INTERNAL DEFENCE MECHANISMS

IN PATELLA

by

ANWYL COOPER-WILLIS

Zoology Department, Bedford College

Thesis presented to the University of London for the degree of

Doctor of Philosophy

May 1978

ABSTRACT

The haemocytes of Patella were examined living and fixed and stained, by light microscopy and scanning electron microscopy. Similarities between haemocytes and vertebrate macrophages are probably a result of their similar functions, not of homology.

The activity of the haemolymph was examined and found to contain no lytic factors or agglutinins to vertebrate red cells or to bacteria. A haemagglutinin-inhibition factor was found to be present, this inhibited the agglutination of human red blood cells (HRBC) by human anti-sera, but not that of sheep red cells by guinea-pig anti-sheep serum.

Cultured in vitro, the haemocytes were found to lyse HRBC. This was quantified and found to be unaffected by prior opsonisation of the HRBC in Patella haemolymph. The haemocytes of pre-injected animals were found to be more avid.

The haemocytes were able to phagocytose bacteria, when cultured with them in vitro. Opsonisation in haemolymph depressed bacterial uptake, but the haemolymph of pre-injected animals slightly enhanced uptake.

Acid phosphatase was found to be naturally present in the haemocytes and haemolymph. Injection with bacteria resulted in elevated acid phosphatase levels and haemocyte numbers. Sea water injection did not cause elevation of haemocyte acid phosphatase or haemocyte number. Enhanced lysin synthesis is possibly a response to the foreign particles. Culture of haemocytes in vitro with bacteria did not cause detectable changes of acid phosphatase in the culture medium, although cellular levels changed. It is suggested that the haemocytes are not the primary source of this enzyme in the haemolymph. Since the haemolymph is not bactericidal it is suggested that haemolymph lysins may have an opsonic function.

The results are discussed with reference to similar work which has been done on other molluscs.

ACKNOWLEDGEMENTS

Acknowledgements and thanks for assistance with the work must go to the following:-

to Professor R.P. Dales, for supervising the work and for all the advice freely given whenever required

to Miss Linda Caskie, for all her encouragement and constructive discussion

to all the technical staff of the Zoology Department, particularly Mr. D.J.S. Field and Mr. Z. Podhorodecki

to the Marine Biological Association of the United Kingdom, in who's Plymouth laboratories most of the work in Section 6 was done

to University College Hospital Haematology Department, for giving samples of human blood and anti-sera

to Miss Vivian Cowper, for assistance with the Scanning Electron Microscope work

to Miss Elizabeth Storer, for devoting so much of her time to typing the work

and to the Science Research Council for the research studentship during the tenure of which this research was done.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

TABLE OF PLATES

SECTION 1

GENERAL INTRODUCTION

The organised body of a multicellular animal provides a more suitable environment for bacterial growth than does the external environment, this is evident from the fast decomposition which occurs after the death of the animal. To maintain the organisation all animals must have some way of preventing colonisation by potential pathogens.

The birds and mammals, with their constant and elevated body temperature, are the most vulnerable in that their internal environments are the most conducive to the growth of bacteria. The immune systems of these two classes are the most complex, but the vertebrates from the cyclostomes to the mammals show steadily increasing elaboration of their immune responses, particularly of the humoral aspects of the responses. The vertebrates also show a trend of increasing individual size, of increasing parental commitment to the subsequent generation and of lower reproductive potential and so, of low recruitment. All of these trends require that to reproduce successfully the individual lifespan must also increase, so an effective immune system is a necessity.

Members of the invertebrate phyla must also be able to resist pathogens, but the mechanisms of internal defence of multicellular invertebrates are much less specific than those of the vertebrates. The invertebrates are, on the whole, smaller in size, with high

reproductive potential, high rates of recruitment and low parental commitment to the next generation. So for successful reproduction an invertebrate need not live for very long and a highly efficient internal defence system is of very much less selective advantage to the animal.

Like the vertebrate immune response the internal defence mechanisms of the invertebrate species which have been studied, can be divided into cellular and humoral responses. But the cellular seems to be of much greater importance and invertebrates seem to depend largely on the phagocytic action of free amoebocytic cells in the haemocoel or coelom. Many invertebrates have been found to contain agglutinating and bactericidal factors in solution in their body fluids, these are probably not the major line of defence. Scott (1971) suggests that, as with the vertebrates, it is possible that there is an evolutionary trend towards increasing dependence on the humoral responses for recognition of foreigness by the phagocytes of the invertebrate phyla. But the species which have been studied in any one phylum are few and often do not represent very different evolutionary levels within that phylum. Any evolutionary trends which may exist are by no means easily discernible in the light of current knowledge of invertebrate internal defence mechanisms.

Knowledge of molluscan internal defence is restricted to the small number of species which have been studied in any depth. These are either pulmonates - Lymnaea stagnalis, Biomphalaria glabrata, Helix aspersa, Helix pomatia and Otala (Helix) lactea - or lamellibranchs - Mytilus edulis, Crassostrea virginica, Mercenaria mercenaria and Tridacna maxima. Work has also been done on two octopoid species -

Eledone cirrosa and Octopus dofleini - on the sea hare, Aplysia californica and on the chiton Liolophura gaimardi. These species are not evenly distributed among the molluscan groups and to draw generalised conclusions from the results obtained from these species about molluscan internal defence mechanisms would seem to be unwise. A comparison of the internal defence mechanisms of a more primitive species with those of the more advanced gastropod orders could possibly give some indication of the direction of the evolution of these mechanisms within the class. With the exception of Liolophura, the species mentioned above are members of the more advanced and specialised molluscan groups, so it was thought that a more primitive species might provide an interesting comparison. Patella vulgata was chosen as a member of the most primitive gastropod order, the Archaeogastropoda. The archaeogastropods are thought to be ancestral to the prosobranchs, from which the other two sub-classes, the opisthobranchs and the pulmonates probably arose. The chitons, though thought to be more primitive than the gastropods, are a more specialised group. Patella is a common and abundant species and is easily collected, even during neap tides.

It was thought that the internal defence mechanisms found in Patella could give an indication of the more primitive aspects of molluscan internal defence. More specialised groups might be expected to show increasing sophistication of the mechanisms. Pulmonates which have been studied possess a wide range of lytic enzymes, agglutinins and opsonic factors in the haemolymph, which probably aid in maintaining a sterile internal environment. And though Patella appears to show less involvement of soluble factors in the haemolymph in its internal defence the haemolymph is, none the less, sterile.

SECTION 2

GENERAL METHODS

i. Animal collection and maintenance.

Most of the animals used were collected at Hastings, Rock-a-noor, or at Wembury Bay. Collections were occasionally made at other sites.

Animals were brought back to the laboratory and placed under sea water circulation in the aquarium within 24 hours of collection. The aquarium is maintained at $14^{o}C \pm 2^{o}C$, with a 12 hour night/day cycle.

It was impossible to feed the animals once collected since in the wild Patella grazes algal sporelings from the rock surface.

All animals were used within a week of capture as it was found that cell counts of the haemolymph fell markedly during captivity, and it was felt that starvation might induce other, less evident changes in the animals' physiology.

ii. Haemolymph extraction and treatment.

Patella has an open, haemocoelic circulation and the organs are directly bathed in haemolymph. Haemocoelic volume varies with the size of the animal. Those used weighed not less than 10g, live wet weight with shell. Such animals would have an internal volume

of about 2000 mm^3. Some of the animals used were, however, larger than this.

The internal volume was not determined, except in specific cases, as there did not seem to be any satisfactory parameter of the living animal to which it could be related. Shell weight, being such a large proportion of the total weight, would cause wide apparent variations in internal volume in animals of similar size but slightly different shell thickness. Shell diameter is equally unsatisfactory in that animals from more exposed parts of the shore tend to have lower shells with proportionately wider openings, whereas those from sheltered areas may grow quite high with smaller openings.

The haemolymph contains free, amoeboid cells, which number about 1000 mm^{-3} and so make up only a small proportion of the total volume. If haemolymph is left to stand the cells very soon settle out and spread over the substratum. Davies and Partridge (1972) found that settling out was delayed by lowering the temperature of the haemolymph.

Haemolymph was collected from the circumpalial arterial sinus, which carries haemolymph to the mantle from the heart. Using a hypodermic syringe with a 25 gauge needle, haemolymph was aspirated from the artery just where it leaves the heart. The haemolymph was then emptied into a centrifuge tube and kept on ice until required. Alternatively the haemolymph was collected by cutting the artery where it leaves the heart and around the mantle edge and allowing the animal to bleed directly into the centrifuge tube.

As soon as possible after extraction cell counts were made on samples of the fluid. These were done, in an Improved Neubauer haemocytometer, (Hawksley) using phase contrast illumination.

When cell free haemolymph was required the whole haemolymph was centrifuged at 600 x g for 5 minutes. This left a little pellet of cells at the bottom of the tube, which was easily separable from the fluid.

The haemolymph is clear and generally straw coloured, though it may vary from almost colourless to quite dark amber. Its osmolarity was determined using an electronic, semi-micro osmometer (Knauer, Berlin) and was found to be close to that of sea water, 976 $\pm$ 36 μOsmoles l^{-1} (n = 10). After heating at 56^{o}C for 20 minutes the osmolarity was found to rise slightly, 1005 $\pm$ 39 μOsmoles l^{-1} (n = 7).

Preliminary measurements of the haemolymph pH were made on anaerobic samples. A capillary microelectrode (Radiometer BMS 2), held at 15^{o}C and attached to a pH analyser (Radiometer PHM 17) was used (after the method of Wells (1973)). Two animals were tested and gave results of 7.50 and 7.12 pH, which accord well with the results obtained by Davies (1977, personal communication) of 7.2 - 7.4 pH. When the pH was measured the readings barely settled down which suggests that the haemolymph is poorly buffered.

Where sterile haemolymph was required, it was drawn through a small membrane filter chamber into a sterile hypodermic syringe. Membranes with a pore size of 0.2 μm were used (Sartorius, membrane filter).

The cell pellets were used in experiments detailed in section 6.ii and for setting up cell migration experiments. The pellets were introduced into haematocrit tubes, the ends of which were sealed in a flame. They were then spun at 12,000 x g for 3 to 4 minutes in a micro-haematocrit centrifuge (Hawksley). The resulting cell column could then be used as required.

iii. Treatment of sea water and use of fixed delivery pipettes.

The sea water used in all experiments was artificial, taken from the aquarium. Prior to use it was filtered through a membrane, pore size 0.2 μm (Sartorius). When sterile sea water was required the filtered sea water was autoclaved for 15 minutes at 15 lbs in^{-2}.

In all cases where volumes of 25 μl, 100 μl and 500 μl were needed fixed delivery micrometer pipettes were used (Excalibur). These delivered to an accuracy of about 1%, 25 $\pm$ 0.25 μl, 100 $\pm$ 0.75 μl and 500 $\pm$ 3.00 μl.

iv. The viability of the haemocytes *in vitro*.

Sea water was used as a culture medium for the haemocytes *in vitro* as the osmolarity is close to that of the haemolymph. Haemocytes cultured in sea water appeared to be quite normal and seemed to remain viable over several hours. No long term culture of the cells was attempted.

Haemocyte viability was assessed over a period of $3\frac{1}{2}$ hours, following the method of Ford and Hunt (Wier, 1973). The exclusion

of Trypan blue was scored for 100 cells at 3 different temperatures, (see table 2.iv.1).

v. Haemocyte monolayers.

Where haemocytes were to be used to quantify phagocytic rates, in vitro cell monolayers were set up. Equal volumes of whole haemolymph were put into all the culture dishes to be used. These were 5 cm diameter plastic Petri dishes (Sterilin). They were left at room temperature for 20 minutes to allow the haemocytes to settle out and attach to the dish. When phagocytic rate was to be scored visually, glass coverslips were put into the Petri dishes for the cells to settle on. After 20 minutes the haemolymph was drawn off with a Pasteur pipette, leaving only the attached cells. The haemolymph was replaced with sterile sea water, in which the test particles were suspended.

Where haemocytes were to be observed under the microscope (section 3.iii.), drops of whole haemolymph were placed on slides. The slides were incubated on moist filter paper, in Petri dishes at room temperature while the cells settled out.

vi. Fixation of the haemocyte monolayers.

Having settled for an appropriate length of time, the cell monolayers were fixed by adding one drop of Baker's formol calcium - 40% formaldehyde buffered with calcium carbonate - to the liquid on the slide. They were left to fix for one minute, then rinsed in distilled water.

TABLE 2.iv.1.

temp	culture time hrs.	n	% viable
37°C	1	100	100
	1.5	100	95
	2	100	94
	2.5	100	96
	3.5	100	68
20°C	1	100	99
	1.5	100	98
	2	100	100
	3.5	100	96
15°C	1	100	100
	2	100	100
	3.5	100	98

LEGEND The proportion of haemocytes found to be viable, assessed by their ability to exclude Trypan blue dye, after *in vitro* culture at 3 different temperatures.

(n = number of cells scored)

Where phagocytosis was to be scored the slides, or coverslips, were rinsed in sterile sea water to remove all unattached particles, before fixing.

vii. The bacteria used, their culture and treatment.

The following strains of bacteria were used:

1. Escherichia coli K12 CL265 (National collection of type cultures, Colindale).
2. Micrococcus luteus NCTC 2665 (National collection of type cultures, Colindale).
3. Bacterium megaterium NCIB 8291 (National collection of Industrial Bacteria, Torrey, Aberdeen).
4. Serratia marinorubra (National collection of marine bacteria, Torrey, Aberdeen).
5. 'A', a gram negative rod, isolated from the sea water in the aquarium at Bedford College. It seems to be common in natural sea water also.

The bacteria were grown in broth, innoculated from slopes maintained in the laboratory and incubated overnight at 37°C. ZoBell's marine medium (Moebus, 1972) was used for the marine species and yeast extract peptone broth (YEP) for the terrestrial species (Moebus, 1972).

The broths were sterilised when made up by autoclaving for 15 minutes at 1 bar.

When bacteria were to be incubated in cell free haemolymph

dilutions of the overnight cultures were made using sterile broth as a diluent, (section 4.iii.). These dilutions were then added to the haemolymph samples and to the controls.

If to be used for injection, the live bacteria were washed three times in sterile sea water by centrifugation and resuspension. They were then resuspended in sterile sea water to the required concentration using a McFarland nephelometer (Campbell, Garvey, Cremer & Sussdorf, 1963).

