella abortus equi and Escherichia coli 086 using as an assay system the reversal of blockade. These results indicated that there was an apparent lack of specificity of the opsonins in crayfish serum for these strains of bacteria. This dilemma was resolved however through the following experiment. Twenty millilitres of normal crayfish serum were pooled from 10 crayfish. Four millilitres of this serum were adsorbed for 3h at 4°C with $3 \times 10^{10}$/ml Salmonella abortus equi. The serum was centrifuged at 3,000 g for 20 min and passed finally through a 0.45 u millipore filter (Gelman Filters, USA.) to remove the bacteria. The titre of the opsonins for Escherichia coli 086 was determined in both adsorbed and unadsorbed serum using the method outlined on p. 76 of this Chapter. The data in Table 4.7a show that adsorption of crayfish serum with Salmonella abortus equi resulted in only a partial removal of opsonic activity against Escherichia coli 086. Such adsorption effected a complete removal of the opsonins against Salmonella abortus equi (Table 4.7b). Similar results were obtained using serum adsorbed with Escherichia coli 086 and titred against Salmonella abortus equi. In view of this data it appears that there may be opsonins specific for both Salmonella abortus equi and Escherichia coli 086 and opsonins which cross react with these strains. These cross reacting opsonins being in highest titre.


**TABLE 4.7a** Titration of the opsonic activity for Escherichia coli 086 in normal crayfish serum and serum adsorbed with Salmonella abortus equi.

<table>
<thead>
<tr>
<th>Dilution of serum</th>
<th>Bacteria opsonised with</th>
<th>Percent phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal serum</td>
<td>Adsorbed serum</td>
</tr>
<tr>
<td>1/2</td>
<td>31</td>
<td>29</td>
</tr>
<tr>
<td>1/4</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>1/8</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>1/10</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>1/16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1/20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**TABLE 4.7b** Opsonic activity for Salmonella abortus equi in normal crayfish serum and serum adsorbed with Salmonella abortus equi.

<table>
<thead>
<tr>
<th>Dilution of serum</th>
<th>Bacteria opsonised with</th>
<th>Percent phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal serum</td>
<td>Adsorbed serum</td>
</tr>
<tr>
<td>Neat</td>
<td>35</td>
<td>2</td>
</tr>
</tbody>
</table>
The effect of pretreatment of Salmonella abortus equi with a specific rabbit antiserum on its uptake by trypsin treated haemocytes

It has been shown by McKay and Jenkin 1970(a), that the uptake of sheep erythrocytes by haemocytes was dependent upon opsonins in the serum of the crayfish. Furthermore these opsonins showed phylum specificity in that the pretreatment of sheep erythrocytes with antibody raised in rabbits or sera from other invertebrates of different phyla would not enhance the uptake of sheep red blood cells by crayfish haemocytes. In the light of these observations it seemed pertinent to determine whether Salmonella abortus equi pretreated with specific antibody could be phagocytosed by trypsin treated haemocytes.

An antiserum raised in rabbits to Salmonella abortus equi was used in these experiments. The agglutination titre of the antiserum for Salmonella abortus equi was determined by diluting serially an aliquot (0.4 ml) of the antiserum in 0.4 ml volumes of physiological saline in 2 ml glass test tubes. Then aliquots (0.4 ml) of a suspension of $1 \times 10^9$/ml washed Salmonella abortus equi were added to each tube and mixed manually. The tubes were incubated at 37°C for 60 min and then left overnight at 6°C. The last dilution showing visible signs of agglutination of the bacteria was taken as representing the end point. In the experiments to be described
below the antiserum had an agglutination titre of \( {\frac{1}{8},000} \).

Salmonella abortus equi at a concentration of \( 2 \times 10^7 \) organisms per ml was opsonised with a \( {\frac{1}{4},000}, {\frac{1}{8},000} \) and \( {\frac{1}{16},000} \) dilution of the antiserum for 60 min at 4°C. The organisms were pipetted vigorously to break up any agglutinates and diluted in van Harrevald's buffered salt solution to give a final concentration of \( 2 \times 10^3 \) organisms per ml. Aliquots (0.5 ml) of this suspension was added to each tube containing monolayers of trypsin treated haemocytes. The percentage uptake of these organisms was determined at the end of 60 min. It was found that the uptake of Salmonella abortus equi by trypsin treated haemocytes could not be restored by opsonisation of the bacteria with a specific rabbit antiserum.

Experiments were carried out also to determine whether pretreatment of Salmonella abortus equi with specific antibody inhibited it's uptake by normal crayfish haemocyte monolayers. The bacteria were treated with \( {\frac{1}{4},000}, {\frac{1}{8},000} \) and \( {\frac{1}{16},000} \) dilutions of the antiserum for 60 min at 4°C. After pipetting vigorously to break up any agglutinates they were diluted in van Harrevald's buffered salt solution to a final concentration of \( 2 \times 10^3 \) organisms per ml. Aliquots (0.5 ml) were added to each tube containing monolayers of normal haemocytes. The percentage uptake of these bacteria was determined at the end of 60 min. As a control the uptake by normal haemocytes of Escherichia coli 086 opsonised with the same dilutions of anti-
serum was determined using the same procedure as above.

The data in Table 4.8 show that opsonisation of *Salmonella abortus equi* with specific antiserum resulted in the inhibition of uptake by normal haemocytes. However the uptake of *Escherichia coli* 086 was unaffected by opsonisation of the bacteria with the same dilutions of this antiserum. It is possible that the antibody to *Salmonella abortus equi* masks antigenic determinants which might otherwise enable the bacteria to bind to the recognition factors on the surface of the haemocytes. In view of the data presented above it is conceivable that the partial inhibition of phagocytosis of *Pseudomonas CP* and *Salmonella abortus equi* by pretreatment with crayfish serum (see Table 4.1) may be caused by recognition factors masking antigenic determinants on these bacteria which might otherwise enable the binding of the bacteria to the cell bound opsonins.

**DISCUSSION**

The data presented in this Chapter indicate that the recognition of bacteria by circulating haemocytes maintained in a tissue culture system is mediated by opsonins which may be associated either with the cell membranes or free in the haemolymph as is the case with cytophilic antibody in the vertebrates. Preliminary experiments also suggest that the membrane bound opsonins are identical to those present in the haemolymph.
The uptake of *Salmonella abortus equi* and *Escherichia coli 086* pretreated with antiserum raised against *Salmonella abortus equi* by normal haemocytes from the crayfish.

<table>
<thead>
<tr>
<th>Dilution of antiserum</th>
<th>Percent phagocytosis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Salmonella abortus equi</em></td>
</tr>
<tr>
<td>1/4000</td>
<td>20</td>
</tr>
<tr>
<td>1/8000</td>
<td>21</td>
</tr>
<tr>
<td>1/16000</td>
<td>8</td>
</tr>
<tr>
<td>No antiserum</td>
<td>60</td>
</tr>
</tbody>
</table>

* Mean of 2 separate experiments.
At first these data appear to conflict with the observations on the recognition and uptake of erythrocytes by crayfish haemocytes where it was shown that phagocytosis or binding of the red cell to the membrane did not take place unless the erythrocyte had been pretreated with serum (McKay and Jenkin, 1970a). Results of similar experiments in the vertebrates have shown that the amount of antibody required to facilitate the uptake of erythrocytes by phagocytes is greater than that required for bacteria due to the differences in surface area between the two particles, (Miescher, Spiegelberg and Benacerraf, 1963). It is possible therefore that the concentration of the opsonins on the membrane of the haemocyte is insufficient to bind the red cell but is sufficient to facilitate the uptake of bacteria. Further investigations revealed that there were opsonins specific for both Salmonella abortus equi, Escherichia coli 086, and Salmonella typhimurium C5 and opsonins which cross reacted with these strains. This will be commented on further in Chapter 6.

The interesting experiments of Scott, 1971, with haemocytes from the cockroach, (Periplaneta americana) show that the recognition of erythrocytes by these cells appear to be independent of recognition factors in the haemolymph. However, the capacity of these cells to take up erythrocytes was destroyed by treating the haemocytes with trypsin and could not be restored by opsonisation of the erythrocytes with haemo-
lymph. It seems not improbable that the crayfish and cockroach have similar recognition mechanisms but in the case of the cockroach the recognition factors have a much higher affinity for cell membranes so very few of these molecules appear free in the haemolymph, the concentration being insufficient to enhance adhesion and ingestion of erythrocytes by trypsin treated haemocytes.
CHAPTER 5

Studies on the destruction of tumour cells by haemo-
cytes from the crayfish (Paracharaeraps bicarinatus).

It has been established in vertebrates that both
lymphocytes and macrophages are capable of recognising and
destroying foreign tissue cells. Brunner, Mauel, Rudolf and
Chapius, 1969, have found that lymphocytes from mice (C3H/BL)
immunised against DBA/2 mastocytoma cells were cytotoxic for
these tumour cells in vitro. The cytotoxicity was measured
by the release of $^{51}$Cr label from the tumour cells. Reports
by Evans and Alexander, 1970; 1972 have suggested that macr-
ophages also may be cytotoxic for target cells. They found
that macrophages harvested from mice (CBA) immunised with SL2
(DBA/2) lymphoma cells inhibited the growth of these tumour
cells in vitro. Similar observations have been recorded by
Lohmann-Matthes, Schipper and Fischer, 1972. These findings
suggest that macrophages may play an important role in cell
mediated immunity (Mackaness, 1971) although their mechanism
of action is far from clear.

However not all cell mediated cytotoxic reactions
manifested by spleen cells and macrophages depends upon these
cells having been harvested from animals immunised with the
target cells. The phenomenon of "allogeneic inhibition" has
been described recently by Hellstrom and Hellstrom, 1965.
They found that lymphomas, carcinomas and sarcomas from mice (C₅₇BL) grew better in the syngeneic strain of mice (C₅₇BL) than in F₁ allogeneic strains of mice (C₅₇BL x DBA, C₃H x C₅₇BL). The inhibition of growth of such transplants in F₁ strains of mice was termed "allogeneic inhibition". This inhibition of growth is due to the contact of tumour cells with cells of the host containing H-2 isoantigens foreign to those present on the surface of the tumour cells. This phenomenon has been described also in vitro. Hellstrom, Hellstrom and Motet, 1969, found that the growth of BaLB/C sarcoma cells in tissue culture on Falcon plastic petri dishes could be inhibited by lymph nodes cells from an allogeneic strain of mice (C₃H). This inhibition of growth depended upon the presence of phytohaemagglutinin and occurred with either intact or disintegrated lymph node cells.

Recently Cooper, 1970, has shown that the earthworms, *Lumbricus terrestris* and *Eisenia fetida* are capable of recognizing and destroying reciprocal xenografts. Bailey, Miller and Cooper, 1971, have transferred successfully xenograft reactivity to normal earthworms with coelomic fluid from worms actively rejecting xenografts from the same donor strain. These results suggest that xenograft reactivity is a property of cells in the coelom of earthworms.

In the light of these observations it appears that cells from both vertebrates and invertebrates are capable of manifesting cytotoxic reactions against various target cells.
It seemed pertinent therefore to determine whether crayfish haemocytes were cytotoxic for tumour cells. Such information would extend our knowledge of the recognition of foreignness by cells from an invertebrate and these findings might be of significance in relation to the defense mechanisms of the crayfish.

The cytotoxic action of crayfish haemocytes on $^{51}$Cr-labelled Ehrlich ascites tumour cells

A series of experiments were carried out to determine whether monolayers of crayfish haemocytes were cytotoxic for $^{51}$Cr-labelled Ehrlich ascites tumour cells. Aliquots (0.5 ml) of van Harrevald's buffered salt solution containing $1 \times 10^4$ $^{51}$Cr-labelled Ehrlich ascites tumour cells were added to Leighton tubes containing monolayers of crayfish haemocytes or control Leighton tubes containing no haemocytes. The release of $^{51}$Cr label from the tumour cells was assayed at regular time intervals following the protocol outlined in Chapter 2 p. 35. The data in Fig. 5.1 show that Ehrlich ascites tumour were killed extremely rapidly by crayfish haemocytes. The maximum release of label occurred over the first 90 min. and corresponded to approximately 60% of the label introduced. When $^{51}$Cr-labelled Ehrlich ascites tumour cells were killed by immersion in chloroform they released approximately 85% of the total label. It appears therefore that greater than 70% of the Ehrlich ascites tumour cells
FIGURE 5.1

The cytotoxic action of crayfish haemocytes on $^{51}$Cr-labelled Ehrlich ascites tumour cells.