Bacteria were heat killed by boiling the overnight cultures for about 2 hours, then washing and resuspending as above.

viii. The making up of sterile plates for bacterial growth.

A 1.5 w:v solution of agar was made up in either the ZoBell or the YEP broth. This was autoclaved, then poured onto sterile Petri dishes. These were left to cool, then dried with both lid and base inverted, at 37°C for about 2 hours. The plates were kept at room temperature overnight before use, so that any contaminated ones could be rejected.

All plating out was done after the method of Miles and Misra (1938). Samples were first diluted by 10^{-1} and then 6 25 μl samples were dropped onto 6 plates. Each plate then had one drop of each of 6 samples.

To score, the number of colonies were counted under a binocular microscope after overnight incubation of the plates at 37°C, or

room temperature for 'A'. From the counts the mean number of bacteria in the experimental culture could be calculated.

ix. The vertebrate red blood cells and their treatment.

Human red blood cells (HRBC) were given by University College Hospital. They were in whole, heparinised blood and were kept at $4^{o}C$ for up to 2 weeks.

Prior to use the HRBC were washed 3 times in phosphate buffered saline (PBS) (Weir, 1973 p20.6). They were then made up to the required dilution with either PBS, modified barbitone buffer (MBB) (Campbell, Garvey, Cremer and Sussdorf, 1963), or sterile sea water, depending on the experiment.

To allow opsonisation of the HRBC in cell free haemolymph, washed HRBC were made up to an approximately 10% v:v suspension in the haemolymph. They were left for one hour at room temperature, then washed in PBS 3 times. For the experiments the washed HRBC were made up to a 1% v:v suspension in one of the 3 media used.

Human antisera, anti-A and anti-B, were also given by University College Hospital. They were stored at $-20^{o}C$. After thawing, the antisera were stored at $4^{o}C$ and used within 48 hours.

Sheep red blood cells (SRBC) were preserved in Alsever's solution (Flow Laboratories, Ayreshire), and kept at $4^{o}C$ for up to 6 weeks. For use the SRBC were washed and resuspended as above.

The antiserum used against the SRBC was rabbit anti-SRBC,

preserved 50% v:v with glycerol (Flow Laboratories, Lot R87,4020). This was stored at $4^{o}C$, and diluted for use with PBS.

Chicken blood was given, fresh and heparinised, by University College Hospital. Washed as above, the cells were used fresh.

Dogfish blood, given by The Marine Biological Association, Plymouth, was fresh and heparinised. The cells were washed and resuspended in dogfish ringer (Hale, 1958, after Young).

SECTION 3

A DESCRIPTION OF THE FREE CELLS VIEWED BY LIGHT AND SCANNING ELECTRON MICROSCOPY

i. Introduction

As has been observed in all other molluscs examined, the haemolymph of Patella contains free, phagocytic cells. They occur in a concentration of about 10^3 mm^{-3} of haemolymph. The cells are of two morphological types, but whether these represent two distinct cell lines is unknown.

The more common cell type is the amoebocyte, so named by Davies and Partridge (1972) because of its mode of locomotion. The other type makes up at most 1%, but often much less, of the population. These cells were called macrophages by Davies and Partridge (1972) from their superficial resemblance to vertebrate macrophages when spread in culture. Where no distinction is to be made between these two types the free cells are referred to as haemocytes.

ii. The living cells viewed by phase contrast illumination method.

Method

Whole haemolymph was placed on slides and the cells left to settle out, at room temperature, for times ranging from 0 to 20 minutes. To view the cells a coverslip was placed over the drop and the slides examined by phase contrast illumination on a Zeiss Photomicroscope II using oil immersion.

Description

Immediately on removal from the animal the cells appear circular, and to have a diameter of 8.10 ± 1.37 μm (n = 20). About 30 seconds after withdrawal from the animal the cells start to develop cytoplasmic extensions (Davies and Partridge, 1972). Over a period of minutes the cells gradually spread over the substratum. Before they are fully spread the cells have a strong phase halo, no internal structure is discernible, and they do not photograph well (Plates 3.ii.1. and 3.ii.2.)

As the spikes extend the cells flatten and become attached to the slide and loose the strong phase halo (Plate 3.ii.3.)

The fully spread cells have a central region of dense, fairly granular cytoplasm containing vacuoles and phase-dense bodies. Davies and Partridge (1972) found that the cells were positive to Gomori's stain, and suggested that the acid phosphatase might be located in the phase-dense bodies.

The nucleus is ovoid and the cells mononucleate. Occasionally cells appear to be binucleate (Plate 3.ii.4.), but in some cases this could be two closely attached cells (Plate 3.ii.5.). No mitotic figures have been observed, though in some cells chromatid-like threads are visible in the nuclei (Plate 3.ii.6.). The spikes project from the region of dense cytoplasm and seem to support the fine cytoplasmic veils, which are non-granular and have no inclusions or vacuoles (Plate 3.ii.7.). In this state the cells typically have an elongated, stellate outline and measure 37.90 ± 9.75 x

Plate 3.ii.1. LEGEND

Living haemocytes just removed from the animal, with spikes starting to develop (phase contrast).

Plate 3.ii.2. LEGEND

Living haemocytes with spikes further developed (phase contrast).

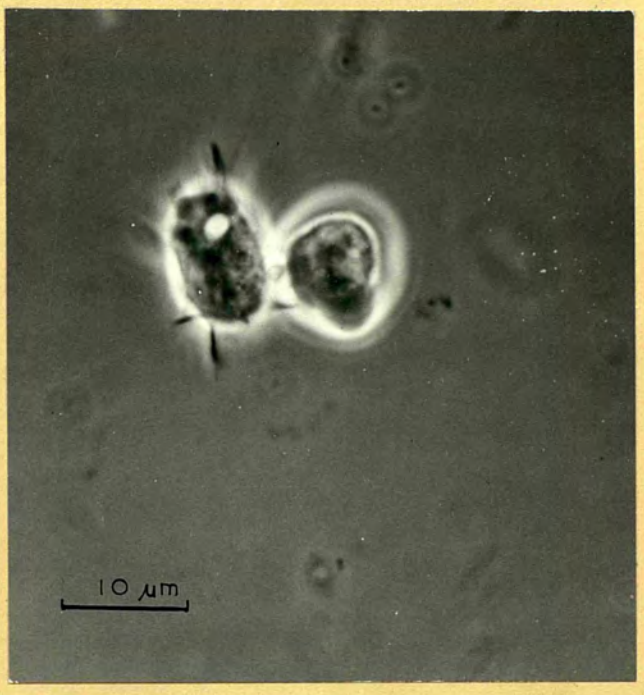


PLATE 3.ii.1.

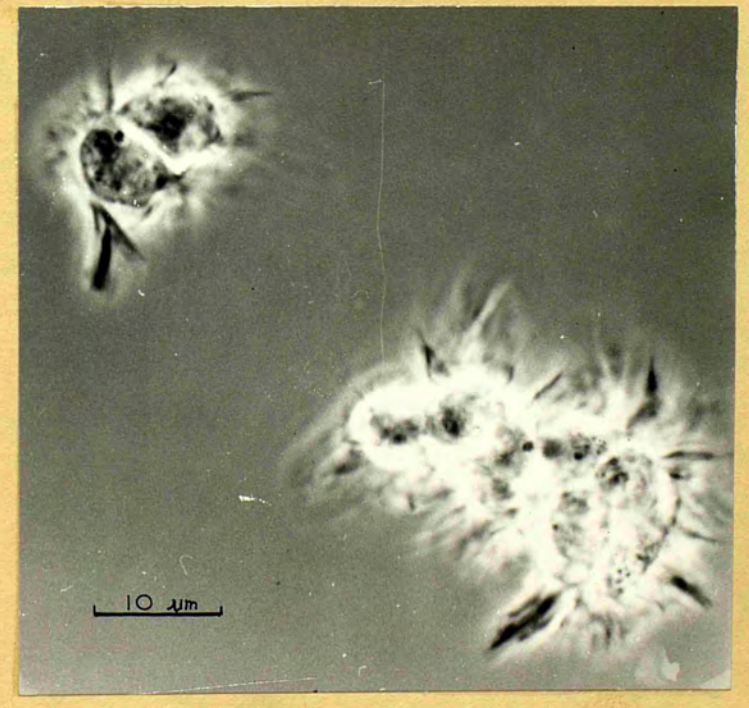


PLATE 3.ii.2.

Plate 3.ii.3. LEGEND

Living amoebocytes, partially spread on the slide (phase contrast).

Plate 3.ii.4. LEGEND

Living amoebocyte, fully spread, possibly binucleate (phase contrast).

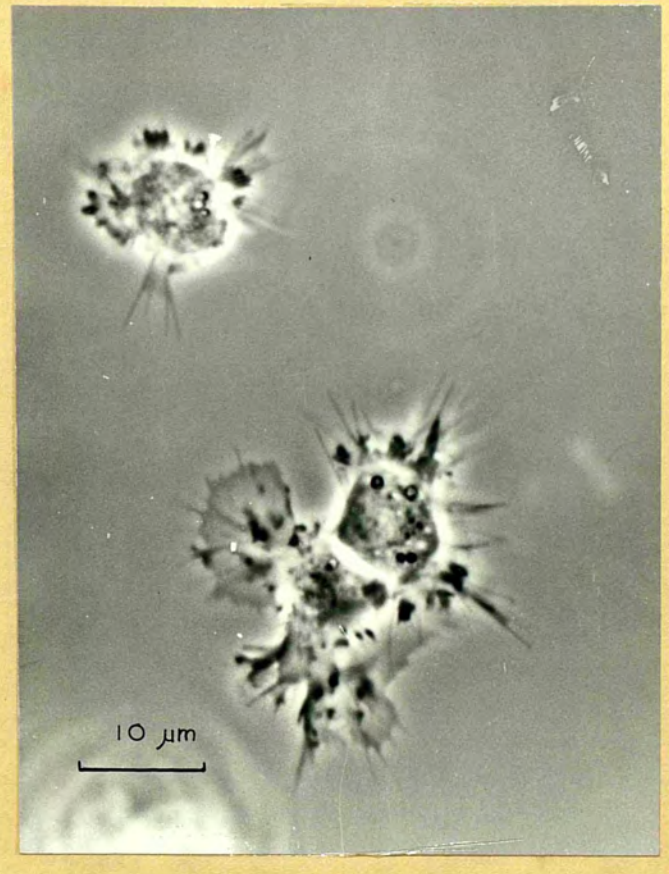


PLATE 3.ii.3.

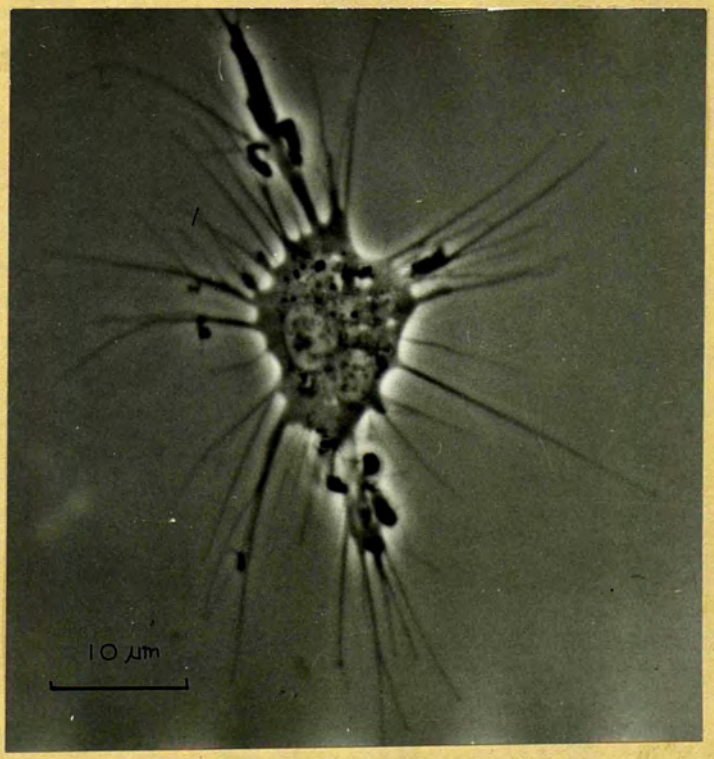


PLATE 3.ii.4.

Plate 3.ii.5. LEGEND

Living amoebocytes in close juxtaposition giving the appearance of a single, binucleate cell (phase contrast).

Plate 3.ii.6. LEGEND

Living amoebocyte, fully spread with chromatin threads visible in the nucleus (phase contrast).

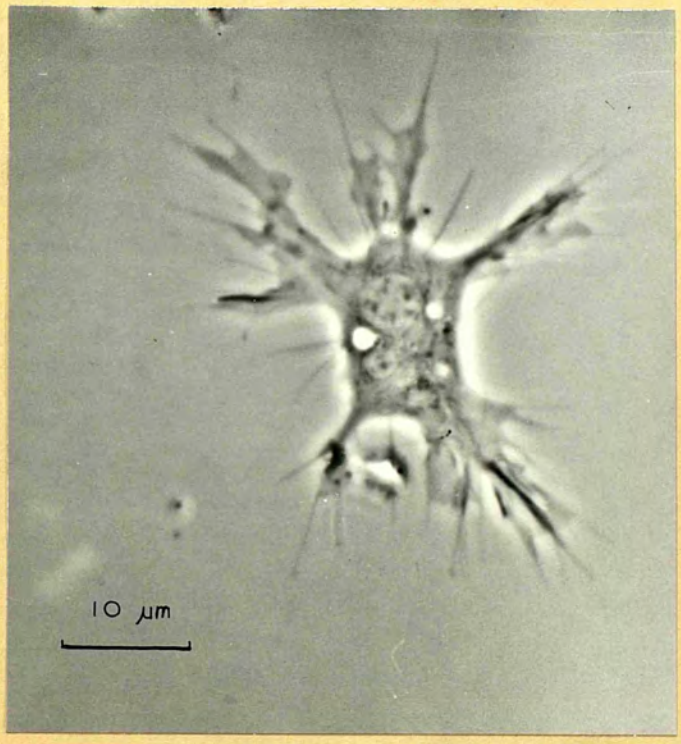


PLATE 3.ii.5.

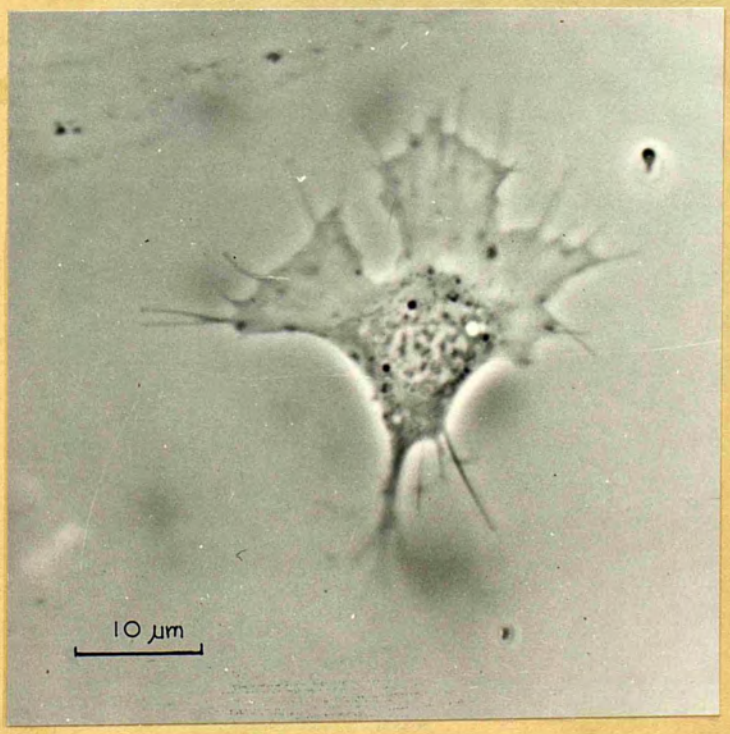


PLATE 3.ii.6.

Plate 3.ii.7. LEGEND

Living amoebocytes, fully spread, showing spikes and well developed veils (phase contrast.).

Plate 3.ii.8. LEGEND

Living 'macrophage', showing large numbers of inclusions (phase contrast).

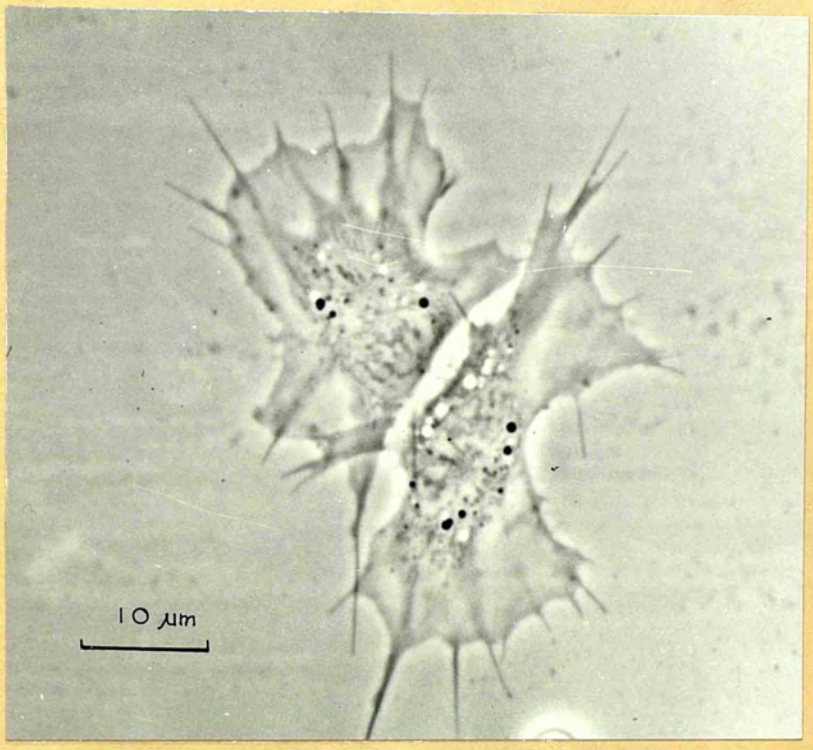


PLATE 3.ii.7.

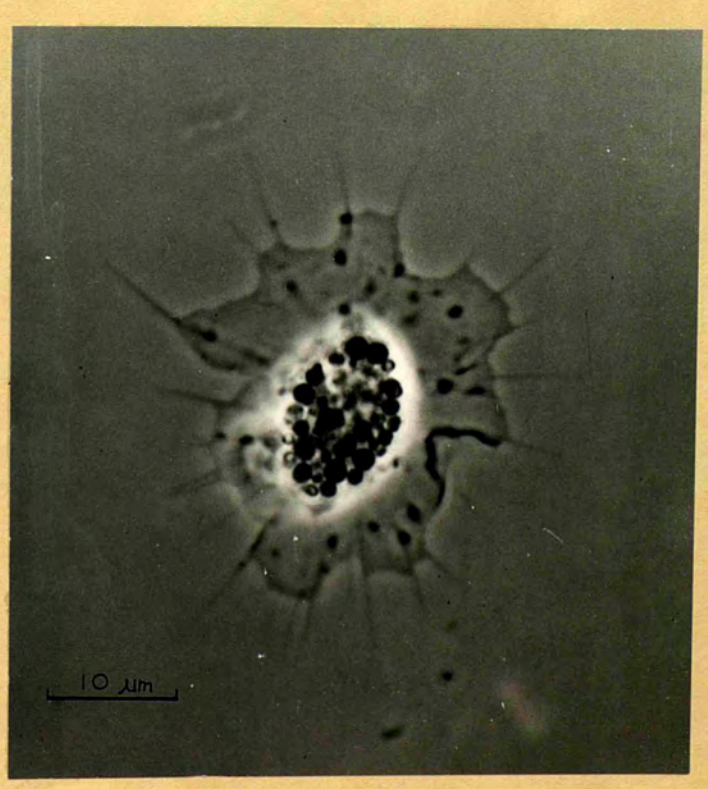


PLATE 3.ii.8.

Plate 3.ii.9. LEGEND

Living 'macrophage', showing extensive cytoplasmic spikes (phase contrast).

Plate 3.ii.10. LEGEND

Living 'macrophage', showing spikes and intra-cellular inclusions (phase contrast).

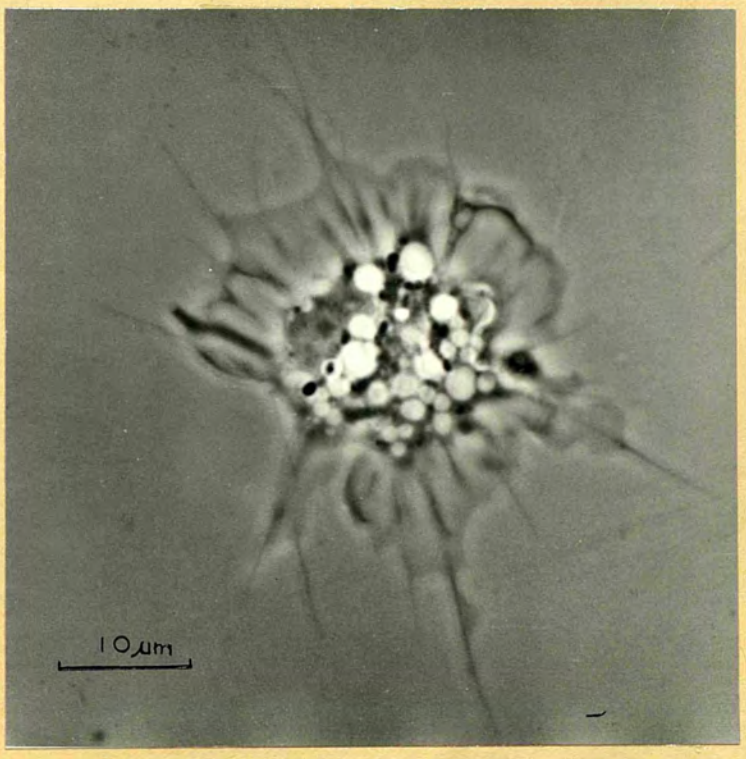


PLATE 3.ii.9.

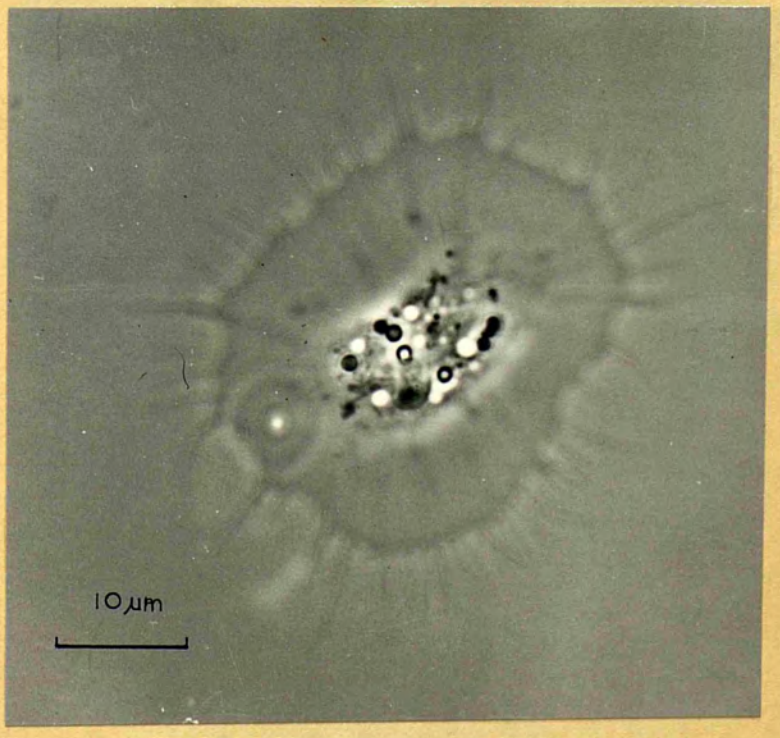


PLATE 3.ii.10.

Plate 3.ii.11. LEGEND

Living amoebocytes spread from the periphery of a cell clot (phase contrast).

Plate 3.ii.12. LEGEND

Living amoebocytes, fully spread (phase contrast).

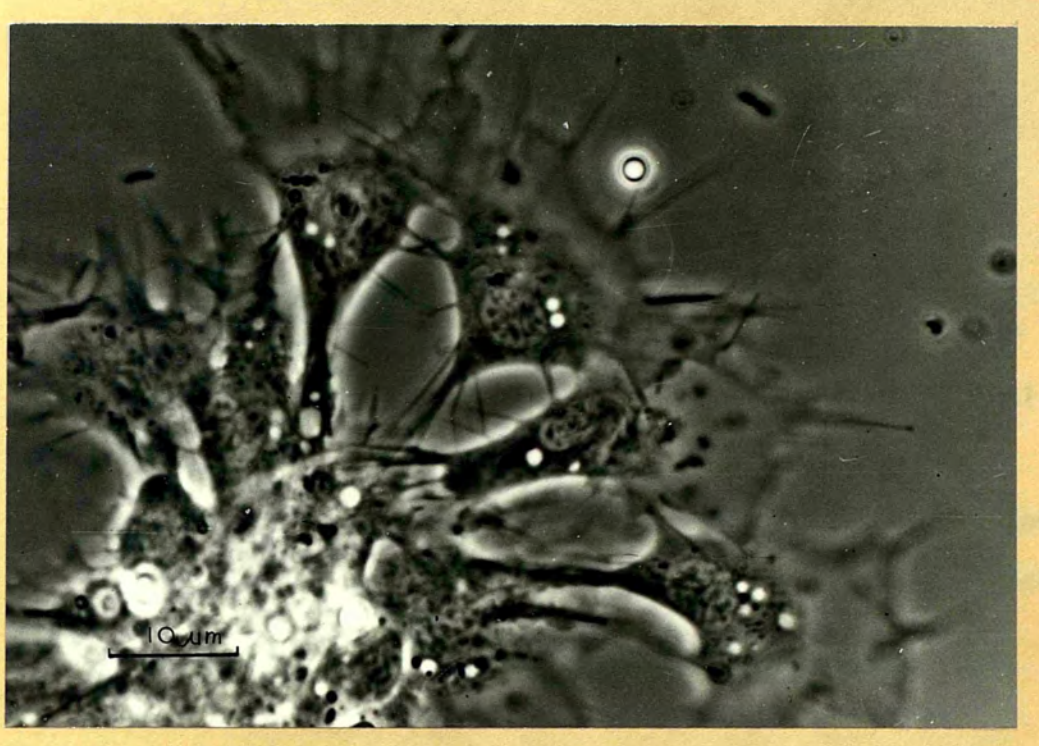


PLATE 3.ii.11.

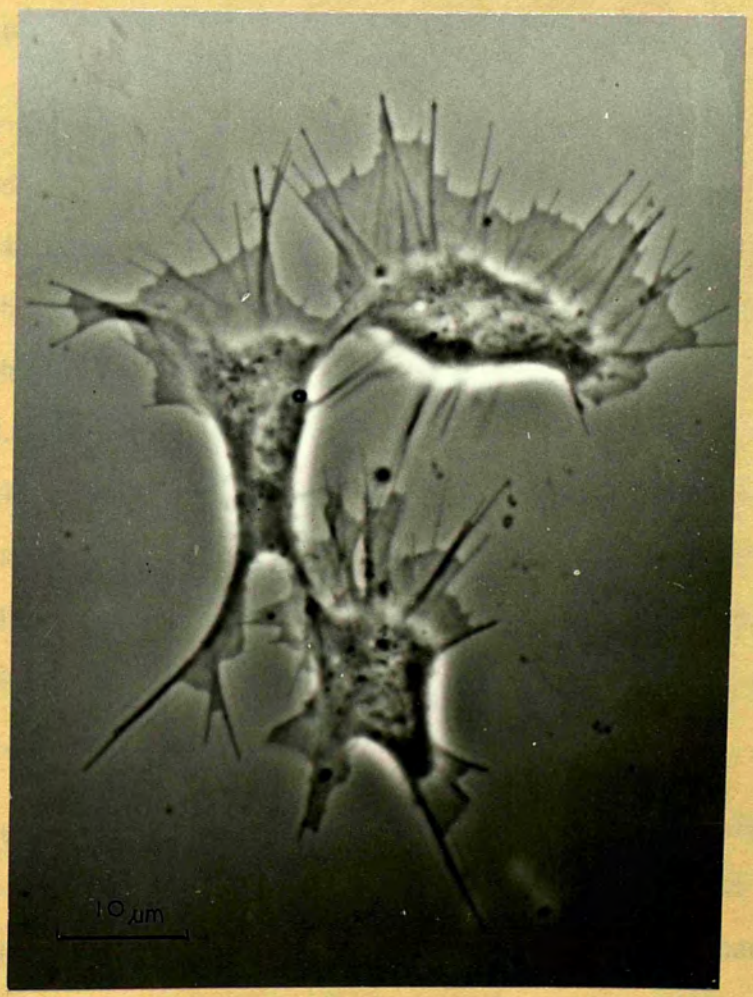


PLATE 3.ii.12.