- Leighton tubes containing monolayers of crayfish haemocytes

- Leighton tubes containing no haemocytes
were killed by crayfish haemocytes over the first 120 min. These observations were confirmed by trypan blue exclusions tests. Haemocyte monolayers were treated with 0.2% trypan blue at various time intervals after the introduction of the Ehrlich ascites tumour cells. Although the haemocytes remained 90-95% viable over the 240 min. incubation period the majority of tumour cells were scored as dead after 120 min. No phagocytosis of either viable or dead intact tumour cells by crayfish haemocytes was observed over the incubation period.

The dependence of cytotoxicity on the presence of viable haemocytes

In the introduction of this Chapter it was pointed out that the growth in vitro of sarcoma cells from mice (Balb/C) could be inhibited by homogenised tissue fragments of lymph node cells from an allogeneic strain of mice (C3H). It was of interest therefore to determine whether the cytotoxicity of crayfish haemocytes for Ehrlich ascites tumour cells depended upon viable haemocytes. Haemocyte monolayers were killed by either freezing and thawing or by treatment for 15 min. with 75% alcohol. After the treatment with 75% alcohol the monolayers were washed 3 times with 3 ml of van Harrevald's buffered salt solution prior to the addition of 51Cr-labelled Ehrlich ascites tumour cells. The cell monolayers were dead after such treatments as judged by staining with trypan blue. The data in Figs. 5.2, 5.3, show that in both cases dead
The effect of treatment with 75% alcohol on the cytotoxicity of crayfish haemocytes for $^{51}$Cr-labelled Ehrlich ascites tumour cells.

- Leighton tubes containing monolayers of haemocytes treated with 75% alcohol
- Leighton tubes containing monolayers of viable haemocytes
- Leighton tubes containing no haemocytes
FIGURE 5.3

The effect of freezing and thawing haemocyte monolayers on their cytotoxicity for $^{51}$Cr-labelled Ehrlich ascites tumour cells.

- Leighton tubes containing monolayers of crayfish haemocytes
- Leighton tubes containing frozen and thawed monolayers of crayfish haemocytes
- Leighton tubes containing no haemocytes
haemocyte monolayers had lost completely their ability to kill
$^{51}$Cr-labelled Ehrlich ascites tumour cells. It seems therefore that this \textit{in vitro} cytotoxicity is a property of viable
haemocytes.

\textbf{Assay for the release of soluble cytotoxic substances}

It has been shown by Weiser, Heise, McIvor, Granger and Han, 1969, that macrophages harvested from mice (C$_{57}$BL/Ks)
immunised with the ascitic tumour SAI inhibit the growth of
these tumour cells \textit{in vitro}. This growth inhibition was
attributed in part to soluble cytotoxic substances released by
the macrophages after contact with the tumour cells. Other
reports of cytotoxins released from macrophages in contact
with target cells have appeared recently in the literature
(McIvor and Weiser, 1971(a), 1971(b)). As a result of these
reports the destruction of Ehrlich ascites tumour cells by
crayfish haemocytes was examined for the possible involvement
of soluble cytotoxic substances. Twelve haemocyte monolayers
were each incubated for 4h at room temperature (19$^\circ$C) with
$1 \times 10^4$ Ehrlich tumour cells in 0.5 ml of tissue culture
medium. Following incubation the supernatants were drawn off
with a Pasteur pipette and pooled. Ehrlich ascites tumour
cells labelled with $^{51}$Cr were diluted to a concentration of
$1 \times 10^4$/ml in the pooled supernatants or in van Harrevald's
buffered salt solution and their viability followed over 4h.
In neither case was there any release of $^{51}$Cr-label which
suggests that the killing of Ehrlich ascites tumour cells is not mediated by soluble cytotoxic materials released into the medium.

The effect of varying the ratio of haemocytes to tumour cells on the cytotoxic reaction

The effect of varying the ratio of haemocytes to tumour cells was investigated to determine whether this had any influence on the rate and total release of $^{51}$Cr-label from Ehrlich ascites tumour cells. Aliquots (0.5 ml) of van Harrevald's buffered salt solution containing either $1 \times 10^5$, $1 \times 10^6$, or $2 \times 10^6$ $^{51}$Cr-labelled Ehrlich ascites tumour cells were added to monolayers containing approximately $2 \times 10^6$ haemocytes. The protocol for these experiments followed the procedure outlined in Chapter 2, p.35. It can be seen from Fig. 5.4 that there was only a slight depression in the release of $^{51}$Cr-label in tubes where there were approximately equal numbers of tumour cells and haemocytes compared to tubes where there were smaller numbers of tumour cells. It appears likely that the majority of crayfish haemocytes are involved in this cytotoxic action against the tumour cells. This is in contradiction to what has been found in vertebrates where decreasing the ratio of immune lymphocytes to target cells causes a corresponding depression in the rate and total amount of $^{51}$Cr released from target cells. (Brunner, Mauel, Cerottini and Chapius, 1968).
The effect of varying the ratio of haemocytes to $^{51}$Cr-labelled Ehrlich ascites tumour cells on the cytotoxic reaction.

- Leighton tubes containing approximately 1 haemocyte : 1 tumour cell
- Leighton tubes containing approximately 2 haemocytes : 1 tumour cell
- Leighton tubes containing approximately 20 haemocytes : 1 tumour cell
The effect of immunisation of crayfish with Ehrlich ascites tumour cells on the cytotoxicity of the haemocytes

It was mentioned in the introduction that lymphocytes or macrophages from mice immunised with allogeneic tumour cells were cytotoxic for these same cells in vitro whereas normal cells were not unless phytohaemagglutinin was present in the system. In the light of these observations it was of interest to see whether haemocytes from crayfish immunised with Ehrlich ascites tumour cells possessed an altered cytotoxic action against these tumour cells in vitro. Groups of crayfish were immunised with $1 \times 10^7$ Ehrlich ascites tumour cells at weekly intervals over 5 weeks. Haemocytes were tested from these animals for activity against $^{51}$Cr-labelled Ehrlich ascites tumour cells at regular intervals after each injection of tumour cells. The data shown in Fig. 5.5 is pooled from the results obtained from all the groups of crayfish tested over the period of immunisation. The cytotoxic activity of the respective cell populations at 90 min, 120 min and 240 min were found to differ not significantly as determined by students "T" test. It appears therefore that immunisation of crayfish with Ehrlich ascites tumour cells does not influence the cytotoxicity of haemocytes from these animals.
FIGURE 5.5

The cytotoxic activity of haemocytes harvested from crayfish immunised with Ehrlich ascites tumour cells.

Leighton tubes containing monolayers of haemocytes collected from immunised crayfish

Leighton tubes containing monolayers of haemocytes collected from normal crayfish

Leighton tubes containing no haemocytes
The effect of crayfish serum on the cytotoxic action of haemocytes for Ehrlich ascites tumour cells

McKay and Jenkin, 1970a, demonstrated that crayfish serum possessed opsonins for sheep erythrocytes which promoted their uptake by haemocytes. It was of interest therefore to determine whether pretreatment of Ehrlich ascites tumour cells with crayfish serum enhanced their killing by crayfish haemocytes. Ehrlich ascites tumour cells labelled with $^{51}$Cr were opsonised for 60 min at 19°C with a 1/2 dil. of normal crayfish serum. The cells were washed 3 times by centrifugation at 270g for 2 min respectively with 20 ml of Hank's buffered salt solution and finally resuspended in van Harrevald's buffered salt solution to give a concentration of $2 \times 10^4$ cells/ml. An aliquot (0.5 ml) was added to each haemocyte monolayer. The data in Fig. 5.6 show that treatment of tumour cells with crayfish serum did not influence the rate at which the haemocytes kill the tumour cells.

The effect of treating haemocyte monolayers with trypsin on their cytotoxicity for Ehrlich ascites tumour cells

Weiser, Heise, McIvor, Granger and Han, 1969, established that macrophages from mice (C$_{57}$BL/Ks) immunised with the ascitic tumour SAI were cytotoxic for these tumour cells in vitro. In further work it was found that this activity could be inhibited by treatment of the macrophages with trypsin.
FIGURE 5.6

The cytotoxicity of haemocytes for $^{51}$Cr-labelled Ehrlich ascites tumour cells pretreated with crayfish serum.

- Leighton tubes containing monolayers of crayfish haemocytes with opsonised Ehrlich ascites tumour cells

- Leighton tubes containing monolayers of crayfish haemocytes with unopsonised Ehrlich ascites tumour cells

- Leighton tubes containing no haemocytes
It is thought that this enzyme removes cytophilic antibody specific for the target cell from the surface of the macrophage. The cytophilic antibody plays an important role in facilitating the binding of the target cell to the macrophage. Activity could be restored by incubating the trypsin treated macrophages with immune serum raised against the target cells. Lohmann-Matthes, Schipper and Fischer, 1972, have found that trypsin treated macrophages from mice (C57BL/6J/BOM) immunised with DBA/2 mastocytoma cells recover their cytotoxicity for these tumour cells after prolonged incubation in tissue culture. They have suggested that this may be due to the production of an antibody-like factor exocytosed from the macrophage pool.

In view of the above and our previous experiments with bacteria experiments were carried out to determine whether the recognition and binding of tumour cells to the surface of crayfish haemocytes was dependent on trypsin labile factors associated with the cell membrane. Initially haemocyte monolayers were treated for 2h at 19°C with 0.002% trypsin which had been found to be effective in the suppression of the uptake of bacteria. However this treatment did not suppress consistently the cytotoxic reaction between haemocytes and tumour cells. Consequently haemocyte monolayers were incubated for 30 min at 19°C with 0.05% trypsin. They were then washed 3 times with 3 ml of van Harrevald's buffered salt solution. Aliquots (0.5 ml) of van Harrevald's buffered salt solution containing 2 x 10^6 51Cr labelled Ehrlich ascites tumour cells were added to each of
these trypsin treated monolayers and samples were taken at regular time intervals as outlined in Chapter 2. p.35. The results shown in Fig. 5.7 demonstrate that these higher levels of trypsin suppressed the cytotoxic reaction between haemocytes and tumour cells.

In order to ensure that the trypsin treatment had not damaged the haemocytes, the following experiments were carried out. Haemocyte monolayers were prepared and treated with trypsin as described above. Aliquots (0.5 ml) of van Harrevald's buffered salt solution containing $2 \times 10^3 \text{Salmonella abortus equi}$ pre-opsonised with normal crayfish serum was added to each of 6 trypsin treated monolayers, while unopsonised \text{Salmonella abortus equi} was added as controls to the other 6 haemocyte monolayers which had been trypsin treated. It was found that while only 2% of unopsonised \text{Salmonella abortus equi} were taken up by the trypsin treated haemocytes whereas approximately 63% of the opsonised bacteria were removed by these cells. It appears therefore that treatment of crayfish haemocytes with 0.05% trypsin does not damage these cells. This was confirmed by trypan blue exclusion tests which indicated that the haemocytes in the trypsin treated monolayers were 85-90% viable.

The results of these experiments with trypsin suggested that the recognition of tumour cells as for bacteria was mediated by recognition factors associated with the haemocyte membrane. In the case of bacteria it was found that the
The effect of treating haemocyte monolayers with trypsin on their cytotoxicity for $^{51}$Cr-labelled Ehrlich ascites tumour cells.

- Leighton tubes containing untreated haemocyte monolayers
- Leighton tubes containing trypsin treated haemocyte monolayers
ability of trypsin treated haemocytes to take up bacteria could be restored by incubation with normal crayfish serum. In the light of this observation experiments were carried out in an attempt to restore the cytotoxic activity of trypsin treated haemocytes by incubation with crayfish serum. Haemocyte monolayers were incubated with trypsin as described above and then treated for a further 60 min at 19°C with normal crayfish serum dialysed against van Harrevald's buffered salt solution. Aliquots (0.5 ml) of the same medium containing 2 x 10^6 ^{51}Cr-labelled Ehrlich ascites tumour cells were added then to each of the monolayers and samples were taken at regular time intervals over 4h. The results indicated that crayfish serum could not restore the cytotoxic activity to trypsin treated haemocytes. Similar results were obtained if the Ehrlich ascites tumour cells were pre-opsonised with crayfish serum before being added to trypsin treated haemocyte monolayers. It appears therefore that the recognition factors associated with the membrane of the haemocyte are either not present in the haemolymph or in insufficient quantity to "re-arm" trypsin treated haemocytes.

Attempts to block the cytotoxic reaction between crayfish haemocytes and Ehrlich ascites tumour cells by using tumour cell fragments.

The data above suggested that the cytotoxicity of crayfish haemocytes for ^{51}Cr-labelled Ehrlich ascites tumour cells
was mediated by cell associated recognition factors. Experiments were carried out therefore to block these recognition factors using fragments of tumour cells. Ehrlich ascites tumour cells were adjusted to a concentration of $1 \times 10^8$ cells/ml in van Harrevald's buffered salt solution. Five millilitres of this suspension was sonicated for 10 min at 4°C in a MSE ultra-sonicator which resulted in the destruction of all intact cells. The sonicate was centrifuged at 2,000 g for 10 min, the supernatant withdrawn and the pellet resuspended in 5 ml of van Harrevald's buffered salt solution. Each of the haemocyte monolayers was incubated with 1 ml of either the supernatant or residue for 60 min and then washed 4 times in situ with van Harrevald's buffered salt solution. Ehrlich ascites tumour cells labelled with $^{51}$Cr were adjusted to $1 \times 10^8$/ml and aliquots (0.5 ml) were added to each monolayer. The data in Fig. 5.8 show that the supernatant had no inhibitory effect while the residue completely abolished the cytotoxic action of crayfish haemocytes. It is conceivable that this inhibition was caused by antigenic determinants on the tumour cell fragments in the pellet fraction binding to the receptors (recognition factors) associated with the membrane of the haemocytes.

**Specificity of the cytotoxic reaction**

Other cell types were labelled with $^{51}$Cr and tested in vitro with crayfish haemocytes in an attempt to determine the specificity of the cytotoxic action.
The inhibitory effect of a sonicate prepared from Ehrlich ascites tumour cells on the cytotoxicity of crayfish haemocytes.

- Leighton tubes containing haemocyte monolayers treated with the residue fraction from sonicate
- Leighton tubes containing haemocyte monolayers treated with the supernatant fraction from sonicate
- Leighton tubes containing no haemocytes
The cytotoxic action of crayfish haemocytes on $^{51}$Cr-labelled Krebs II ascites tumour cells

Krebs ascites tumour cells were labelled with $^{51}$Cr as described in Chapter 2, p.33. Aliquots (0.5 ml) of van Harrevald's buffered salt solution containing $1 \times 10^4$ tumour cells were added to monolayers of crayfish haemocytes and samples were taken at regular intervals over 4h as outlined in Chapter 2, p.35. It can be seen from Fig. 5.9 that crayfish haemocytes are cytotoxic for $^{51}$Cr-labelled Krebs tumour cells although not to the same extent as for Ehrlich ascites tumour cells. In all the experiments carried out there appeared to be a lag period of between 90-120 min before there was any detectable release of $^{51}$Cr-label from these tumour cells.

The effect of immunisation of crayfish with Krebs II ascites tumour cells on the cytotoxicity of the haemocytes

The previous experiment showed that crayfish haemocytes were not as cytotoxic for Krebs ascites tumour cells as for Ehrlich ascites tumour cells. In view of these observations experiments were designed to determine whether immunisation of crayfish with Krebs II ascites tumour cells could enhance the cytotoxicity of the haemocytes for these tumour cells. Crayfish were immunised at weekly intervals over 4 weeks with either $1 \times 10^7$ Krebs cells or $1 \times 10^7$ Krebs cells together with
The cytotoxic activity of crayfish haemocytes against $^{51}$Cr-labelled Krebs II ascites tumour cells.

- Leighton tubes containing monolayers of crayfish haemocytes
- Leighton tubes containing no haemocytes
5μg of endotoxin from Salmonella typhimurium C5. Endotoxin was used in the immunisation schedules since McKay and Jenkin, 1970a, had established that treatment of crayfish with endotoxin resulted in an enhancement of the phagocytic activity of the haemocytes. Haemocytes harvested from these animals at the end of the first and the fourth week after immunisation failed to be any more reactive against ⁵¹Cr-labelled Krebs cells than haemocytes taken from normal control animals.

The cytotoxic action of crayfish haemocytes on ⁵¹Cr-labelled Hela cells

Hela cells were used as the target cells in order to investigate further the specificity of the cytotoxic activity of crayfish haemocytes. Hela cells were labelled with ⁵¹Cr as outlined in Chapter 2, p.33. Aliquots (0.5 ml) of van Harrevald's buffered salt solution containing 1 x 10⁴ tumour cells were added to haemocyte monolayers and samples were taken at regular intervals over 4h. It was found that crayfish haemocytes were not cytotoxic when tested against ⁵¹Cr-labelled Hela cells and this was found to be the case even when the incubation period was extended over 8h.

Treatment of Hela cells with crayfish serum

Since crayfish haemocytes lacked activity against Hela cells it was necessary to determine whether prior treatment of Hela cells with crayfish serum would promote a cytotoxic
reaction. Hela cells labelled with $^{51}$Cr were preopsonised for 60 min at 19°C with 1/2 dil. of normal crayfish serum and then washed 3 times by centrifugation at 270g for 10 min with 30 ml of van Harrevald's buffered salt solution. Aliquots (0.5 ml) containing $1 \times 10^4$ cells/ml in the same medium were added to each of the haemocyte monolayers. There was no release of $^{51}$Cr-label from the opsonised Hela cells over a 4h incubation period. It was possible that the reason why Hela cells were not killed by crayfish haemocytes was due to the fact that the haemocytes lacked receptors for these tumour cells which were needed for cell to cell contact mandatory for cell lysis. This possibility was investigated as follows.

(a) **The effect of phytohaemagglutinin on the reaction between Hela cells and crayfish haemocytes**

It has been shown that normal lymphocytes from humans and mice in the presence of phytohaemagglutinin are cytotoxic for both syngeneic and allogeneic target cells *in vitro* (Pearlmann and Holm, 1969; Hellstrom, Hellstrom and Motet, 1969). The phytohaemagglutinin causes adherence of lymphocytes to target cells which is followed by blastogenesis of the lymphocytes, both of which contribute to this cytotoxicity.

Crayfish haemocytes were incubated with $^{51}$Cr-labelled Hela cells in the presence of 40 µg/ml, 80 µg/ml, 200 µg/ml or 800 µg/ml phytohaemagglutinin. None of these treatments resulted in the induction of a cytotoxic reaction between Hela cells and crayfish haemocytes.
(b) Pretreatment of Hela cells with alkali-treated lipopolysaccharide

If the supposition is correct, i.e. haemocytes are not cytotoxic for Hela cells because they lack the necessary receptor sites, then it might be possible to demonstrate a cytotoxic reaction if we impose on the Hela cell an antigen that the haemocyte can recognise. Our previous data have established that haemocytes have on their membranes recognition factors capable of reacting with Salmonella abortus equi. It was not unreasonable to assume that these factors may be directed against the lipopolysaccharide component of the cell wall of these bacteria. Hela cells were coated therefore with lipopolysaccharide from *Salmonella abortus equi* (for experimental details see Chapter 2, p.35) to provide a means by which the haemocytes might recognise and destroy these cells. Aliquots (0.5 ml) of van Harrevald's buffered salt solution containing $1 \times 10^4 \text{Cr}^{51}$-labelled Hela cells sensitised with lipopolysaccharide were added to monolayers of haemocytes from normal crayfish. As shown in Fig. 5.10 haemocytes from normal crayfish were not cytotoxic for Hela cells coated with lipopolysaccharide. In view of this lack of activity similar experiment were carried out with haemocytes harvested from crayfish which had been immunised with Hela cells. The data in Fig. 5.10 show that haemocytes from immunised crayfish were cytotoxic for Hela cells coated with lipopolysaccharide. Evidently the lipopolysaccharide enables the Hela cells to be recognised and
The cytotoxic action of crayfish haemocytes against $^{51}$Cr-labelled Hela cells pretreated with lipopolysaccharide from *Salmonella abortus equi*.

- Leighton tubes containing monolayers of haemocytes from normal crayfish
- Leighton tubes containing monolayers of haemocytes from crayfish immunised with Hela cells
- Leighton tubes containing frozen and thawed monolayers of haemocytes from normal crayfish
destroyed by haemocytes from immunised animals. The mechanism by which immunisation of crayfish with Hela cells results in an "activated" population of haemocytes is unknown at this stage. Similar results were obtained using Hela cells sensitised with lipopolysaccharide from Salmonella typhimurium C5.

**DISCUSSION**

In this Chapter the cell mediated cytotoxicity of crayfish haemocytes for Ehrlich and Krebs II ascites tumour cells has been described. This activity appears to be a property of haemocytes from normal crayfish since immunisation with tumour cells did not influence the cytotoxic activity of these cells. In this way it differs from most vertebrate systems where cell mediated cytotoxicity is a property of either lymphocytes or macrophages taken from animals immunised against the target. It differs also from "allogeneic inhibition" in that it is a property of viable haemocytes and shows specificity between the different foreign tumour cells. Thus while Ehrlich and Krebs II ascites tumour cells are killed by haemocytes of the crayfish these cells show no reaction against Hela cells. However if Hala cells are coated with lipopolysaccharide from Salmonella abortus equi they are killed by crayfish haemocytes from immunised animals. It is possible that the lipopolysaccharide facilitates the binding of Hela cells to haemocytes due to the cell associated recognition factors directed against antigenic determinants on the lipopolysaccharide of Salmonella abortus.
equi and Salmonella typhimurium C5.

Treatment of monolayers of haemocytes with trypsin partly abolished their cytotoxicity for Ehrlich ascites tumour cells, without altering their viability. These results indicated that receptors associated with the membrane of haemocytes were involved in this cytotoxic process. Incubation with crayfish serum did not restore activity and this is in contrast to that which has been observed for the phagocytosis of bacteria by crayfish haemocytes. In this case the phagocytic activity of trypsin treated haemocytes could be restored by incubation with crayfish serum.

This cell mediated cytotoxicity demonstrated by crayfish haemocytes may represent an in vivo defense mechanism ridding the crayfish of cell mutants which might otherwise develop into neoplasms. The circulating haemocytes might therefore play a role in "immune surveillance" a function of the thymus derived lymphocytes in the vertebrates.
CHAPTER 6

The Purification of the Opsonic and Haemagglutinating Activities in Crayfish Haemolymph

Data presented in the previous chapters have shown that the uptake of bacteria by haemocytes of the crayfish depends upon recognition factors which may be either free in the haemolymph or associated with the membrane of the phagocytic cell. McKay and Jenkin, 1970a, have suggested that the opsonins for erythrocytes from various vertebrates may be related to the haemagglutinins found in crayfish serum.

In this chapter the purification of the opsonins for bacteria and sheep erythrocytes is described using the technique of affinity chromatography.

Purification of the opsonins in crayfish serum for bacteria and sheep erythrocytes using affinity chromatography

Because of the low levels of haemagglutinating activity (1/32), and the opsonic activity for bacteria (1/20) in crayfish serum, gel filtration and ion exchange chromatography could not be used in the initial purification of these activities. However the technique of affinity chromatography offered a means of obtaining active material which could be used for further purification.

The antisera for the affinity columns were raised in rabbits against sheep erythrocytes or bacteria which had been
opsonised with crayfish serum (for details of protocol see Chapter 2, pp.36-38). These antisera were coupled to Biogel A 50M agarose beads using cyanogen bromide (for details see Chapter 2, pp.41-43). The affinity columns were found to retain all of the haemagglutinating and opsonic activity of crayfish serum irrespective of whether the antisera coupled to these columns came from rabbits immunised with opsonised sheep erythrocytes or bacteria. The significance of this finding is commented on below.