24.98 ± 5.49 μm (n = 20).

The 'macrophages' are initially indistinguishable from the amoebocytes, but when fully spread their outline is circular rather than elongate and the spikes tend to be few, or absent. The central region of denser cytoplasm in these cells has a phase halo, unlike that of the amoebocytes. The peri-nuclear region has more inclusions and particularly numbers of large, pale yellowish bodies. The phase-dense granules are larger than those seen in the amoebocytes. The nucleus is usually obscured by these intra-cellular bodies (Plates 3.ii.8. and 3.ii.9.). Owing to their scarcity only four of these cells could be measured. They seem to be slightly smaller than the amoebocytes, 25.90 ± 5.60 μm x 22.90 ± 5.20 μm.

The macrophage described by Davies and Partridge (1972) lacked spikes and had a lobulate margin. They found no intermediate types and felt that the two cell types might stem from different cell lines. Though the spikeless forms were observed the 'macrophages' photographed in this case all seem to be of an intermediate type (Plates 3.ii.10. and 3.ii.9.). The cytoplasm is spread in a circle and has all the typical 'macrophage' inclusions, but spikes are abundant. That the macrophage cell type appear to be more numerous in the haemolymph of animals which have been starved for some time, may suggest that these are a different stage of development in a single cell line.

In vitro the cells are inclined to form clumps of varying numbers. Once resting on a substratum the cells spread out from the periphery of the clot (Plate 3.ii.11.). Cooling the haemolymph delays the formation of clots (Davies and Partridge, 1972). Cells

from animals recently stressed by injection seem to clot more readily and slightly fewer single cells are found in the monolayers of attached, spread cells. In no case are all the cells involved in the formation of clots.

The spread cells move slowly over the surface (2.06 μm min^{-1}, Partridge and Davies, 1974), making and breaking contact with other cells in their paths. Contact may be between cytoplasmic extensions (Plate 3.ii.12) or may be very close (Plate 3.ii.5.).

iii. The cells fixed and stained, viewed by light microscopy.

Method

Haemocytes, which had been allowed to settle onto slides for 20 minutes at room temperature, were fixed in Baker's formol calcium (see Section 2.vi.).

The fixed cell monolayers were stained with Ehrlich's haematoxylin and eosin, Leishman's stain and Best's carmine. The methods of Gurr (1960) were used for all of the stains.

Description

In some of the fixed preparations the nuclei of the cells appeared to be lobate, or indented. In living cells the nuclei are a smooth ovoid shape. Binucleate cells have been observed after fixation. This condition is not found in the living cells, though cells in close contact (Plate 3.ii.5.) could be mistaken for single binucleate cells, for after fixation cell boundaries

are no longer distinguishable (see Plates 3.iii.5., 3.iii.2. and 3.iii.4.).

Stained with Ehrlich's haematoxylin and eosin the nuclei appear dark red to purple and are highly granular, to the extent of seeming blotchy in less strongly stained cells. Some of the nuclei stain too darkly to show any structure (see Plates 3.iii.1., 3.iii.2. and 3.iii.3.).

The cytoplasm stains pink and has an even, grainy appearance. In some cells the cytoplasm is quite dense, in others it seems diffuse and highly vacuolated. Living cells never show such diffuse cytoplasm. The differing intensities of cytoplasmic staining could be due to different extents of cell spreading. None of the cells show differentially staining granules in the cytoplasm. Nor are any 'macrophages' distinguishable, as structurally differing cells, or as differently staining cells.

Using Leishman's stain the nuclei stain pink to violet and are granular. The ground material of the nucleus stains bright pink, but the overall depth of colour depends on the amount of chromatic material, which stains purple, present (see Plate 3.iii.4.). The nuclei of trypanosomes stain pink in Leishman's stain, which is thought to be due to large quantities of the strongly basic protein protamine present in them.* Nucleoli are visible in the nuclei of some of the cells (Plates 3.iii.4. and 3.iii.7.). The staining of the nuclei in both haematoxylin and eosin and in Leishman's stain suggest that they are less strongly basophilic than the nuclei of mammalian leucocytes.

*(Unna and Tieleman, 1918, in Barker 1958)

Plate 3.iii.1. LEGEND

Fixed haemocyte clot, stained with Ehrlich's haematoxylin and eosin, after 1 hour of *in vitro* culture with human red blood cells (HRBC) showing intra-cellular HRBC.

Plate 3.iii.2. LEGEND

Haemocyte after 1 hour of *in vitro* culture showing attached and intra-cellular HRBC. Stained with Ehrlich's haematoxylin and eosin.

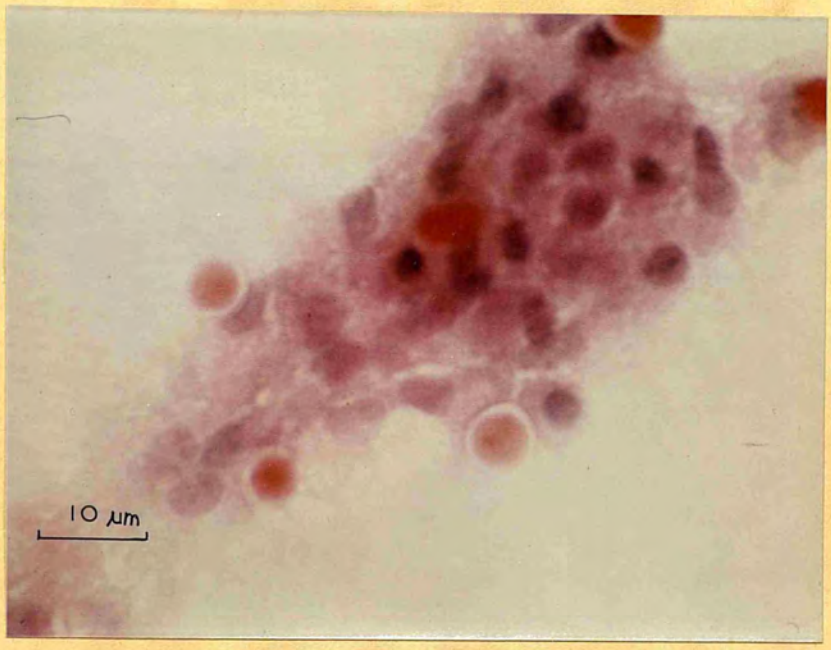


PLATE 3.iii.1.

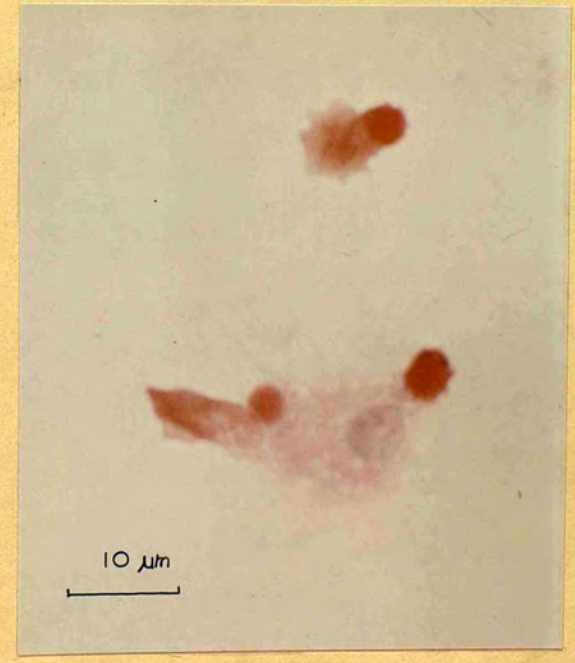


PLATE 3.iii.2.

Plate 3.iii.3. LEGEND

Haemocytes after 2 hours of *in vitro* culture with intra-cellular HRBC. Stained with Ehrlich's haematoxylin and eosin.

Plate 3.iii.4. LEGEND

Haemocytes after 30 minutes of *in vitro* culture with heat-killed bacteria and stained in Leishman's stain.

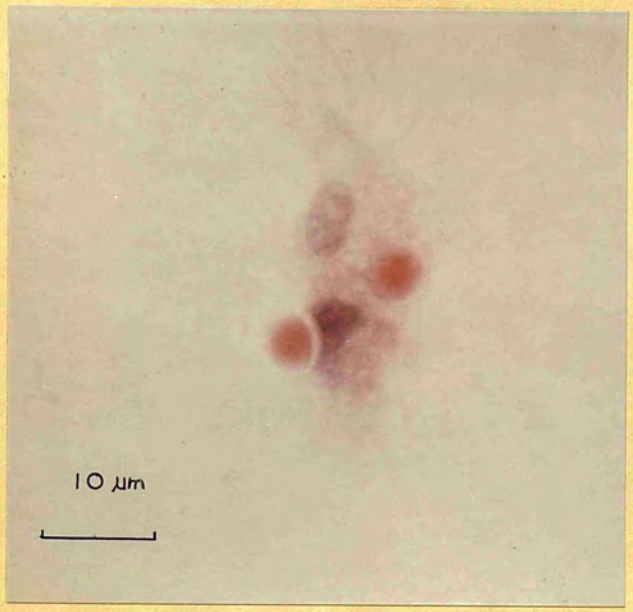


PLATE 3.iii.3.

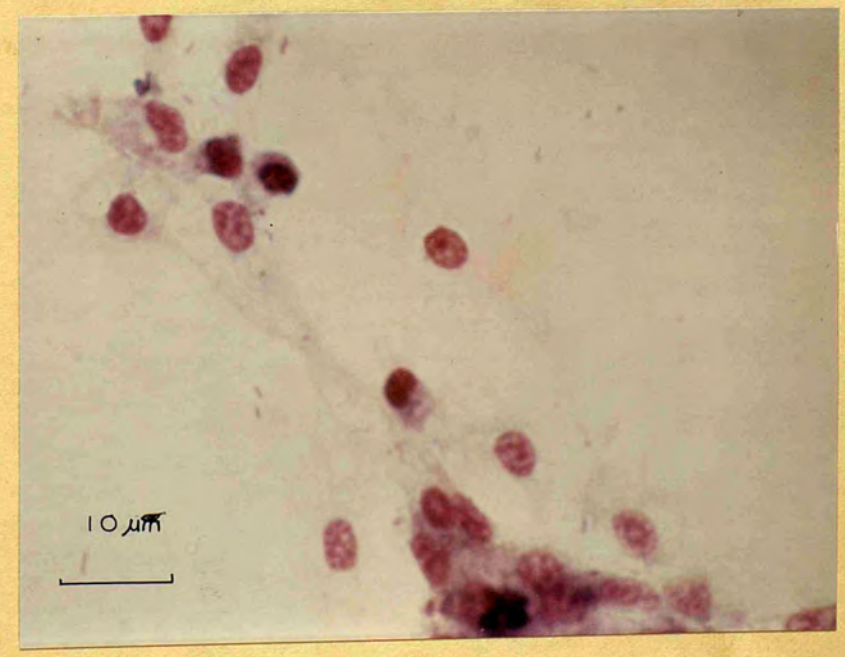


PLATE 3.iii.4.

Plate 3.iii.5. LEGEND

Haemocytes from a naive animal fixed and stained in Leishman's stain.

Plate 3.iii.6. LEGEND

Haemocyte from an animal previously injected with heat-killed bacteria, fixed and stained in Leishman's stain. One cell has nucleoli visible in the nucleus (n) (phase contrast).

PLATE 3.iii.5.

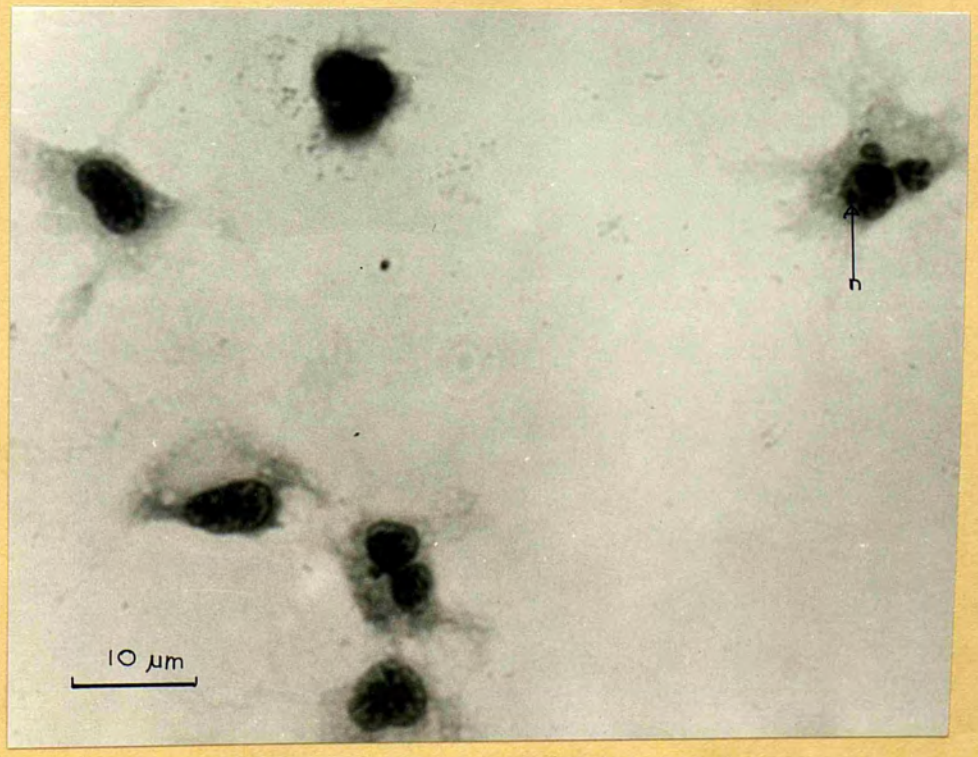


PLATE 3.iii.6.