The rabbit antiserum to be coupled to the agarose beads was found however by immunodiffusion to contain antibodies to haemocyanin the major protein component in crayfish serum. This arose from the non specific adsorption of haemocyanin onto the rabbit erythrocytes during sensitisation with crayfish serum. The antiserum was run through therefore a haemocyanin column (for details see Chapter 2, p.43) to remove these contaminating antibodies. All of the antibody against the crayfish opsonins to sheep erythrocytes was recovered in the excluded material as assayed by the modified Coomb's technique (Table 6.1). The excluded material had no detectable antibodies to haemocyanin as judged by immunodiffusion.

The properties of the antisera raised against the opsonins for the various strains of bacteria

It has been shown in Chapter 4 that crayfish serum
The recovery of antibody activity against the opsonins in normal crayfish serum after passage through an affinity column of Haemocyanin.

<table>
<thead>
<tr>
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<th>Haemolytic titre*</th>
<th>volume</th>
<th>total units</th>
</tr>
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<tbody>
<tr>
<td>Original antiserum</td>
<td>1/160</td>
<td>7 ml</td>
<td>1120 units</td>
</tr>
<tr>
<td>Excluded material</td>
<td>1/24</td>
<td>50 ml</td>
<td>1200 units</td>
</tr>
<tr>
<td>Eluate **</td>
<td>0</td>
<td>23 ml</td>
<td>0 units</td>
</tr>
</tbody>
</table>

* expressed as the dilution showing approximately 50% lysis of sheep erythrocytes as assayed by the modified Coomb's technique.

** the material in the eluate was eluted with 3M sodium thiocyanate and dialysed overnight at 0°C against 0.05M phosphate buffered saline pH 7.1.
possesses opsonins for \textit{Salmonella abortus equi}, \textit{Salmonella typhimurium C5}, \textit{Escherichia coli O86} and \textit{Pseudomonas CP} as well as for sheep erythrocytes. These opsonic factors are either free in the haemolymph or associated with the membrane of phagocytic cells. It was of interest therefore to raise antibodies against these various opsonins in order to see if they were antigenically related. The details for the immunisation schedule are given in Chapter 2 pp.36-38. The data shown in Table 6.2 indicate that all the antisera including that raised against crayfish haemocytes promoted haemolysis of sheep erythrocytes presensitized with crayfish serum. Furthermore as has been stated above affinity columns made from antisera raised against the opsonins for \textit{Salmonella abortus equi} and \textit{Escherichia coli O86} were as efficient as the columns made from antisera raised against the opsonins for sheep erythrocytes in purifying the haemagglutinins from normal crayfish serum. It appears likely therefore that the opsonins for the bacteria and the haemagglutinins in crayfish serum are either antigenically related or are the properties of the same molecule.

\textbf{Affinity chromatography using normal crayfish serum}

Initially, affinity chromatography was carried out using normal crayfish serum in the purification of the opsonins for sheep erythrocytes and bacteria. Forty to sixty millilitres of normal crayfish serum in 0.01M tris buffered saline
TABLE 6.2 The titre of antibody against opsonins for various strains of bacteria and those associated with crayfish haemocytes in rabbits following immunisation with pre-sensitised bacteria or haemocytes, as measured by modified Coomb's technique.

<table>
<thead>
<tr>
<th>Antisera against</th>
<th>Haemolytic titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella abortus equi</em></td>
<td>$\frac{1}{500}$</td>
</tr>
<tr>
<td><em>Escherichia coli 086</em></td>
<td>$\frac{1}{2000}$</td>
</tr>
<tr>
<td><em>Salmonella typhimurium C5</em></td>
<td>$\frac{1}{20} - \frac{1}{50}$</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>$\frac{1}{50}$</td>
</tr>
<tr>
<td><em>Pseudomonas CP</em></td>
<td>$\frac{1}{50}$</td>
</tr>
<tr>
<td><em>Rabbit erythrocytes</em></td>
<td>$\frac{1}{500}$</td>
</tr>
<tr>
<td><em>Crayfish haemocytes</em></td>
<td>$\frac{1}{200}$</td>
</tr>
</tbody>
</table>

* dilution showing 50% haemolysis of sheep erythrocytes.
at pH 7.1 was passed through the column and the protein bound to the column after extensive washing with the same buffer was eluted with 3M sodium thiocyanate. The eluate was dialysed overnight against 0.01M tris buffered saline pH 7.1 at 4°C. It was found necessary to titrate the eluate in the presence of 0.01M Ca\textsuperscript{++} to yield quantitative recoveries of haemagglutinating activity. The dependence of the haemagglutinating activity in normal crayfish serum and eluate on Ca\textsuperscript{++} will be examined in greater detail later in this chapter. Other eluting agents such as 3M MgCl\textsubscript{2}, IM urea, 3.08% sodium citrate were tried but these failed to give quantitative recoveries of haemagglutinating activity in the eluate. The results of an experiment using 30 ml of normal crayfish serum is shown in Table 6.3. From these figures it can be estimated that 10% of the haemagglutinating activity and about 1.7% of the protein was eluted from the column. This represents a 42 fold increase in the haemagglutinating activity/mg protein compared with normal crayfish serum.

Affinity chromatography was carried out also on crayfish plasma to determine whether there was a similar retention of haemagglutinating activity after passage through the column. The crayfish plasma was prepared according to the method outlined in Chapter 2, p.25. Twenty millilitres of crayfish plasma were prepared from 8 crayfish and dialysed overnight at 4°C against 0.01M tris buffered saline pH 7.1. The crayfish plasma was chromatographed then on an affinity column following
TABLE 6.3 The purification of the haemagglutinins and opsonins in crayfish serum by affinity chromatography.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Haemagglutination titre</th>
<th>Total units</th>
<th>Total protein</th>
<th>Activity/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original serum</td>
<td>30 ml</td>
<td>$1/32$</td>
<td>960 units</td>
<td>2010 mg</td>
</tr>
<tr>
<td>Eluate</td>
<td>25 ml</td>
<td>$1/4$</td>
<td>100 units</td>
<td>4.80 mg</td>
</tr>
<tr>
<td>Excluded material</td>
<td>63 ml</td>
<td>0</td>
<td>0 units</td>
<td>1723 mg</td>
</tr>
</tbody>
</table>
the procedure outlined above. From the data in Table 6.4 it can be seen that all the haemagglutinating activity was retained on the column and 10% eluted with 3M sodium thio-cyanate. These figures agree closely with those obtained using normal crayfish serum.

The purification of opsonins against Salmonella abortus equi

In the previous two chapters it was shown that cray-fish serum possessed opsonins for Salmonella abortus equi, and other bacteria tested. It was of interest therefore to see if the eluate recovered from affinity columns of antibodies raised against the haemagglutinins possessed opsonic activity for Salmonella abortus equi. Salmonella abortus equi at a concentration of $1 \times 10^7$ organisms/ml was opsonised for 60 min at 19°C with either eluate or excluded material in the presence of 0.01M Ca++. The bacteria were diluted subsequently in van Harrevald's buffered salt solution to a concentration of $2 \times 10^3$ organisms/ml and aliquots (0.5 ml) were added to each monolayer of trypsin treated haemocytes. Four monolayers of trypsin treated haemocytes and 4 control Leighton tubes containing no haemocytes were used for each group of bacteria. The data in Table 6.5 demonstrate that the opsonic activity for Salmonella abortus equi was recovered in the eluate but not in the excluded material. These results correlate with the observations made earlier in this chapter that the antisera
TABLE 6.4 Purification of the haemagglutinins for sheep erythrocytes in crayfish plasma using affinity chromatography.

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
<th>Haemagglutination titre</th>
<th>Total units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original plasma</td>
<td>20 ml</td>
<td>1/32</td>
<td>640 units</td>
</tr>
<tr>
<td>Eluate</td>
<td>16 ml</td>
<td>1/4</td>
<td>64 units</td>
</tr>
<tr>
<td>Excluded material</td>
<td>38 ml</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
The effect of eluate and excluded material in the presence of 0.01M Ca\textsuperscript{++} on the uptake of *Salmonella abortus equi* by trypsin treated haemocytes.

<table>
<thead>
<tr>
<th>Treatment of bacteria</th>
<th>Percent phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluate</td>
<td>45</td>
</tr>
<tr>
<td>Excluded material</td>
<td>2</td>
</tr>
<tr>
<td>Saline (control)</td>
<td>3</td>
</tr>
</tbody>
</table>
raised against the opsonins for sheep erythrocytes and
Salmonella abortus equi cross react as determined by the modi-
fied Coomb's technique. Therefore it appears that the opsonic
activity for Salmonella abortus equi and the opsonic and
haemagglutinating activity for sheep erythrocytes may be
properties of the same or an antigenically related group of
molecules.

**Affinity chromatography carried out on crayfish serum**
in the presence of 0.01M EDTA

Affinity chromatographs were done on crayfish serum
dialysed against 0.01M EDTA for the following reasons. In
experiments to be outlined in Chapter 7 it was found that the
haemagglutinins and other proteins in crayfish serum were
capable of binding to polysaccharide substrates containing
galactose such as in the agarose making up the structure of
the Biogel A50M beads. However it was found that if the cray-
fish serum was dialysed against 0.01M EDTA then this retention
was inhibited although the antibody-antigen reaction involving
the uptake of the haemagglutinins onto antibodies bound to the
column still occurred. Furthermore in the chromatographs using
normal crayfish serum only 10% of the total haemagglutinating
activity bound to the column was eluted with 3M sodium thio-
cyanate. Affinity chromatographs were carried out therefore in
the presence of 0.01M EDTA in order to prevent the nonspecific
binding of the proteins in crayfish serum to the agarose beads
and to recover greater yields of haemagglutinating activity in the eluate.

It was found that dialysis of crayfish serum against 0.01M EDTA overnight at 4°C resulted in the loss of haemagglutinating activity. However this effect could be reversed by diluting out in 0.01M CaCl₂ resulting in a 50% recovery of haemagglutinating activity (Table 6.6). Therefore crayfish serum dialysed against 0.01M EDTA was passed through an affinity column pre-equilibrated with 0.01M EDTA in 0.01M tris buffered saline pH 7.1. The serum was washed through with 0.01M EDTA, and the column re-equilibrated with 0.01M tris buffered saline pH 7.1. Finally the bound material was eluted with 3M sodium thiocyanate. The eluate was dialysed overnight against 0.01M tris buffered saline pH 7.1 at 4°C. The results for the recovery of haemagglutinating activity and total protein are shown in Table 6.7.

Forty percent of the total haemagglutinating activity was recovered from the column when the eluate was diluted out 0.01M Ca++. This was significantly better than the recovery of haemagglutinating activity in eluates obtained from affinity chromatographs using crayfish serum in the absence of 0.01M EDTA. Furthermore as distinct from chromatographs using normal crayfish serum in the absence of EDTA, smaller amounts of protein were estimated as binding to the column and all of this was eluted with 3M sodium thiocyanate. There was a 158 fold increase in the haemagglutinating activity/mg of protein in the
TABLE 6.6 The effect of Ca\(^{++}\) on the haemagglutinating activity of crayfish serum for sheep erythrocytes.

<table>
<thead>
<tr>
<th>Treatment of crayfish serum</th>
<th>Diluting medium</th>
<th>Haemagglutination titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td>TBSA *</td>
<td>1/32</td>
</tr>
<tr>
<td>Normal serum</td>
<td>TBSA + 0.01M Ca(^{++})</td>
<td>1/64</td>
</tr>
<tr>
<td>Serum dialysed against 0.01M EDTA</td>
<td>TBSA</td>
<td>0</td>
</tr>
<tr>
<td>Serum dialysed against 0.01M EDTA</td>
<td>TBSA + 0.01M Ca(^{++})</td>
<td>1/16</td>
</tr>
</tbody>
</table>

*0.01M tris buffered saline pH 7.1.
TABLE 6.7 The purification of the haemagglutinins and opsonins in crayfish serum by affinity chromatography carried out in the presence of 0.01M EDTA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume</th>
<th>Haemagglutination titre</th>
<th>Total units</th>
<th>Total protein (mg)</th>
<th>Activity/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original serum</td>
<td>44.7 ml</td>
<td>1/32</td>
<td>1430 units</td>
<td>3263 mg</td>
<td>0.41</td>
</tr>
<tr>
<td>Eluate</td>
<td>18 ml</td>
<td>1/32</td>
<td>576 units</td>
<td>8.8 mg</td>
<td>65</td>
</tr>
<tr>
<td>Excluded material</td>
<td>48.4 ml</td>
<td>1/4</td>
<td>196 units</td>
<td>3267 mg</td>
<td>0.06</td>
</tr>
</tbody>
</table>
eluate as compared with normal crayfish serum.

It is interesting to note that the excluded material showed an agglutination titre of between $\frac{1}{2}$-4 in the presence of Ca\(^{++}\) although there was no haemolytic titre as assayed by the modified Coomb's technique. Attempts to remove this residual activity by recycling through the same column were unsuccessful.

The opsonic activity for Salmonella abortus equi in the eluate obtained from affinity chromatography done in the presence of 0.01M EDTA

The eluate and excluded material from an affinity chromatograph was tested for opsonic activity against Salmonella abortus equi. Salmonella abortus equi at a concentration of $5 \times 10^7$ organisms/ml was opsonised for 60 min at 19°C with either normal eluate or excluded material in the presence of 0.01M Ca\(^{++}\). The bacteria were diluted subsequently in van Harrevald's buffered salt solution to a concentration of $2 \times 10^3$ organisms/ml and aliquots (0.5 ml) were added to each Leighton tube. Four Leighton tubes containing trypsin treated haemocytes and four Leighton tubes containing no haemocytes were used for each group of bacteria. It is apparent from the data (Table 6.8) that all the opsonic activity for Salmonella abortus equi was recovered in the eluate and not the excluded material. These results taken together support the concept that the opsonins for Salmonella abortus equi and sheep
TABLE 6.8  The effect of the eluate and excluded material in the presence of 0.01M Ca\textsuperscript{++} on the uptake of Salmonella abortus equi by trypsin treated haemocytes.

<table>
<thead>
<tr>
<th>Treatment of bacteria</th>
<th>Percent phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluate</td>
<td>21</td>
</tr>
<tr>
<td>Excluded material</td>
<td>0</td>
</tr>
<tr>
<td>Saline (control)</td>
<td>0</td>
</tr>
</tbody>
</table>
erythrocytes are properties of an antigenically related group of molecules. The range of activity of the opsonins in the eluate for *Salmonella abortus equi* and other strains of bacteria was investigated in the following section.

The range of activity of the opsonins in the eluate obtained from affinity chromatographs carried out in the presence of 0.01M EDTA

Since the opsonins against *Salmonella abortus equi*, *Salmonella typhimurium C5* and *Escherichia coli 086* in whole crayfish serum appeared to cross react it was necessary to determine whether the eluate obtained from affinity chromatography which possessed opsonic activity against *Salmonella abortus equi* also possessed activity against the other two strains of bacteria. The protocol for the opsonisation of bacteria was similar to that described above and four haemocyte monolayers treated with trypsin was used for each strain of bacteria. As can be seen from the data in Table 6.9 not only does the eluate possess opsonic activity against *Salmonella abortus equi* but also it possesses activity against *Escherichia coli 086* and *Salmonella typhimurium C5*. This data suggests that the opsonic activities for these three strains of bacteria and as will be shown later for sheep erythrocytes may be related functions of the same molecular species. These conclusions were supported further by the results of the experiments cited below.
### TABLE 6.9

The opsonic activity of the eluate from affinity chromatography carried out in the presence of 0.01M EDTA.

<table>
<thead>
<tr>
<th>Strain of bacteria used in these studies</th>
<th>Percent phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unopsonised</td>
</tr>
<tr>
<td><strong>Salmonella abortus equi</strong></td>
<td>0</td>
</tr>
<tr>
<td><strong>Salmonella typhimurium C5</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>Escherichia coli 086</strong></td>
<td>14</td>
</tr>
</tbody>
</table>
Immunodiffusion using whole crayfish serum or eluate from affinity chromatography carried out in 0.01M EDTA.

Immunodiffusion was carried out on either the eluate or normal crayfish serum to confirm that the opsonins for Salmonella abortus equi, Escherichia coli 086 and sheep erythrocytes were antigenically similar. The technique of immunodiffusion has been outlined in Chapter 2, pp.48-49. It was found that with eluate in the absence of Ca** two lines of complete identity were formed against the antisera raised against the opsonins for Salmonella abortus equi, Escherichia coli 086 and the haemagglutinins. However there were no precipitin lines formed in the presence of 0.01M Ca**. This difference is due to the fact that in the presence of Ca**, the opsonins in the eluate bind to the agarose in the gel and are thus prevented from forming a precipitin line with the antisera. When normal crayfish serum was used in the absence of Ca** then two precipitin lines of complete identity were seen with each of the antisera cited above. The precipitin line next to the antisera wells was due to haemocyanin since it was found to be present also when the excluded material from affinity chromatography was used instead of whole crayfish serum. The excluded material contained no haemagglutinating, haemolytic or opsonic activity for sheep erythrocytes or bacteria. The other precipitin line next to the antigen well was due to the opsonins for bacteria since
it was absent from immunodiffusion plates using excluded
material from affinity chromatographs instead of whole crayfish
serum. Therefore the opsonins for *Salmonella abortus equi*,
*Escherichia coli* 086 and sheep erythrocytes both in normal
crayfish serum and eluate from affinity chromatography appear
to be antigenically similar.

The biological properties of the eluate in the presence
and absence of 0.01M-Ca$^{++}$. 

Since it was found that the restoration of the haemagglutinating activity of the eluate obtained from affinity chromatographs in the presence of EDTA was dependent on Ca$^{++}$ it was of interest to determine whether the haemagglutinins in the eluate were capable of binding to sheep erythrocytes in the absence of Ca$^{++}$. 

An aliquot (0.2 ml) of eluate was diluted serially in g
tubes containing vols. (0.2 ml) of 0.01M tris buffered saline
either in the presence or absence of 0.01M Ca$^{++}$. To each of
these tubes was added 0.2 ml of 1% (V/V) washed sheep erythro-
cytes and the mixture incubated for 1h at $19^\circ$C with occasional
shaking. The cells were then washed 3 times with 3 ml of
physiological saline by centrifugation at 1,000g for 5 min
respectively and resuspended in 0.4 ml of the same medium. An
aliquot (0.2 ml) of a $^{1}/30$ dilution in saline of an antiserum
raised in rabbits against the haemagglutinins was added to
each tube. Finally 0.2 ml of a $\frac{1}{10}$ dilution of fresh guinea pig serum in physiological saline was used as a source of complement. The rabbit antiserum had been adsorbed with an equal vol of sheep red blood cells overnight to remove natural antibody to sheep erythrocytes. The tubes were incubated at 37°C for 30 min and the end-point of haemolysis was determined as above.

It was found that if the eluate was titrated in 0.01M tris buffered saline pH 7.1 in the absence of Ca$^{++}$ then there was the same order of haemolytic activity (titre $\frac{1}{64}$-$\frac{1}{128}$) relative to the eluate being diluted in the presence of Ca$^{++}$ (titre $\frac{1}{128}$) as assayed by the modified Coomb's technique. Furthermore when the eluate in the absence of Ca$^{++}$ was adsorbed with $\frac{1}{5}$ packed vol. sheep erythrocytes for 3h at $4^\circ$C then the activity of the haemagglutinins as assayed by the modified Coomb's technique was removed. If this adsorbed eluate was diluted then in 0.01M Ca$^{++}$ there was no recovery of haemagglutinating activity.

Taken together these data indicate that although the agglutinating activity of the haemagglutinins for sheep erythrocytes is dependent on Ca$^{++}$ they are still capable of binding to sheep red blood cells in the absence of Ca$^{++}$. Further investigations were carried out to determine whether the opsonic activity of the eluate for sheep erythrocytes and Salmonella abortus equi was dependent on Ca$^{++}$. 
The opsonic activity of the eluate obtained from affinity chromatography was carried out in 0.01M EDTA.

The opsonic activity of the eluate against sheep erythrocytes and bacteria was determined both in the presence and absence of 0.01M Ca\(^{++}\).

The opsonic activity of the eluate for sheep erythrocytes was determined using a method similar to that described by McKay and Jenkin, 1970a. Washed erythrocytes (5.0 x 10\(^8\) cells were added to tubes containing aliquots (1.0 ml) of eluate in suitable dilutions of 0.01M tris buffered saline pH 7.1 in the presence or absence of Ca\(^{++}\). The red cells were incubated at 19\(^\circ\)C for 60 min and washed three times by centrifugation with 30 ml of the same medium. The washed cells were resuspended in 0.5 ml of van Harrevald's buffered salt solution and aliquots (0.1 ml) were added to haemocyte cultures in Leighton tubes. The haemocytes and erythrocytes were incubated for 90 min at 6\(^\circ\)C. Coverslips were removed from the tubes and gently washed in van Harrevald's salt solution to remove loosely adherent cells. Microscopic fields were chosen at random until approximately 400 haemocytes had been counted. Results were expressed as the total number of erythrocytes associated with 400 haemocytes. Data given in Table 6.10a show that opsonic activity of the eluate for sheep erythrocytes was dependent on Ca\(^{++}\)ions since very low numbers of sheep erythrocytes opsonised with eluate in the absence of
Ca\textsuperscript{++} ions were taken up by crayfish haemocytes.

The opsonic activity of the eluate against \textit{Salmonella abortus equi} was determined both in the presence and absence of Ca\textsuperscript{++}. The protocol for these experiments followed the procedure outlined on p.106 of this chapter. Four Leighton tubes of trypsin treated haemocytes and four Leighton tubes containing no haemocytes was used for each group of bacteria. As can be seen from Table 6.10b the opsonic activity of the eluate for \textit{Salmonella abortus equi} was dependent on Ca\textsuperscript{++}. The modified bactericidal test outlined below investigated the reason for this dependence of opsonic activity on Ca\textsuperscript{++}.

**Modified bactericidal test for detecting the opsonins against \textit{Salmonella abortus equi}**

The binding of crayfish opsonins to \textit{Salmonella abortus equi} could be assayed conveniently by the following bactericidal test. \textit{Salmonella abortus equi} at a concentration of $1 \times 10^9$ organisms/ml was incubated for 1h at 19°C with 1 ml of suitable dilutions of either normal crayfish serum or eluate in the presence and absence of Ca\textsuperscript{++}. They were diluted then in saline to give a concentration of $4 \times 10^3$ bacteria/ml and aliquots (0.5 ml) were added to glass tubes followed by 0.35 ml of saline containing 0.002M Mg\textsuperscript{++} together with 0.1 ml of \textsuperscript{1/100} dilution of rabbit antiserum and 0.05 ml of fresh guinea pig serum. The rabbit antiserum was raised against rabbit red cells that had been sensitised with normal crayfish serum (for details see Chapter 2, p.36). Both the rabbit antiserum and
**TABLE 6.10a.** The opsonic activity of the eluate for sheep erythrocytes in the presence and absence of Ca$^{++}$.

<table>
<thead>
<tr>
<th>Treatment of erythrocytes</th>
<th>RBC$^\ast$/400 haemocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat Eluate</td>
<td>368</td>
</tr>
<tr>
<td>$1/2$ Eluate</td>
<td>88</td>
</tr>
<tr>
<td>$1/4$ Eluate</td>
<td>-</td>
</tr>
<tr>
<td>$1/8$ Eluate</td>
<td>37</td>
</tr>
<tr>
<td>$1/16$ Eluate</td>
<td>48</td>
</tr>
<tr>
<td>$1/32$ Eluate</td>
<td>40</td>
</tr>
<tr>
<td>Saline control</td>
<td>35</td>
</tr>
<tr>
<td>$1/8$ Eluate in the presence of 0.01M Ca$^{++}$ ($=2$ H.A. units)</td>
<td>1040</td>
</tr>
</tbody>
</table>

$^\ast$ RBC = sheep red blood cells  
$^{**}$2H.A. = 2 haemagglutinating units

**TABLE 6.10b.** The opsonic activity of the eluate for *Salmonella abortus equi* in the presence and absence of Ca$^{++}$.