Plate 3.iii.7. LEGEND

Haemocytes from an animal previously injected with heat-killed bacteria. Bacteria (b) are evident in some cytoplasmic vacuoles (phase contrast).

Plate 3.iii.8. LEGEND

Haemocytes from an animal previously injected with heat-killed bacteria, a cell which could be a 'macrophage' (m) is present (phase contrast).

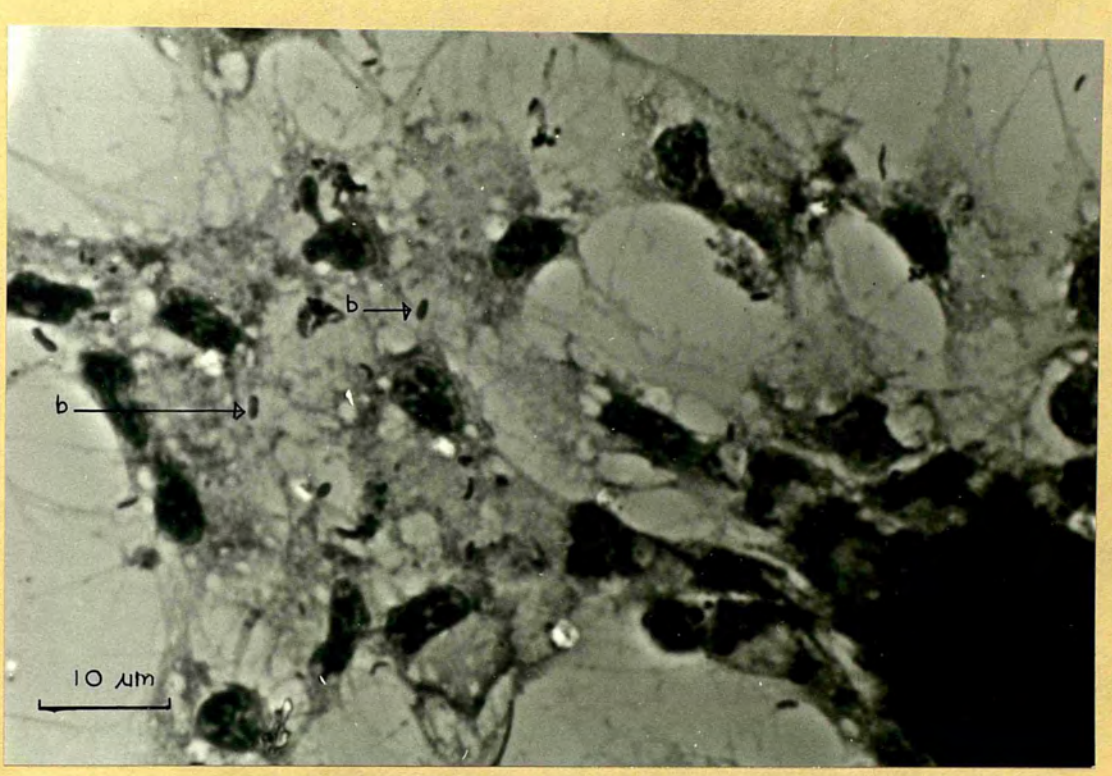


PLATE 3.iii.7.

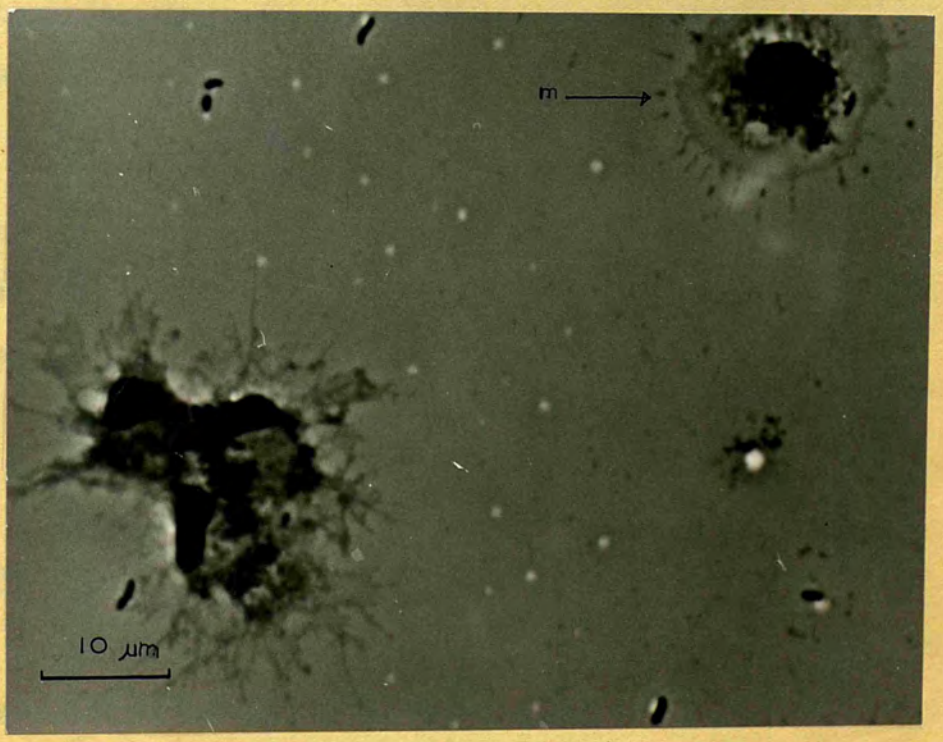


PLATE 3.iii.8.

The cytoplasm stains mauve to grey and in most cases seems diffuse and highly vacuolated. No granules are evident in the cytoplasm (Plate 3.iii.4.). No counterpart of the denser peri-nuclear cytoplasm seen in the living cell is apparent.

In preparations where the cells are incubated with bacteria, the bacteria can often be seen lying in vacuoles in the cytoplasm (Plate 3.iii.7.). The intensity of nuclear staining does not seem to correlate with the absence or presence of bacteria in the cytoplasm. More strongly staining nuclei should be the more active ones, and Cheng, Rodrick, Foley and Koehler (1975) have suggested that release of acid phosphatase into the surrounding medium by Mercenaria cells is a direct result of phagocytosis.

What may be a macrophage is shown in Plate 3.iii.8. This cell does have a denser peri-nuclear region, it can be seen to have taken up bacteria, its staining however does not differ from that of the amoebocytes.

Results using Best's carmine stain for the identification of glycogen were uniformly negative, even after celloidin coating. Davies and Partridge (1972), however, identified large quantities of glycogen in electronmicrographs of Patella haemocytes.

iv. The cells viewed in the scanning electron microscope.

Method

Drops of fresh haemolymph on coverslips were incubated for 15 and 20 minutes with O group human red blood cells (HRBC). To

fix, the haemolymph was drawn off and replaced with a drop of 1% glutaraldehyde in a balanced salt solution. The preparations were left to fix for 30 minutes at room temperature, then rinsed in fresh salt solution and post fixed in osmium vapour for 30 minutes, also at room temperature. As much fluid as possible was then removed from the coverslips and they were serially dehydrated in acetone. Prior to examination the specimens were critical point dried in carbon dioxide and vacuum-coated with gold palladium alloy.

Description

The structure of the cells seen under the scanning electron microscope (SEM) correlates well with that of the living cells seen by phase contrast with the light microscope. The central region of denser cytoplasm (Plate 3.ii.6.). can be seen as the main body of the cell (Plates 3.iv.1. and 3.iv.2.), from this the veils and spikes project. From these pictures it can be seen that the spikes do not only spread over the slide but also rise up vertically into the surrounding medium (Plate 3.iv.3.). The relationship of veil and spike is well shown in Plates 3.iv.4. and 3.iv.5., the spikes appear to be denser than the veils.

Both colchicine and cytochalasin B are known to inhibit the formation and maintenance of microtubules in cells. Jones and Partridge (1974), using Patella haemocytes, found that in colchicine the cells lost their polarity and oriented movement, though the spikes were not disrupted, over a period of hours. Cytochalasin B in very low concentrations caused rapid collapse of fully formed spikes. Cells suspended in sea water containing cytochalasin B did not

Plate 3.iv.1. LEGEND

Haemocyte after 15 minutes incubation with O HRBC showing projecting spikes and veils (Scanning Electron Microscope (SEM) x 5900).

Plate 3.iv.2. LEGEND

Haemocytes after 15 minutes incubation with O HRBC, showing projecting spikes and veils (SEM x 2300).

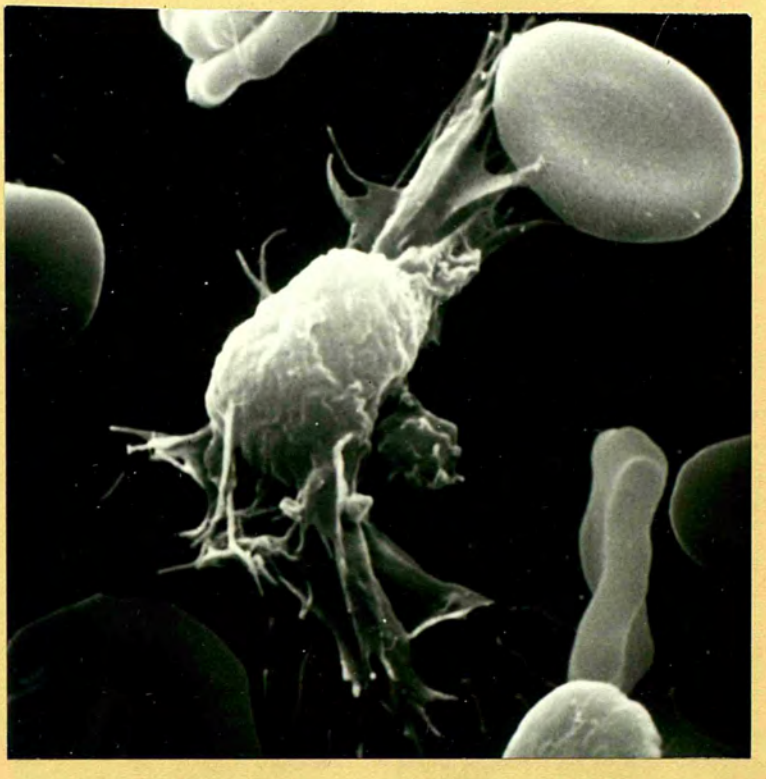


PLATE 3.iv.1.

PLATE 3.iv.2.

Plate 3.iv.3. LEGEND

Haemocyte clot after 15 minutes incubation with O HRBC, showing vertical and horizontal spreading (SEM, x 2400).

Plate 3.iv.4. LEGEND

Haemocytes after incubation with Micrococcus, showing spread veil and spikes (SEM, x 2950).

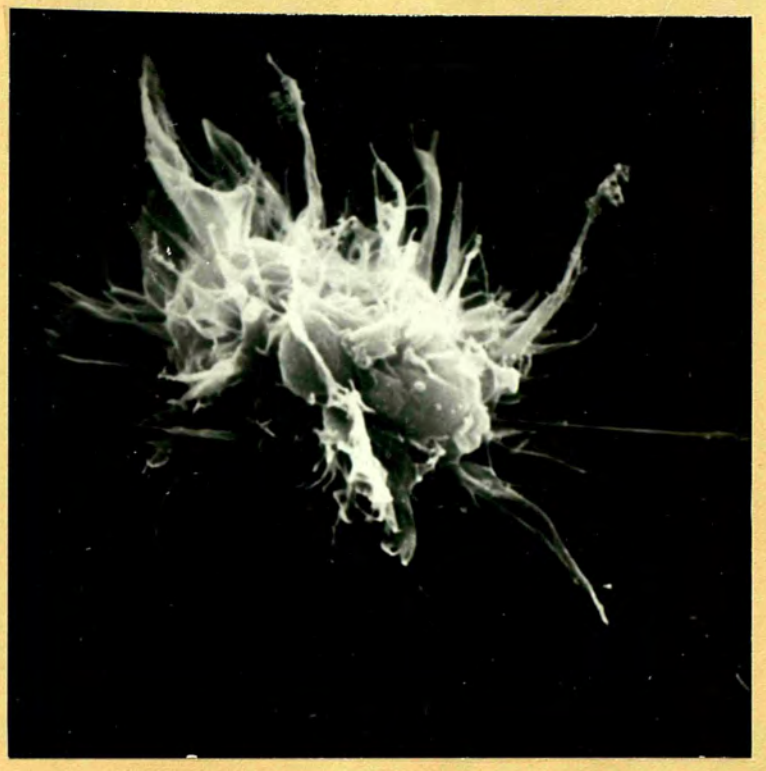


PLATE 3.iv.3.

PLATE 3.iv.4.

Plate 3.iv.5. LEGEND

Haemocytes after incubation with Micrococcus, showing spread veil and spikes (SEM, x 2950).

Plate 3.iv.6 LEGEND

Haemocytes after 15 minutes incubation with O HRBC, showing some fillopod-like projections (SEM, x 2400).

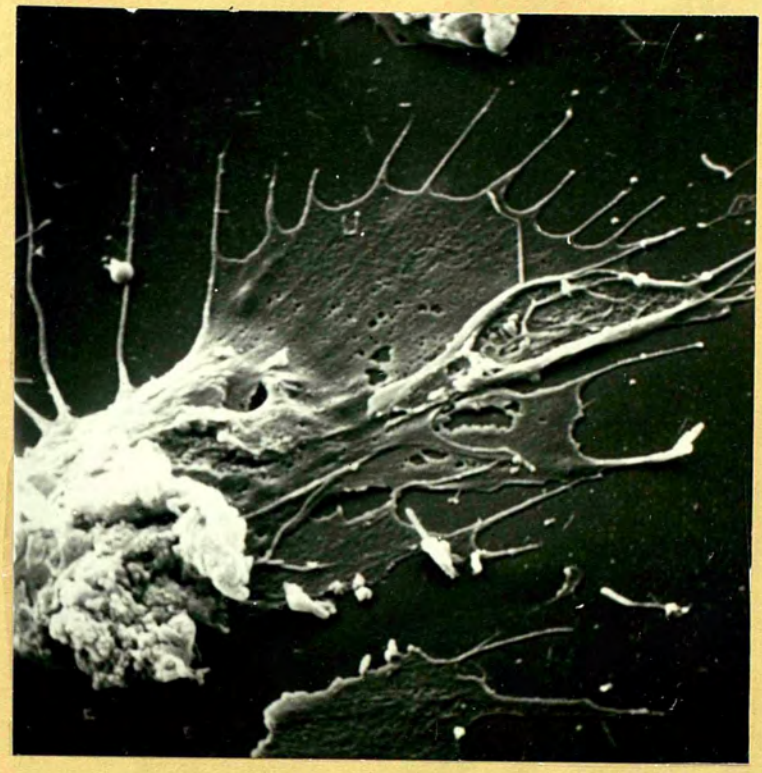


PLATE 3.iv.5.