<table>
<thead>
<tr>
<th>Treatment of bacteria</th>
<th>Percent phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluate</td>
<td>23%</td>
</tr>
<tr>
<td>Eluate in the presence of 0.01M Ca$^{++}$</td>
<td>50%</td>
</tr>
<tr>
<td>Saline (control)</td>
<td>22%</td>
</tr>
</tbody>
</table>
guinea pig serum had been adsorbed previously for 2h at 4°C with 2 x 10^{10}/ml acetone dried Salmonella abortus equi to remove natural antibodies against this organism. Duplicate aliquots (0.1 ml) were taken from the tubes at 0 min, 30 min, 60 min and 120 min and plated onto agar. The end point was taken as the dilution which killed 90% of the bacteria in 60 min.

The data in Table 6.11 show that the opsonins for Salmonella abortus equi in normal crayfish serum and eluate in the presence of Ca^{++} bound to the bacteria and yielded an end point titre of 1/32. They did not however in the absence of Ca^{++} attach to the bacteria. It appears therefore that the inactivity of the opsonins for Salmonella abortus equi in the absence of 0.01M Ca^{++} is due to their inability to bind to the bacteria.

**DISCUSSION**

In this Chapter the purification of the haemagglutinating and opsonic activities in normal crayfish serum for sheep erythrocytes and bacteria has been described using the technique of affinity chromatography. Such a technique was extremely useful since it enabled us to purify biologically active material from normal crayfish serum. The best enrichment in biological activity was achieved by passing the serum through the affinity column in the presence of 0.01M EDTA. The eluates from these column chromatographs not only contained all of the haemagglut-
TABLE 6.11  The bactericidal assay for the presence of opsonins against *Salmonella abortus equi*.

<table>
<thead>
<tr>
<th>Treatment of bacteria</th>
<th>Bactericidal titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal crayfish serum</td>
<td>1/32</td>
</tr>
<tr>
<td>Eluate</td>
<td>0</td>
</tr>
<tr>
<td>Eluate in the presence of 0.01M Ca²⁺</td>
<td>1/32</td>
</tr>
</tbody>
</table>
inating activity for the sheep erythrocytes but also the opsonic activity for sheep erythrocytes and bacteria. Therefore it appears likely that the haemagglutinins and opsonins in normal crayfish serum are an antigenically related group of molecules. This conclusion was supported further by immunodiffusion studies which showed that the opsonins for bacteria and sheep erythrocytes were antigenically similar.

The haemagglutinating and opsonic properties of the eluate was found to be dependent on Ca\textsuperscript{++}. Thus eluate in the absence of Ca\textsuperscript{++} was found to possess neither opsonic nor haemagglutinating activities. However the haemagglutinins could still bind to sheep erythrocytes as assayed by the modified Coomb's technique in contrast to bacteria. The significance of these findings will be discussed further in the next chapter.

The dependence of haemagglutinating activity on Ca\textsuperscript{++} appears to be a common feature of many of the haemagglutinins studied, (Marchalonis and Edelman, 1968; Acton, Bennett, Evans and Schrohenloher, 1969; Finstad, Litman, Finstad and Good, 1972). The importance of Ca\textsuperscript{++} ions for stabilizing the biological activity and structure of the haemagglutinins for sheep red blood cells in crayfish serum will be discussed further in Chapter 7.
CHAPTER 7

Further purification and characterisation of the molecules responsible for the opsonic and haemagglutinating activities of crayfish serum

In the previous chapter the technique of affinity chromatography was described as the initial step in the purification of haemagglutinins for sheep erythrocytes. Fractions obtained by these means possessed opsonic activity for different strains of bacteria. Data presented suggested that these biological activities were properties of an antigenically related group of molecules. It seemed to us desirable to purify further these molecules in order to determine their molecular weight and to see if they possessed a sub-unit structure analogous to that which has been described for the haemagglutinins obtained from other invertebrates, (Marchalonis and Edelman, 1968; Finstad, Litman, Finstad and Good, 1972).

The use of gel filtration in the purification of the haemagglutinins for sheep erythrocytes

Previously the purification of material possessing opsonic activity for bacteria and haemagglutinating activity for a variety of erythrocytes has been followed conveniently by assaying fractions for activity against sheep red blood cells using either direct haemagglutination or a modified
Coomb's type technique. In view of its convenience the latter technique was used for assaying fractions obtained by gel chromatography. However, as indicated in the text, investigations were carried out also to see if there were any dissociation of haemagglutinating and opsonic properties during these procedures. Prior to gel-filtration fractions possessing both haemagglutinating and opsonic activities were concentrated with aquacide 11 (Calbiochem, U.S.A.).

**Gel filtration using Biogel P-150**

A glass column (100 cm x 1.3 cm) was packed with Biogel beads P-150 (200-400 mesh) in 0.1M tris buffered saline pH 8.0. An alkaline pH was chosen to ensure that none of the protein was retarded non-specifically on the polyacrylamide beads.

The calibration of the column was carried out using the following markers viz., human gamma-globulin, bovine serum albumin and ovalbumin. Two millilitres of 0.1M tris buffered saline pH 8.0 containing 5 mg of each of the respective markers was loaded onto the column. The flow rate of the column was 3 ml/h and the whole procedure was carried out at 6°C. The optical adsorption at 280 nm of the fractions collected from the column was assayed using a Schimadzu Spectrophotometer (Schimadzu, Japan) in order to determine the elution vol. of the respective markers. Human gammaglobulin having a mol.wt. of 150,000 daltons was excluded from the column and came through in the
void volume (Eo) which corresponded to 51.5 ml. Biogel P-150 excluded proteins of mol.wt. greater than or equal to 150,000. Bovine serum albumin (mol.wt. 67,000) had an elution vol. (Ev) of 61.5 ml while ovalbumin (mol.wt. 43,000) had an elution vol. of 70.4 ml. The calibration graph of the column was determined by plotting the ratio $E_v/E_o$ of the respective markers against log. mol. wt. (Fig. 7.1). For Biogel P-150 there is a linear relationship of log. mol. wt. to the ratio $E_v/E_o$ for globular proteins of mol.wt. between 15,000 and 140,000. (BIO-RAD laboratories price list and technical information booklet, pp. 42-43, 1968).

Two millilitres of eluate obtained from affinity chromatography containing 2 mg of protein was loaded onto the column. The flow rate of the column was 3 ml/h and the whole procedure was carried out in a cold room (6°C). A total of 54% of the protein was recovered following gel filtration. The data in Fig. 7.2 show that the peak of activity of the haemagglutinins for sheep erythrocytes as assayed by the modified Coomb's technique corresponds to an elution vol. of 58.3 ml. The mol.wt. as estimated from the calibration line (Fig. 7.1) gives a value of 81,000 daltons.

This same procedure was carried out also using crayfish serum to determine whether the haemagglutinins for sheep erythrocytes were of the same mol.wt. as those isolated from the material purified by affinity chromatography. The data
FIGURE 7.1

The calibration of the Biogel P-150 column using bovine serum albumin (67,000 mol.wt.) and ovalbumin (43,000 mol.wt.)
The graph shows the relationship between the log of molecular weight (log. mol. wt.) and increasing elution volume. The graph includes data points for Ovalbumin and Bovine Serum Albumin. The y-axis represents the ratio of Ev/Evo, and the x-axis represents log. mol. wt. The data points are marked with circles.

- **Ovalbumin** is represented by a data point at approximately log. mol. wt. 4.4 and elution volume of 1.5.
- **Bovine Serum Albumin** is represented by a data point at approximately log. mol. wt. 4.9 and elution volume of 0.5.
FIGURE 7.2

The elution pattern on Biogel P-150 of the haemagglutinins for sheep erythrocytes purified by affinity chromatography.

- Protein profile of the material purified by affinity chromatography.

Profile of the haemagglutinating activity as assayed by the modified Coombs technique.

▲ Protein of marker proteins.

Peak 1: Human gamma globulin, mol.wt. 150,000.
Peak 11: Bovine serum albumin, mol.wt. 67,000.
Peak 1ll: Ovalbumin, mol.wt. 43,000.
are shown in Fig. 7.3 where it may be seen that the elution vol. of the peak of activity as assayed by modified Coomb's technique corresponds to 58.5 ml which gives a mol.wt. of 81,000 daltons. Thus the haemagglutinins for sheep erythrocytes purified by affinity chromatography corresponds to that which is present in whole crayfish serum. It is interesting to note that the peak of haemolytic activity is distinct from the major protein peak (haemocyanin).

It was found that the haemagglutinating activity in normal crayfish serum could be enhanced 2 fold by dialysis against 0.01M Ca++. It was of interest therefore to determine whether this potentiation of haemagglutinating activity correlated with a change in the mol.wt. of the haemagglutinins. An aliquot (2.4 ml) of crayfish serum which had been dialysed overnight against 0.01M Ca++ was passed through a Biogel P-150 column which had been pre-equilibrated with 0.01M Ca++. The elution profile is shown in Fig. 7.4 from which it can be seen that the elution vol. of the peak of activity (58 ml) corresponds to that from normal crayfish serum. Therefore, while the addition of 0.01M Ca++ caused a 2 fold increase in activity it did not alter the mol.wt. of the haemagglutinins.

Investigations were carried out also to determine whether opsonic activity for Salmonella abortus equi could be recovered in the active fractions from Biogel P-150. Thirty millilitres of eluate from affinity chromatography were con-
FIGURE 7.3

The elution pattern of normal crayfish serum on Biogel P-150.

○---○ Protein profile of crayfish serum.

□---□ Profile of the haemagglutinating activity as assayed by the modified Coombs technique.

▲---▲ Protein of marker proteins.

Peak 1; Human gamma globulin, mol.wt. 150,000.
Peak II; Bovine serum albumin, mol.wt. 67,000.
Peak III; Ovalbumin, mol.wt. 43,000.
FIGURE 7.4

The elution pattern of crayfish serum in the presence of 0.01M Ca\(^{++}\) on Biogel P-150.

- Protein profile of crayfish serum.

Profile of the haemagglutinating activity as assayed by the modified Coombs technique.

Protein of marker proteins

Peak 1; Human gamma globulin, mol.wt. 150,000.
Peak II; Bovine serum albumin, mol.wt. 67,000.
Peak III; Ovalbumin, mol.wt. 43,000.
centrated with acquicide 11 (Calbiochem, USA) to a final volume (2 ml). This material was dialysed against 0.01M Ca\textsuperscript{++} and chromatographed on Biogel P-150 in the presence of 0.01M Ca\textsuperscript{++}. The fractions containing activity as assayed by the modified Coomb's enhancement technique were pooled and concentrated with acquicide 11 to a final volume (3 ml). Such material had a haemagglutinating titre of 1/4 for sheep erythrocytes. An aliquot (1.5 ml) of the material was dialysed against 0.01M Ca\textsuperscript{++} in physiological saline. *Salmonella abortus equi* at a concentration of 1 x 10\textsuperscript{6} organisms/ml was opsonised with 0.5 ml of the above material for 1 hr at 19°C. They were diluted then in van Harrevald's buffered salt solution to a concentration of 2 x 10\textsuperscript{3} organisms/ml and aliquots (0.5 ml) were added to Leighton tubes containing either trypsin treated haemocytes or no haemocytes. Percentage phagocytosis was determined at the end of 60 min. As can be seen from the data in Table 7.1 the purified material enhanced the uptake of *Salmonella abortus equi* by trypsin treated haemocytes. This data supports further the contention that the haemagglutinins for sheep erythrocytes and the opsonins for bacteria may be properties of a related group of molecules.

**Gel filtration using Biogel A0.5M**

It was found that the material purified on Biogel P-150 gave 10 bands on polyacrylamide gel electrophoresis ranging from 77,000 to 12,000 mol.wt. when carried out in the presence
TABLE 7.1  The uptake of *Salmonella abortus equi* pretreated with material purified on Biogel P-150 by trypsin treated haemocytes.