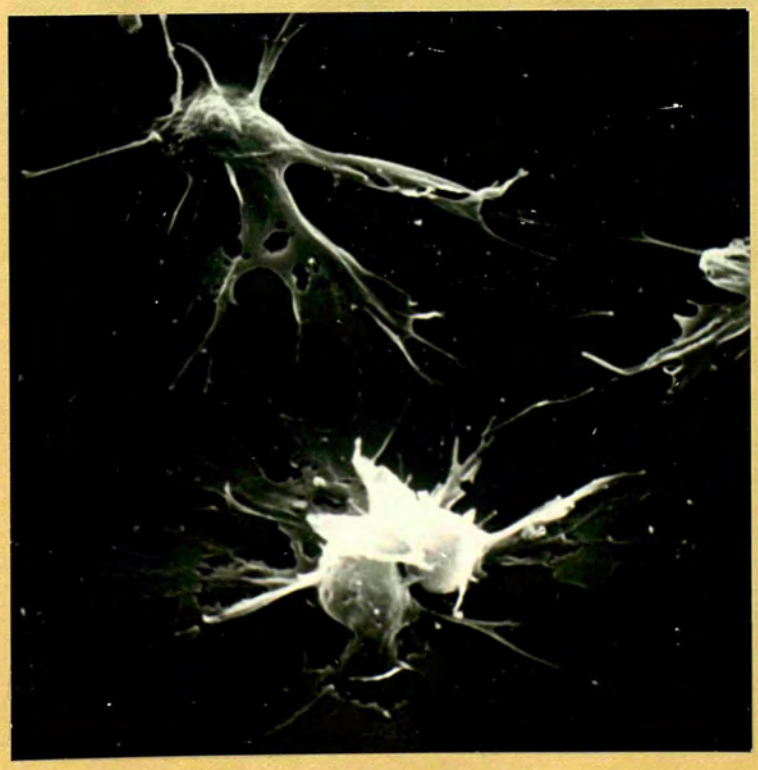


PLATE 3.iv.6.

Plate 3.iv.7. LEGEND

Haemocyte clot after 20 minutes incubation with O HRBC, with adhering HRBC (SEM, x 2400).

Plate 3.iv.8. LEGEND

Surface of a haemocyte clot after 20 minutes incubation with O HRBC, showing attached HRBC (SEM, x 6000).

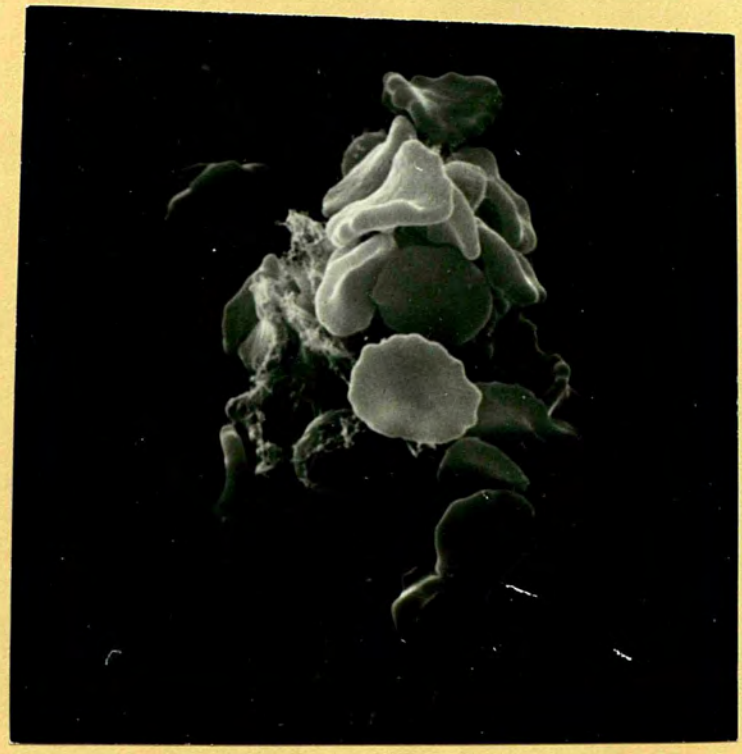


PLATE 3.iv.7.

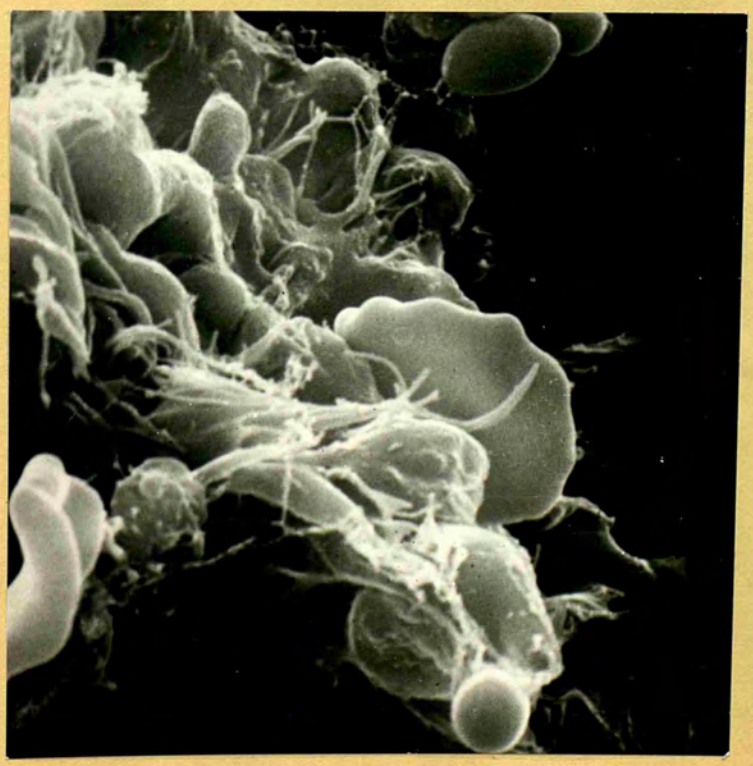


PLATE 3.iv.8.

Plate 3.iv.9. LEGEND

Haemocytes after 20 minutes incubation with O HRBC, with attached HRBC and what may be a phagocytosed HRBC (SEM, x 2600).

Plate 3.iv.10. LEGEND

Haemocyte clot after 15 minutes incubation with O HRBC in which individual cell bodies are distinguishable (SEM, x 2400).

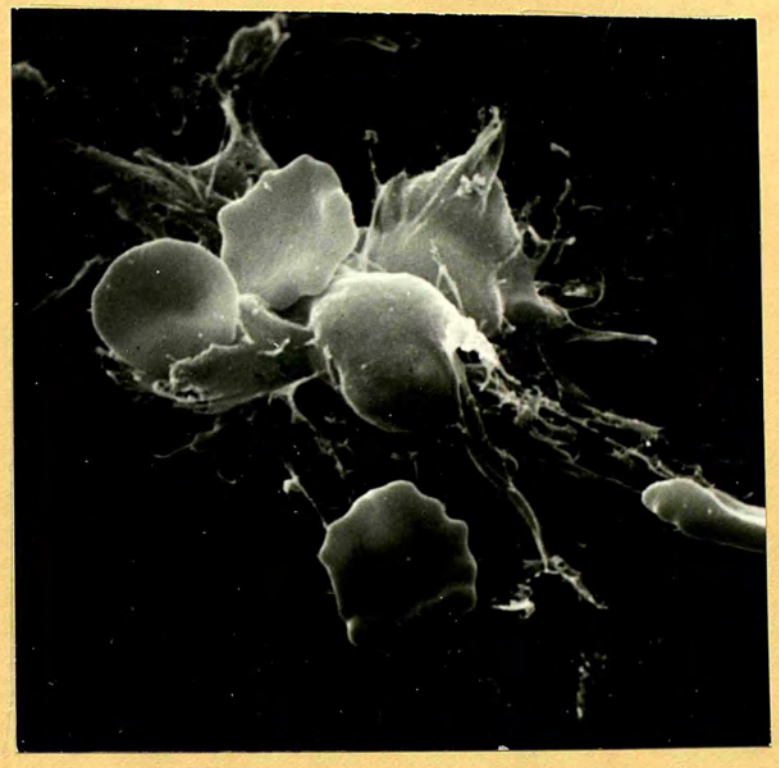


PLATE 3.iv.9.

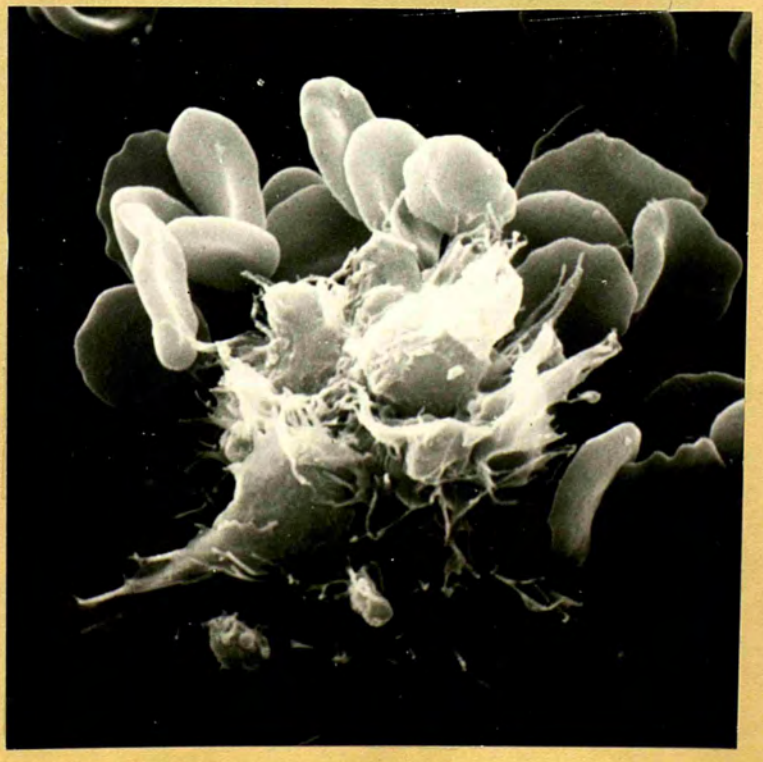


PLATE 3.iv.10.

Plate 3.iv.11. LEGEND

Haemocyte clot after 20 minutes incubation with O HRBC, with a possible HRBC ghost (g) attached (SEM, x 2600).

Plate 3.iv.12. LEGEND

Haemocyte clot after 15 minutes incubation with O HRBC, showing a thread attaching to HRBC (SEM, x 1200).

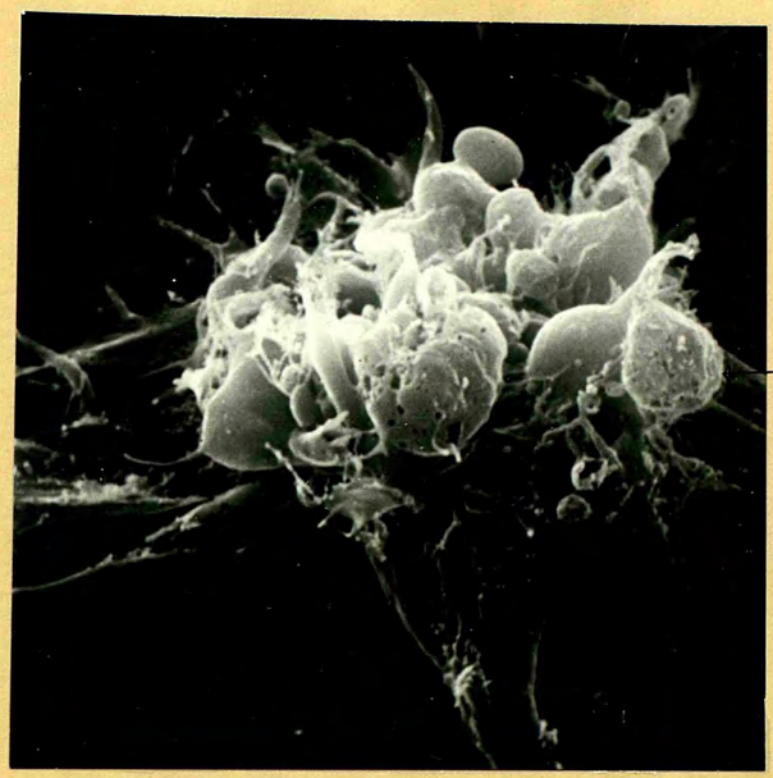


PLATE 3.iv.11.

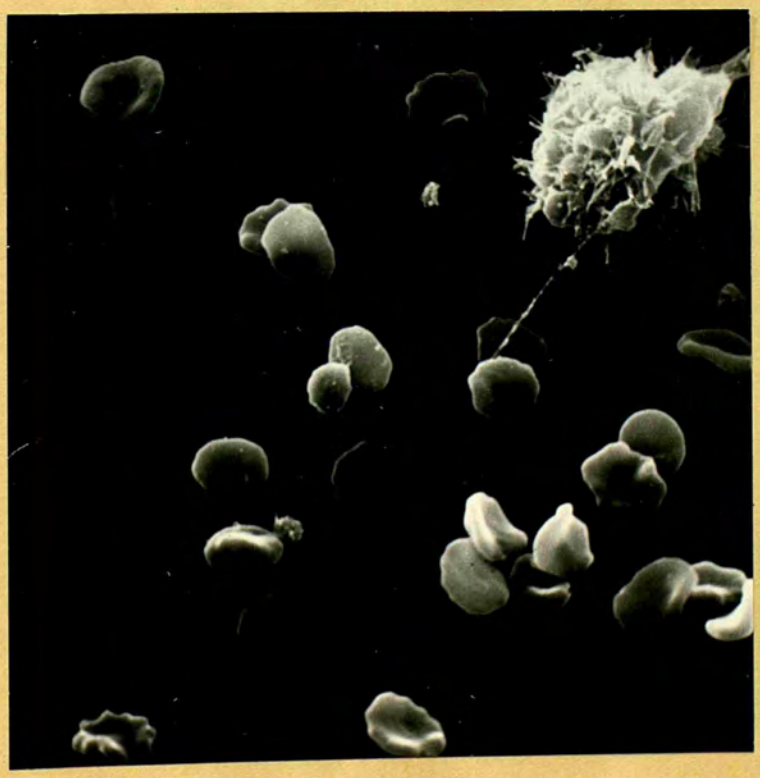


PLATE 3.iv.12.