<table>
<thead>
<tr>
<th>Treatment of bacteria</th>
<th>Percent phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal crayfish serum</td>
<td>45</td>
</tr>
<tr>
<td>Material purified on Biogel P-150</td>
<td>38</td>
</tr>
<tr>
<td>Saline (control)</td>
<td>5</td>
</tr>
</tbody>
</table>
of sodium dodecyl sulphate and B mercapto ethanol. These impurities were due to the fact that the fractions containing haemolytic activity as assayed by the modified Coomb's technique ran very close to the fractions containing small amounts of haemocyanin. Such levels of haemocyanin contaminated eluates from affinity chromatographs due to non-specific adsorption of this protein onto the Biogel A50M beads composing the affinity columns. In order to achieve a clearer separation of the haemagglutinins from haemocyanin gel filtration was carried out on material from affinity chromatographs using Biogel A 0.5M (200-400 mesh). It was found that if either the eluate from affinity chromatography or whole crayfish serum was passed through the Biogel A 0.5M column all the activity as assayed by the modified Coomb's technique was retarded on the agarose beads. This was confirmed by passing 3 ml of normal crayfish serum through a small column (30 mm x 1.1 mm) of Biogel A 0.5M beads in the absence or presence of 0.01M EDTA. In the absence of 0.01M EDTA only 30% of the haemagglutinating activity against sheep erythrocytes could be recovered in the excluded material after passage through the column, however 100% of the activity could be recovered in the excluded material if the serum was dialysed previously against 0.01M EDTA (Table 7.2).

Therefore chromatographs on Biogel A 0.5M were carried out in the presence of 0.01M EDTA to inhibit the retardation
**TABLE 7.2**  Recovery of haemagglutinating activity after passage of normal crayfish serum in the absence or presence of 0.01M EDTA through a column (30 m.m x 1.1 m.m.) of Biogel A0.5M beads.

**Normal serum**

<table>
<thead>
<tr>
<th>haemagglutination titre</th>
<th>vol.</th>
<th>Total units</th>
</tr>
</thead>
<tbody>
<tr>
<td>original serum</td>
<td>( \frac{1}{64} )</td>
<td>3 ml</td>
</tr>
<tr>
<td>excluded material</td>
<td>( \frac{1}{2} )</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

**Serum dialysed against 0.01M EDTA**

<table>
<thead>
<tr>
<th>haemagglutination titre *</th>
<th>vol.</th>
<th>Total units</th>
</tr>
</thead>
<tbody>
<tr>
<td>original serum</td>
<td>( \frac{1}{128} )</td>
<td>2.3 ml</td>
</tr>
<tr>
<td>excluded material</td>
<td>( \frac{1}{32} )</td>
<td>8.3 ml</td>
</tr>
</tbody>
</table>

* diluted out in 0.01 Ca++.  

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...
of the haemagglutinins on the agarose beads. Calibration was carried out using the following markers viz., human gamma-globulin (150,000 mol.wt.), bovine serum albumin (67,000 mol. wt.) and ovalbumin (43,000 mol.wt.) in the presence of 0.01M EDTA. The procedure followed the protocol outlined previously for the Biogel P-150 column. The calibration graph was determined by plotting the ratio $E_v/E_o$ of the respective markers against log. mol. wt.

Fourteen millilitres of eluate obtained from affinity chromatography were concentrated with acquacide I1 (Calbiochem, U.S.A.) to a final vol. of 1 ml which was dialysed overnight at 6°C against 0.1M tris buffered saline pH 7.1 containing 0.01M EDTA. An aliquot (0.6 ml) of this material containing 3 mg of protein was chromatographed on the Biogel A 0.5M. It was estimated that 75% of the protein was recovered after passage through this column. The results are shown in Fig. 7.5 and the peak of activity as assayed by the modified Coomb's technique has an elution vol. of 64 ml located between the markers bovine serum albumin (elution vol. 60 ml) and ovalbumin (elution vol. 66 ml). By reference to the calibration graph it was estimated that the mol.wt. of the haemagglutinins for sheep erythrocytes in the presence of 0.01M EDTA was 51,200 daltons.

Further experiments were carried out to determine whether the mol.wt. of the haemagglutinins in crayfish serum dialysed against 0.01M EDTA corresponded with the estimate made
FIGURE 7.5

The elution pattern on Biogel A 0.5M in the presence of 0.01M EDTA of the haemagglutinins for sheep erythrocytes purified by affinity chromatography.

- Protein profile of the material purified by affinity chromatography.

- The activity of haemagglutinins as assayed by the modified Coombs technique.

▲ Protein profile of marker proteins.

Peak I; Human gamma globulin, mol.wt. 150,000.

Peak II; Bovine serum albumin, mol.wt. 67,000.

Peak III; Ovalbumin, mol.wt. 43,000.
on the material purified by affinity chromatography. An aliquot (2.6 ml) of crayfish serum dialysed against 0.01M EDTA was loaded onto the Biogel A 0.5M column. It was found that the peak of activity as assayed by the modified Coomb's technique corresponded to a mol.wt. of 52,000 daltons (Fig. 7.6) which agrees closely with the estimate made on the material purified by affinity chromatography.

It appears therefore that treatment of the haemagglutinins for sheep erythrocytes with 0.01M EDTA causes dissociation into a species of smaller mol.wt. However it could be argued that in the presence of 0.01M EDTA the Biogel A 0.5M column was not separating proteins only on the basis of mol.wt. since the fractionation characteristics of this column were observed to change in the presence of 0.01M EDTA.

In order to resolve this problem the eluate obtained from affinity chromatography was dialysed initially against 0.01M EDTA and then 0.1M tris buffered saline pH 8.0 to remove the EDTA and finally chromatographed on a sephadex G-100 column in the same buffer.

Calibration of the G-100 column was carried out using the following markers viz., bovine serum albumin (67,000 mol. wt.), ovalbumin (43,000 mol.wt.) and carbonic anhydrase (29,000 mol.wt.). The protocol involved in the calibration of the column follows the procedure described previously for the Biogel P-150 column. The calibration of the column was deter-
FIGURE 7.6

The elution pattern of crayfish serum in the presence of 0.01M EDTA on Biogel A 0.5M.

- Protein profile of crayfish serum.

- Profile of the haemagglutinating activity as assayed by the modified Coombs technique.

▲ Protein of marker proteins

Peak 1;  Human gamma globulin, mol.wt. 150,000.

Peak II;  Bovine serum albumin, mol.wt. 67,000.

Peak III;  Ovalbumin, mol.wt. 43,000.
mined by plotting the ratio $E_{V}/E_{o}$ of the respective markers against $\log \text{mol. wt.}$.

Twenty millilitres of eluate were concentrated with acquacide 11 (Cal biochem, U.S.A.) to a final vol. of 2 ml which was dialysed consecutively against 0.01M EDTA for 24 h and 0.1M tris buffered saline pH 8.0 for 24 h at 6°C. An aliquot (1.5 ml) of this material was loaded onto a sephadex G-100 column. The peak of activity as assayed by the modified Coomb's technique corresponds to an elution vol. of 45 ml (Fig. 7.7) which is located between the markers bovine serum albumin (elution vol. 40.5 ml) and ovalbumin (elution vol. 51 ml). By reference to the calibration graph the mol.wt. of the haemagglutinins for sheep erythrocytes was estimated at 53,700 daltons. This estimate agrees closely with that obtained on Biogel A 0.5M carried out in the presence of 0.01M EDTA. These observations taken together suggested that the haemagglutinins for sheep erythrocytes could be dissociated from a species of 81,000 mol.wt. to 52,700 mol.wt. by treatment with 0.01M EDTA. Such treatment does not destroy covalent bonds which suggests that the intact molecules might consist of subunits held together by non covalent interactions. This aspect was investigated further by polyacrylamide gel electrophoresis as described below.

**Polyacrylamide gel electrophoresis**

It has been shown above that the fractions containing
FIGURE 7.7

The elution pattern on Sephadex G100 of the haemagglutinins for sheep erythrocytes purified by affinity chromatography.

○——○ Profile of haemagglutinating activity as assayed by the modified Coombs technique.

▲——▲ Protein of marker proteins.

Peak 1; Bovine serum albumin, mol.wt. 67,000.
Peak 11; Ovalbumin, mol.wt. 43,000.
Peak 111; Carbonic anhydrase, mol.wt. 29,000.
activity as assayed by the modified Coomb's technique were separated from the major protein contaminant viz., haemocyanin on Biogel A 0.5M. These fractions were prepared therefore for polyacrylamide gel electrophoresis in the following way. They were dialysed overnight at 4°C against distilled water and then concentrated in the presence of 0.02% sodium dodecyl sulphate by freeze drying in an Edward's Centrifugal freeze dryer (Edwards High Vacuum Ltd., England). This procedure was necessary due to the small amounts of protein associated with haemolytic activity isolated from the Biogel A 0.5M column. The freeze dried material was resuspended in 20 μl of 0.05M Na₂CO₃ with 2 μl of β mercapto ethanol. Such treatment of proteins with sodium dodecyl sulphate and β mercapto ethanol destroys both covalent and non covalent bonds resulting in the dissociation into sub units. These fractions were diluted subsequently with 100 μl of upper gel buffer containing 2% sucrose and 0.02% bromophenol blue. One hundred microlitres of the above solutions containing approximately 60-80 μg of protein was loaded onto the polyacrylamide gels. The protocol for the running of the gels followed the procedure outlined in Chapter 2, pp.44-48.

Each run included the following marker proteins; transferrin (77,000 mol.wt.), bovine serum albumin (67,000 mol. wt.), ovalbumin (43,000 mol.wt.), carbonic anhydrase (29,000 mol.wt.) and lysozyme (13,000 mol.wt.). One hundred microlitres
of upper gel buffer containing 2.5 ug of each of the markers together with 2% sucrose and 0.02% bromophenol blue were loaded onto the polyacrylamide gels. The calibration graph is shown in Fig. 7.8 and was determined by plotting log molecular weight (M) versus relative mobility (Rf) for the respective marker proteins.

Where:  
\[ R_f = \frac{\text{distance of migration of marker protein (m.m)}}{\text{total length of running gel (m.m)}} \]

A photograph of the acrylamide gel on a fraction from the peak of the activity of the haemagglutinins having an elution volume of 64.2 ml on Biogel A 0.5M is shown in Fig. 7.9. This fraction had a titre of \( \frac{1}{512} \) for sheep erythrocytes as assayed by the modified Coomb's technique. There was 1 distinct band of protein and this was estimated from the calibration graph as having a mol.wt. of 13,500 daltons. It is thought that this may represent a sub unit of the intact haemagglutinin. In this gel there appeared also 2 minor bands of protein with mol.wt. estimated at 16,600 and 19,100 daltons. At this stage there is no adequate explanation for these minor bands. However Hammarstrom, 1972, found that the haemagglutinin for human A cells isolated from the albumin glands and eggs of the snail, (Helix pomatia) showed one major band on polyacrylamide electrophesis of 13,000 mol.wt. accompanied by several minor bands. He suggested that these minor bands might be due to
The calibration of polyacrylamide gel electrophoresis using lysozyme, carbonic anhydrase, ovalbumin, bovine serum albumin and transferrin.
TRANSFERRIN (77,000 MOL. WT.)

BOVINE SERUM ALBUMIN (67,000 MOL. WT.)

OVALBUMIN (43,000 MOL. WT.)

CARBONIC ANHYDRASE (29,000 MOL. WT.)

LYSOZYME (13,000 MOL. WT.)
The photograph of the acrylamide gel on a fraction from the peak of activity of the haemagglutinins purified on Biogel A0.5M.

<table>
<thead>
<tr>
<th>Gel</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>The fraction eluted off Biogel A0.5M having an elution volume of 64.2 ml.</td>
</tr>
<tr>
<td>B</td>
<td>Marker proteins, viz., lysozyme, carbonic anhydrase, ovalbumin and transferrin.</td>
</tr>
<tr>
<td>C</td>
<td>Marker proteins, viz., lysozyme and bovine serum albumin.</td>
</tr>
</tbody>
</table>
the heterogeneity in the mol.wt. of the haemagglutinins isolated from an outbred population of snails. It is possible that a similar situation might exist for the haemagglutinins isolated from the crayfish.