Plate 3.iv.13. LEGEND

Detail of the surface of the haemocyte clot in Plate 3.iv.12. (SEM, x 1180).

Plate 3.iv.14. LEGEND

Haemocytes after 20 minutes incubation with O HRBC, showing a possible HRBC ghost (g) attached (SEM, x 2600).

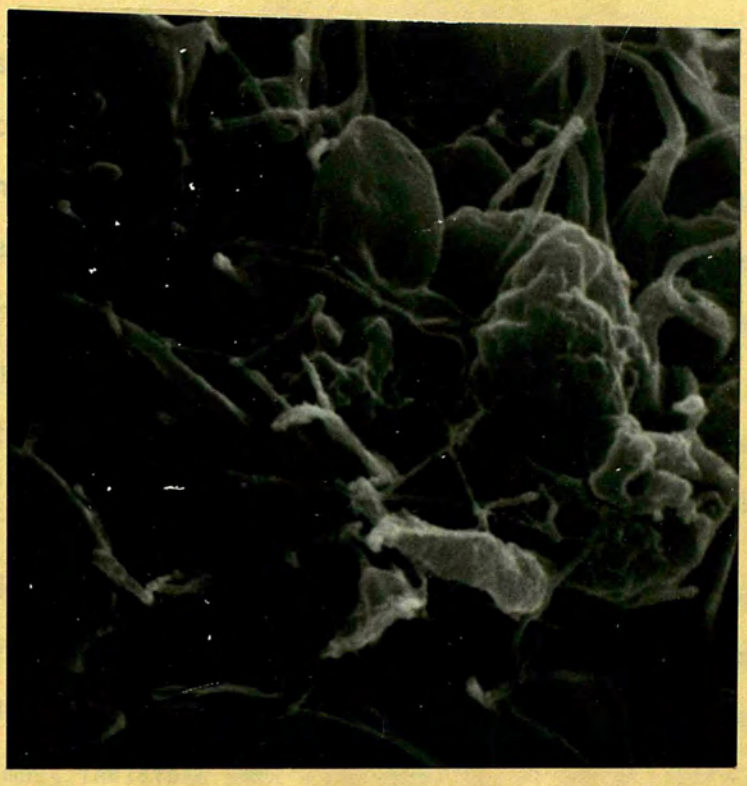


PLATE 3.iv.13.

PLATE 3.iv.14.

develop spikes at all and spreading was greatly retarded. From this it was concluded that microtubules play a part in spike orientation rather than being an intrinsic part of their structure. Initial investigations suggest that the spikes of Patella haemocytes may have a similar structure to the microspikes observed in other cells (Jones and Partridge, 1974). Some of the projections from the cells do slightly resemble the filopodia seen on some fibroblasts in culture (Plates 3.iv.2. and 3.iv.6. and Kessel and Shih, 1974, page 69).

Under phase contrast illumination and in SEM the haemocytes can be observed to form rosettes with RBC. Sheep and human RBC were used and both became attached. In some cases the haemocyte may be almost invisible under the adhering RBC (Plate 3.iv.7.). When formal sheep RBC were used the haemocytes were invisible under the mass of adhering RBC.

Once attached to the cell surface a few HRBC are phagocytosed (Plates 3.iv.8., 3.iv.9. and Plates 3.iii.1., 2, and 3.). Where the cells were allowed to settle for 15 minutes prior to fixation individual cells may be distinguishable in the clots (Plate 3.iv.10), but after 20 minutes in the culture the clots have a very complex surface structure, and it is hard to tell if the smoother, rounded shapes are cell bodies or phagocytosed HRBC (Plates 3.iv.11 and 3.iv.8.).

From light microscope studies very rarely was more than one RBC observed to have been taken up per haemocyte. Most of the haemocytes did not phagocytose any HRBC. Plate 3.iv.9. shows what is probably internal HRBC, one on the left seems to be undergoing the preliminaries of phagocytosis. Plate 3.iv.8. shows one RBC in a similar state and

one largely enveloped. Plate 3.iv.1. shows what could be the first stage of phagocytosis, the spike attached to the RBC surface. Plate 3.iv.12 shows a clot of cells with a single filament extending out and attaching to 5 RBCs. The highly complex surface of the clots is evident in this photograph, Plate 3.iv.13. is a detail of the same clot.

When haemocytes are incubated with HRBC, within about 30 - 40 minutes the cells become surrounded by HRBC ghosts. If intact RBC adhering to a cell are watched their bright yellowish glow under phase contrast gradually dims until the HRBC appears grey, it then looses its contents all together, leaving the attached ghost. A similar phenomenon was observed by Cantacuzene (1919) which he called contact lysis. Bacteria infecting Ascidia mentula were seen to lyse on contact with the blood cells.

No lysed RBC are visible with the cells incubated for 15 minutes but several structures which look like HRBC ghosts are to be seen with those incubated for 20 minutes (Plates 3.iv.14 and 3.iv.11.).

v. Discussion.

The spreading, stellate haemocyte seems to be wide spread among the molluscs. The amoebocytes of Patella are not dissimilar to those which have been described in other species. Similar cells have been observed in Liolophura gaimardi (Crichton, Kilby and Lafferty, 1973), Mercenaria mercenaria (Foley and Cheng, 1974), Crassostrea virginica (Foley and Cheng, 1972; Tripp, 1966) Tridacna maxima (Reade and Reade, 1976), Helix aspersa (Prowse and Tait, 1969), Otala lactea (Anderson and Good, 1976), Biomphalaria glabrata (Cheng

and Auld, 1977) and Lymnæa stagnalis (Sminia, 1974).

Of these species 3 have homogenous cell populations; the chiton Liolophura and 2 gastropods, Lymnæa and Otala.

Tridacna and Biomphalaria both have two different cell lines. One is a spreading, amoebocytic cell and the other a smaller cell. In Biomphalaria the small hyalinocytes do not spread and have no spikes. The smaller cells of Tridacna are polygonal and have short, fine processes, they contain large numbers of phase bright granules which often obscure the nucleus; a situation somewhat similar to that found in the 'macrophage' of Patella.

Both Crassostrea and Mercenaria have 3 cell types; the granulocytes, which have large numbers of differentially staining granules in their cytoplasm; fibrocytes, which appear to be similar to the granulocytes but lack the granules, and the hyalinocytes which are smaller than the other 2 cell types. In Crassostrea the hyalinocytes have very little cytoplasm relative to the size of the nuclei and have no spikes. The hyalinocytes of Mercenaria are rather larger and do not possess spikes either.

Cheng (1975) found that if whole haemolymph of Crassostrea was left to sit, over an hour the proportion of granulocytes in the culture declined sharply, whereas the proportion of fibrocytes rose. Since the haemocytes of Mercenaria are known to release lysozyme during phagocytosis (Cheng, Rodrick, Foley and Koehler, 1975), and its granulocytes have been observed to degranulate in vitro (Foley and Cheng, 1977), it is suggested that the fibrocytes of Crassostrea are probably degranulated granulocytes. Examination

of the haemocytes of Mercenaria by electron microscopy showed that the fibrocytes contained primary phagosomes and accumulations of glycogen, which suggested to Cheng and Foley (1975) that these cells might be aged granulocytes. The accumulation of granules in the Patella 'macrophages' and their apparently greater abundance in starved animals suggests that they could be an aged form of the amoebocytes.

Many features of Patella haemocytes bear a superficial resemblance to those of mammalian phagocytes. For a cell to be an effective phagocyte the requirements are such that a lack of resemblance would be the more surprising situation.

The haemocytes of Patella and mammalian macrophages, monocytes and granulocytes are all mobile cells, able to spread over surfaces by the extension of cytoplasmic veils. All are able to take in particles which they recognise as foreign from their surrounding medium. In the case of macrophages it has been suggested that the process of spreading over a surface is similar to the initial stages of phagocytosis.

Mammalian phagocytes are known to contain a wide range of lytic enzymes, the capacity to break down particles which are phagocytosed is an important property of any phagocyte which is to be at all effective. A wide range of lytic enzymes have been identified in the cells of Mercenaria and Crassostrea (Cheng and Rodric, 1975), Mya arenaria (Cheng and Yoshino, 1976) and Biomphalaria (Yoshino and Cheng, 1977), haemocytes have also been found to possess lytic activity. Gomori staining revealed acid phosphatase in the haemocytes

of Patella (Davies and Partridge, 1972), as did naphthol ASBI phosphate method of Barka (1960, from Bancroft, 1975) (see Plates 6.iii.1. and 6.iii.2, Section 6.). Other lytic enzymes might well be present, since Levvy, Hay and Marsh (1957) found the visceral hump of Patella to be extremely rich in B-glucuronidase using a fluorometric assay method.

Preliminary investigation showed lysozyme to be absent from the haemolymph, before and after injection of heat-killed bacteria. Of 5 samples of packed haemocytes, 3 showed lysozyme present in minute quantities (mean international units lysozyme mm^{-3} packed cells 0.37). But further work would be required to confirm this result.

Mammalian mononuclear cells in culture will form rosettes with antibody-coated RBC. But phagocytosis occurs only limitedly, even in the presence of complement factors. The bound RBC become spherical and osmotically fragile (LoBuglio, Cotran and Jandle, 1967), the unbound, sensitised RBC do not undergo lysis to any extent. A similar process is observed with Patella haemocytes, though no serum factors or pre-treatment of the RBC is necessary. The observed lysis of the attached RBC could be due to osmotic shock as the haemocytes are cultured either in haemolymph or in sea water (see Section 3.iv. and Section 5.ii.). Under these conditions lysis of the RBC is much slower in the control cultures, which lack haemocytes (Section 5.ii.).

The uptake of particles by the vertebrate macrophage is generally mediated by antibody and complement components. Rabinovitch and De Stefano (1970) found that mouse macrophages would take up sheep and horse RBC at a low rate in the absence of any serum factors.

This they termed non-immunological phagocytosis, as none of the mechanisms of classical, mammalian immunology are involved. Non-immunological uptake of RBC is enhanced by treatments which increase the 'foreigness' of the particle, such as fixation in glutaraldehyde, or pre-treatment with chymotrypsin. Treatments which enhance non-immunological uptake by mouse macrophages were also found to enhance uptake by the haemocytes of Galleria mellonella. They suggest that both cell types have features of their cell membranes in common.

Formol sheep RBC were found to adhere much more readily to the haemocytes of Patella than did the fresh ones. Since phagocytes must be able to distinguish self from non-self, where a phagocyte is able to take up particles in the absence of serum factors, any treatment which increases the distinction between self and non-self would be expected to increase the extent of phagocytosis. Phagocytosis in the presence of serum factors might be expected to be less affected. If opsonic factors are present, phagocytosis would depend on how the pre-treatment affected the adsorption of the opsonin onto the test particle rather than on the actual particle surface.

The haemolymph of Patella has been found to contain no opsonic factors (see Section 5) so recognition must depend on receptor sites on the surface of the phagocytes alone. This is a profound difference in mode of function between the haemocytes of Patella and the mammalian macrophage.

Recognition by mammalian phagocytes depends largely on antibody and complement factors. No counterparts to these have been observed in Patella. It has been suggested that the agglutinins, opsonic

factors and inducible bactericidins, found in some invertebrate body fluids might be precursors to the mam malian serum factors (Acton and Weinheimer, 1974; Evans, Cushing and Evans, 1973). But where these factors have been characterised they seem to bear no structural resemblance to antibody (Cornick and Stewart, 1973; Hammarstrom and Kabat, 1969; Marchalonis and Edelman, 1968 ; Miller, Ballback, Pauley and Krassner, 1972; Pauley, Granger and Krassner, 1971).

Viewed either by phase contrast, or when fixed and stained, the morphology of Patella haemocytes is evidently different from that of any mammalian phagocyte. The mammalian cells have nuclei of much more complex shape, their cytoplasm contains differently staining granules and none have any structure resembling the spikes of the haemocytes.

The difference in morphology are highlighted by the SEM photographs. The modes of spreading and surface complexities of macrophages and the haemocytes are quite different. Human white cells spread over the surface much less and retain their spherical shape more than the haemocytes. Small microvilli project from the macrophage surface in all directions, some attach the cell to the slide, or the cell surface may be covered in small ruffles and petaloid flaps (Warfel and Elberg, 1970). By comparison the cell body of the haemocyte is smooth and the projecting veils very large (Plate 3.iv.1.). Mammalian macrophages spread radially and any elongation of the cell tend to be the result of extended cytoplasm left in the wake of the advancing cell. The spikes of the haemocytes are extended in front of the cell body, in the direction of movement.

The haemocytes of Patella may be seen to be fairly typical of molluscan haemocytes. The dissimilarities observed between haemocytes and macrophages are probably indicative of fundamental morphological differences. Any similarities these cells may share are probably no more than might be expected from the comparison of any two phagocytic cell types, and are more probably due to analogy than homology.

SECTION 4

THE REACTION OF THE CELL FREE HAEMOLYMPH IN VITRO

i. Introduction.