**DISCUSSION**

In this Chapter data has been presented to show that the haemagglutinins for sheep red blood cells exist as aggregates made up of sub units. Thus the haemagglutinins found in the haemolymph of the crayfish or in the presence of 0.01M Ca$^{++}$ had a mol.wt. of 81,000 daltons. Furthermore this fraction possessed opsonic activity for *Salmonella abortus equi* since it enhanced the uptake of these bacteria by trypsin treated haemocytes. This finding further confirms the evidence presented in Chapter 6 that the opsonic and haemagglutinating activities in crayfish serum are functions of a related group of molecules. Treatment of the haemagglutinins with 0.01M EDTA resulted in a species of 52,700 mol.wt. It was pointed out in the previous chapter that under these conditions the haemagglutinins lost most biological activity except the ability to bind to sheep erythrocytes as determined by the Coomb's enhancement test.

Under conditions of complete dissociation using sodium dodecyl sulphate and B mercapto-ethanol sub units of 13,500 mol.wt. were isolated on polyacrylamide-gel electrophoresis.
The haemagglutinins in the active biological state (mol.wt. 81,000) may consist therefore of 6 of these sub units of 13,500 mol.wt. while in the presence of 0.01M EDTA they may be composed of 4 such sub units giving a mol.wt. of 52,700 daltons. Recently Hammarstrom, 1972 has established that the mol.wt. of the haemagglutinin for human A erythrocytes extracted from the albumin gland of the snail, (Helix pomatia) is 79,000 daltons and is composed of 6 identical sub units of 13,000 mol.wt. It appears therefore that there may be a similarity between the sub unit structure of this haemagglutinin and the haemagglutinin described and investigated in this chapter.
The work reported in this thesis has shown that haemocytes from the crayfish recognise foreign particles such as bacteria and sheep erythrocytes by a mechanism involving primarily the combination of the foreign particles with certain recognition factors. These recognition factors may be associated either with the cell membranes or free in the haemolymph as is the case with cytophilic antibody in the vertebrates. Preliminary experiments also suggest that the membrane bound opsonins are identical to those present in the haemolymph.

These experiments contrast with the observations of Scott, 1971, who has demonstrated that the adherence of sheep erythrocytes to the haemocytes of the cockroach, (Periplaneta americana) occurs in the absence of serum and depends on a trypsin labile receptor associated with the cell membrane. It seems not improbable that the crayfish and cockroach have similar recognition mechanisms but in the case of the cockroach the recognition factors have a much higher affinity for cell membranes so very few of these molecules appear free in the haemolymph, the concentration being insufficient to enhance adhesion and ingestion of erythrocytes by trypsin treated haemocytes.

Whilst our studies indicate that trypsin treated haemocytes are unable to resynthesise the cell bound recognition factors (opsonins) it is possible that the conditions of culture were sub optimal for such processes.
Obviously it is necessary that more work be carried out to determine whether the circulating haemocytes synthesise and secrete the opsonins or whether there are other sites in the crayfish involved in this process. Further research should be done also in order to delineate the kinetics and regulation of the synthesis of these opsonins.

Although it has been shown from work reported in Chapter 4 that the circulating haemocytes are capable of phagocytosing bacteria in vitro it appears that they may be of minor importance in vivo in the elimination of bacteria from the haemolymph of the crayfish. This has been suggested from experiments which have shown that the bulk of $^{32}$P-labelled bacteria eliminated from the circulation of the crayfish is found in the carcass of the animal even after the removal of all the major organs. It is possible therefore that the phagocytic cells lining the haemal system of the crayfish are the major sites for the removal of bacteria from the circulation of the crayfish. An analogy may be drawn here with the vertebrates where injected foreign particles are removed by the fixed phagocytic cells of the liver and spleen despite the fact that a large population of phagocytic cells are present in the circulation.

In contrast to our findings with bacteria McKay and Jenkin, 1970(a), have found that monolayers of crayfish haemocytes took up sheep erythrocytes only if they were pre-treated with crayfish serum. It appears that there are in-
sufficient opsonins on the surface of haemocytes to enable the uptake of large numbers of sheep erythrocytes unless they have been pretreated with the opsonins in crayfish serum. However there are sufficient opsonins on the membranes of haemocytes to facilitate the uptake of unopsonised bacteria. This difference in the requirement for opsonins may be attributed to the larger surface area of sheep erythrocytes compared with bacteria. Similar experiments in the vertebrates have shown that the amount of antibody required to facilitate the uptake of erythrocytes by phagocytes is greater than that required for bacteria due to the difference in surface area between those two particles, (Miescher, Spiegelberg and Benacerraf, 1963).

Burnet, 1970, has suggested that the haemocytes of the invertebrates show only a crude ability to recognise foreignness. This conclusion was derived from the observations that in the transplantation of tissues between insects, homografts generally survived as well as autograft controls on the recipients although xenografts were rejected.

Data contradictory to the concepts of Burnet have appeared recently which show that the earthworms, *Lumbricus terrestris* and *Eisenia fetida* are capable of distinguishing xenografts (Cooper, 1970; Duprat, 1970). Thus the grafting of epidermal tissue between the worms, *Lumbricus terrestris* and *Eisenia fetida* resulted in an intense cellular reaction by the recipient. However allografts of epidermal tissue between
individual worms of the species, (*Lumbricus terrestris*) were rejected also following a prolonged reaction by the cells of the host (Cooper, 1969a).

In further studies, Cooper, 1970, has shown that the earthworms, (*Eisenia fetida*) primed with first set xenografts from *Lumbricus terrestris* reject second set xenografts in an accelerated manner provided only a short time has elapsed between grafting. This reaction may represent a primitive form of an anamnestic response where the cells in the coelomic fluid of the worm are activated by contact with primary graft and are boosted in activity by confrontation with antigens from the second set graft. This boosting reaction is specific since xenografts from unrelated species of earthworms are rejected on primed animals in a normal first set manner.

Recently Bailey, Miller and Cooper, 1971, have transferred successfully xenograft reactivity to normal earthworms with coelomic fluid from sensitised worms. These results further demonstrate that xenograft reactivity is a property of cells in the coelom of the earthworm.

The response of earthworms to tissue antigenic differences by the rejection of foreign transplants may represent a defense reaction capable of checking or even destroying foreign cancerous cells or neoplasms. It represents the equivalent in the invertebrates of the phenomenon of "immune surveillance" described in the vertebrates (Cooper, 1969b).
The work reported above is of particular interest since it may be related in concept to the studies described in Chapter 5 of this thesis. In this chapter it was shown that cultures of crayfish haemocytes in Leighton tubes were capable of recognising and destroying $^{51}$Cr-labelled Ehrlich ascites tumour cells or Krebs II ascites tumour cells. This cytotoxic reaction depended upon recognition factors associated with the cell surface since treatment of haemocytes with trypsin reduced their capacity to kill tumour cells. There appeared to be specificity in the cytotoxic reaction mediated by crayfish haemocytes since $^{51}$Cr-labelled Hela cells and sheep erythrocytes were not attacked by these cells. It appears likely that the receptors on the surface of haemocytes recognise as foreign certain antigenic determinants associated with the membrane of the tumour cells which results in their destruction.

Later work revealed that $^{51}$Cr-labelled Hela cells coated with a lipopolysaccharide from *Salmonella abortus equi* were killed by crayfish haemocytes provided these cells were harvested from animals which had been immunised with Hela cells. It appears that the lipopolysaccharide provides the means by which Hela cells are able to attach to haemocytes which results in the cytotoxic reaction. This attachment may be mediated by the recognition factors associated with the membranes of haemocytes, which are specific for *Salmonella abortus equi*.
The mechanism by which immunisation of crayfish with Hela cells results in an "activated" population of haemocytes is unknown at this stage. It appears likely however that this may be a non specific process since it has been shown by McKay and Jenkin, 1970(a) that haemocytes harvested from crayfish immunised with a lipopolysaccharide from *Salmonella abortus equi* take up larger numbers of sheep erythrocytes opsonised with crayfish serum than do haemocytes from normal crayfish. Evidently the immunisation with lipopolysaccharide results in an increase in the metabolic activity of these cells.

The cytotoxic system described above might prove useful in elaborating the mechanisms involved in the recognition and rejection of foreign tissue grafts by invertebrates. It has been suggested that the death of a foreign graft is due to encapsulation by host haemocytes which effectively isolate it from its source of nutrients. The interpretation based on the work presented in Chapter 5 is that encapsulation is a measure of the ability of haemocytes to recognise foreign materials and that death of the graft is a result of cell-cell interactions.

The cytotoxic properties of crayfish haemocytes for tumour cells may be of significance *in vivo* in the surveillance against aberrant or cancerous cells. Unfortunately lack of information concerning neoplasms in crayfish prevents further comparison at this stage.
Investigations on the nature of the recognition factors (opsonins) in crayfish serum revealed that there were opsonins both specific for *Salmonella abortus equi*, *Escherichia coli* 086 and *Salmonella typhimurium* C5 and opsonins which cross-reacted with these strains. These results obtained from adsorption of crayfish serum with the respective strains of bacteria revealed that the cross-reacting opsonins were in highest titre. These findings are particularly interesting since previous work on the haemagglutinins and opsonins in sera from a number of different invertebrates have indicated that they have a broad range of specificity (Tyler and Metz, 1945; Prowse and Tait, 1969). It appears therefore that the opsonins in crayfish haemolymph may enable a finer degree of discrimination of foreignness by crayfish haemocytes than has been attributed generally to the invertebrates. Indeed these opsonins may represent functionally a primitive form of antibody found in the vertebrates.

The purification of the haemagglutinins and opsonins for sheep erythrocytes by affinity chromatography resulted in a concomitant purification of the opsonins for *Salmonella abortus equi*, *Escherichia coli* 086 and *Salmonella typhimurium* C5. It seems likely therefore that the opsonins for these cells may be antigenically related as is the case for the immunoglobulins in the vertebrates.

The characterization of the molecules responsible for
opsonic and haemagglutinating activities in crayfish serum revealed that they had a mol.wt. of 81,000 daltons and were composed of 6 sub-units of 13,500 daltons. It seems not unlikely that each of these sub units may possess at least one combinding site for an antigenic specifity on either bacteria or sheep erythrocytes. Thus the opsonins which cross react with Salmonella abortus equi, Escherichia coli 086 and Salmonella typhimurium C5 may be composed of sub units with combinding sites directed against unrelated antigenic determinants on the respective strains of bacteria. Furthermore at least two of these combinding sites in each molecule must be directed against foreign surface groupings on the same strain of bacteria or erythrocyte in order to explain the ability of these opsonins to agglutinate red cells and certain strains of bacteria.

The cross reaction of the opsonins between Escherichia coli 086, Salmonella abortus equi and Salmonella typhimurium C5 may in part be due also to the sub units in the intact molecule possessing combinding sites against determinants common to these strains of bacteria. Indeed it has been established in the vertebrates that a number of different strains of bacteria including Salmonella typhimurium and Escherichia coli share cross reacting antigenic determinants (Springer, Williamson and Brandes, 1961; Minden, McClatchy and Farr, 1972).

More research is required in order to determine the
number and nature of the combining sites on each of the sub units and whether the isolated sub units possess biological activity such as enhancement of phagocytosis of bacteria by trypsin treated haemocytes.

Purification of the haemagglutinins from sera of a number of invertebrates (for details see Chapter I pp. 16-23) has revealed that they are composed of sub units of smaller mol. wt. held together by non covalent bonds as has been described in this thesis. Unfortunately little work has been carried out to investigate the biological activity and specificity of these purified proteins and their sub units.

In conclusion it appears that the discriminatory behaviour of haemocytes from the crayfish, (Parachaeraps bicarinatus) may be as effective as the phagocytic cells in the vertebrates. This discriminatory behaviour is dependent on a series of both specific and cross reactive opsonins. These mechanisms of discrimination may represent the primitive components of the adaptive immune response in the vertebrates.


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