The haemolymph of many molluscs contains humoral factors which are thought to play a part in internal defence. Agglutinins to vertebrate red cells have been found in Otala lactea (Boyd and Brown, 1965), Helix pomatia (Hammerstrom and Kabat, 1969), Aplysia californica (Pauley, Granger and Krassner, 1971), Tridacna maxima (Baldo and Uhlenbruck, 1975), Viviparus malleatur (Cheng and Saunders, 1962), Velesunio ambiguus (Jenkin and Rowley, 1970), Saxidomus giganteus (Johnson, 1964), and Crassostrea virginica (Tripp, 1966; McDade and Tripp, 1967a). Agglutinins to some species of bacteria have also been found in Aplysia californica (Pauley, Krassner and Chapman, 1971) and Mercenaria mercenaria (Arimoto and Tripp, 1977). There is no direct evidence that agglutinins take any part in internal defence, but it is thought that they do contribute. Agglutinins may act as opsonins, since they adsorb onto the surface of foreign particles and so could facilitate their recognition by the phagocytes (Acton and Weinheimer, 1974). It has also been suggested that opsonins may enhance intracellular degradation of phagocytosed particles (Pauley, Krassner and Chapman, 1974). Renwrantz and Cheng (1977a, b) found that the haemocytes of Helix pomatia do possess agglutinin receptors and that various haemagglutinins of plant and animal origin could act as connecting bridges and cause rosetting between HRBC and the haemocytes. In both Aplysia californica (Pauley, Granger

and Krassner, 1972) and Homarus americanus (Cornick and Stewart, 1968) the one species of bacterium found to be pathogenic in each case was not agglutinated by the haemolymph. The authors suggest that the agglutinating factor failed to attach to the pathogen, so the bacteria were less readily phagocytosed and their breakdown once within the cells slower. Agglutination could also physically limit the movement of bacteria within the haemolymph. Innoculation of Limulus polyphemus with endotoxin causes coagulation of the haemolymph (Levin and Bang, 1964; Levin, 1967).

Maia squinado was found to have a natural factor in the haemolymph which agglutinated the cilia of Anophrys sarcophaga, a ciliate parasite of Carcinus maenas (Bang, 1962). About 14% of the Maia tested lacked this agglutinin and when injected with the ciliate succumbed to a fatal infection. Ciliates isolated from these infected crabs failed to have any effect on crabs which possessed the agglutinin, so this factor is probably instrumental in preventing the multiplication of the ciliates within the crabs.

Opsonins are known to be present in the haemolymph of Crassostrea virginica (Tripp, 1966; McDade and Tripp, 1967a), Biomphalaria glabrata (Stanislawski, Renwrantz and Becker, 1976), Aplysia californica (Pauley, Krassner and Chapman, 1971) and Velesunio ambiguus (McKay, Jenkin and Rowley, 1969), and in the albumen gland extracts of Otala lactea (Anderson and Good, 1976) and Helix pomatia (Hammerstrom and Kabat, 1969; Prowse and Tait, 1969). The body fluids of all these species also contain haemagglutinins. But opsonic factors may be present in the haemolymph in the absence of any agglutinins, as in Helix pomatia, Otala lactea and Eledone cirrosa (Stuart, 1968).

Some invertebrate body fluids are known to have anti-bacterial properties; Golfingia gouldii (Bang and Krassner, 1958; Krassner and Flory, 1970), Limulus polyphemus (Johansen, Anderson, Good and Day, 1973; Pistole, 1976; Furman and Pistole, 1976), Homarus americanus (Cornick and Stewart, 1973), Galleria mellonella (Chadwick, 1975), Echinus esculentus (Unkles, 1976) and Ciona intestinalis (Johnson and Chapman, 1970a).

Molluscan haemolymph does not generally seem to possess natural antibacterial factors. Aplysia californica (Johnson and Chapman, 1970), Helix pomatia (Bayne and Kime, 1970) and Octopus dofleini (Bayne, 1973a) were all found to lack such factors. McDade and Tripp (1967b), however, found that Crassostrea virginica haemolymph caused plaques when dropped onto lawns of Bacterium megaterium and B. subtilis, though 17 other species of bacteria were not affected at all. B. megaterium is known to be susceptible to lysozyme, which is found in the haemolymph of Crassostrea (McDade and Tripp, 1967b). The haemolymph of Haliotis rufescens became bactericidal after innoculation with bacteria, though that of the naive animal was not (Cushing, Evans and Evans, 1971). Such inducible bactericidins have been looked for, but not found, in Aplysia californica (Pauley, Krassner and Chapman, 1971), Helix pomatia (Bayne and Kime, 1970) and Crassostrea virginica (Weinheimer, Acton and Evans, 1969).

Vertebrate red cells are commonly used to investigate the agglutinins of invertebrate body fluids as their antigenic sites and surface structure are relatively well known. Antigenic sites with the same binding capabilities are found among the bacteria,

plants and other animals. Human blood group reactive substances are common in invertebrates and plants, where they are called lectins. Many of the molluscan haemagglutinins seem to have a general anti-H specificity, and agglutinate A, B and O HRBC equally (Boyd, Brown and Boyd, 1966). Others have been found which seem to have a high degree of specificity. An extract of whole Saxidomus giganteus, (Johnson, 1964), and albumen gland extracts of both Otala lactea (Boyd and Brown, 1965; Boyd, Brown and Boyd, 1966) and Helix pomatia (Hammerstrom and Kabat, 1969) will agglutinate only A HRBC, B and O-group HRBC being quite unaffected.

Lytic factors against vertebrate red cells are quite common in invertebrate body fluids, but again their significance in internal defence is by no means certain.

Blood group reactive substances have also been found in invertebrate body fluids. They inhibit the agglutination of HRBC by the appropriate antisera by competing with the antigenic sites of the RBC for the antibody. Substances with an A-group reactivity have been found in Loligo vulgaris and Sepia officianalis (Renwrantz and Uhlenbruck, 1974a, b). An aqueous extract of Tubifex rivulorum was also found to have A-group reactivity (Uhlenbruck, Reifenberg and Prokop, 1969). Biomphalaria glabrata haemolymph contains soluble A, B and H blood group reactive substances (Stanislawsky, Renwrantz and Becker, 1976), which produced strong precipitin lines with anti-A reagents in Ouchterlony tests. Pemberton (1970) found that an aqueous extract of whole Patella inhibited the agglutination of A HRBC by anti-A serum.

The cell free haemolymph of Patella was used in vitro to try and elucidate its role in the animal's internal defence system. It was tested for the presence of agglutinins and lytic factors against vertebrate red cells and bacteria. Anti-bacterial effect was assessed by incubating living bacteria in the haemolymph and recording the changes in numbers over 24 hours, using a modification of the method of Shwab and Reeves (1966). Since Haliotis rufescens has been found to possess inducible bactericidins the presence of such factors in Patella was investigated using the haemolymph of previously inoculated animals.

Haemagglutination-inhibition tests were done initially with the haemolymph remaining in the test system. If haemagglutination inhibition resulted, this would suggest either that factors in the haemolymph were successfully competing for the binding sites of the antibody, or that haemolymph factors had masked the antigenic sites on the red cells. To see if this inhibition were due to the latter cause red cells were incubated in cell free haemolymph and washed prior to use. Inhibition in this case would indicate an interaction between red cell surface and factors in the haemolymph. Such a result might be expected were an opsonic factor present. The opsonin would bind to a foreign particle and so render it more liable to phagocytosis. If haemolymph factors were competing with the red blood cells' antigenic sites for the antibody, a positive precipitin test would be expected. Ouchterlony diffusion plates were set up using human anti-A and anti-B sera against Patella haemolymph. Precipitin rings would indicate the presence of a soluble blood group reactive substance.

ii. Interaction of the haemolymph with red blood cells.

Methods

The cell-free haemolymph was tested for lytic and agglutinating factors against dogfish, chicken, sheep and human, A, B, O and AB red cells.

The RBC were all washed 3 times, by centrifugation and resuspension, then made up to a 1% suspension, packed cell to fluid volume before use.

The dogfish cells were washed and resuspended in dogfish Ringer. The other red cells were washed in phosphate buffered 0.9% saline (PBS) and resuspended in modified barbitone buffer (MBB) (see Section 2.xi.).

The cell-free haemolymph of each animal was divided, half was used fresh and half used after heating to 56°C for 20 minutes. Serial double dilutions of both fresh and heated haemolymph were made with MBB in standard 'U' welled microtiter plates (Sterilin). 50 µl of each haemolymph sample was delivered into the first well of each row in the plate, using a fixed delivery micrometer pipette (Excalibur, Section 2.iii.). 25 µl of the diluting medium, either MBB or dogfish saline, was delivered into each succeeding well in the row. 25 µl of haemolymph was then removed from the first well and added to the second, 25 µl of this dilution was then removed and added to the third well. This procedure was followed until a sufficient number of dilutions were made. The last well in each row was a control

and contained only the diluting medium with no haemolymph.

25 µl of one of the red cell suspensions was then added to each well. The titer plates were covered and left overnight at room temperature.

The titer of the haemolymph was recorded as the highest dilution which shows either agglutination or lysis of the red cells.

The ability of cell-free haemolymph to agglutinate bacteria was recorded qualitatively. Washed, heat-killed bacteria were suspended either in sterile sea water or in haemolymph. A drop of each of these suspensions was put on either end of a slide. The bacteria in the haemolymph could then be compared visually with the sea water control for the extent of agglutination.

Results

In no case was any agglutinating or lytic activity observed against any of the red cells used (see Table 4.ii.1.).

Although only 6 specific tests against human red blood cells (HRBC) are recorded here, neither agglutination nor lysis occurred on any occasion when HRBC were incubated in Patella haemolymph in order to opsonise them (see Section 5.ii.).

Viewed under the microscope, bacteria were not visibly agglutinated when suspended in sterile sea water. On comparison of these with bacteria suspended in cell-free haemolymph, it

TABLE 4.ii.1.

RBC	h'lymph	*n	H'Agg	lysis
Dogfish	F	14	-	-
	H	11	-	-
Chicken	F	6	-	-
	H	5	-	-
Sheep	F	16	-	-
	H	16	-	-
Human A	F	6	-	-
B	F	6	-	-
O	F	6	-	-
AB	F	6	-	-

LEGEND The haemagglutinating and lytic activity of fresh (F) and heated (H) cell-free haemolymph when incubated in vitro with vertebrate red cells.

*n = number of trials

RBC = red blood cell, species
H'Agg = haemagglutination

was evident that the haemolymph did not cause any increase in the degree of agglutination of the bacteria which were suspended in it.

Conclusions

The haemolymph of animals which have not previously been challenged apparently contains no factors which are capable of interacting directly with vertebrate RBC to produce either lysis or agglutination, nor were bacteria observed to have agglutinated. Agglutinated masses of bacteria are easily distinguishable under the microscope in cases where agglutination has occurred (Pauley, Granger and Krassner, 1972). Any factors present in the haemolymph probably act in conjunction with the haemocytes, rather than independently.

Since the role of agglutinins in internal defence is unknown, the absence of lytic and agglutinating factors may not necessarily be of any consequence. Though haemagglutinins to vertebrate RBC are widespread among the molluscs, they seem to be more common in lamellibranchs than gastropods. Of 19 gastropods tested for haemagglutinating activity by Boyd, Brown and Boyd (1966), 10 species were completely negative.

The presence of lytic factors in molluscan body fluids is less common than in some other invertebrate groups, notably the sipunculids and the arthropods. Two lamellibranchs, Saxidomus giganteus (Johnson, 1964) and Mytilus edulis (Hardy, Fletcher and Gerrie, 1976) are known to contain haemolytic factors,

and among the gastropods investigated by Boyd, Brown and Boyd (1966) only one showed haemolytic activity. This was Helisoma anceps, a freshwater pulmonate.

The absence of agglutinins and lytic factors from the haemolymph of Patella does not appear to be inconsistent with findings reported from other gastropods.

iii. The interaction of the haemolymph with bacteria:

Method

Cell-free haemolymph from individual animals was membrane filtered to remove any possible contaminants. Half was heated for 20 minutes at 56°C, half was kept on ice to be used fresh.

10^{-4} and 10^{-5} dilutions of overnight broths of bacteria were made with sterile sea water or sterile broth. Yeast extract peptone broth (YEP) was used for the terrestrial species, Escherichia coli and Micrococcus, and ZoBell's marine medium for the marine species, Serratia and 'A' (see Section 2.vii.). Equal volumes of the bacterial dilutions were added to the fresh and heated haemolymph and to a sterile sea water control.

For each experiment 6 cultures were set up. Equal volumes of both bacterial dilutions were incubated with equal volumes of fresh and heated haemolymph and of sterile sea water.

As soon as the bacteria were added to the cultures a 25 μl sample was withdrawn, to give the initial concentration of bacteria. Samples were taken subsequently at 12, 18 and 24 hours. Each sample was diluted by 10^{-1} in sterile sea water for the marine species, or YEP for the terrestrial. One 25 μl drop of the diluted sample was then plated on each of 6 prepared agar plates (see Section 2.viii.). Throughout the experiment the cultures were maintained at 15^{o}C. The plates were incubated overnight at 37^{o}C. Colonies were counted under a dissecting microscope. From the mean count of the 6 drops from each culture at each time, the concentration of bacteria at the time in that culture could be calculated.

Broth controls were used initially but they became so concentrated over the 24 hour period that colony counts could not be made. Chadwick (1975) using Galleria mellonella haemolymph felt that the difference in growth of bacteria between broth controls and haemolymph pointed more to missing growth factors than actual bactericidal factors. Sea water seemed a more real comparison, Patella haemolymph is very close to sea water in osmolarity. Inhibition of bacterial growth in haemolymph over that in a broth control need not be the result of any intrinsic humoral bactericidal agents, but could be due to similarities between it and sea water. Sea water is known have antibacterial properties (Baslow, 1969), probably due to components of organic origin. The organic content of

natural sea water is sufficient to support populations of bacteria much larger than are usually found, and bacterial populations increase significantly in standing sea water (Simon and Oppenheimer, 1968). Scarpino and Pramer (1962) observed that the inhibition of E. coli growth in sea water can be reversed by the addition of small amounts of agents able to chelate metal ions. It was found that over the 24 hours the number of bacteria in the control cultures did not change very much.

In case a short-lived inhibitory effect was present in the haemolymph, one experiment was done using the pooled haemolymph of two animals, which was incubated with E. coli and sampled immediately and 4, 6, 9, 12 and 24 hours after the culture had been set up.

The presence of inducible bactericidal agents in the haemolymph was tested for using a modification of the method developed by Gingrich (1964), using Oncopeltus fasciatus. Animals were injected with heat-killed 'A' and their haemolymph collected 1, 6 and 36 hours post-injection. This and the haemolymph of naive animals, was membrane filtered prior to use, to remove any contaminating bacteria.

Petri dishes were made up with either YEP or ZoBell agar. One central and 5 surrounding wells were cut in each with a sterile cork borer. Each well was then filled with the sterile haemolymph of a different individual, and the central well with sterile sea water. The dishes were left at $4^{o}C$ for 14 hours to allow diffusion to occur. A sterile cotton swab innoculated with an overnight broth

of a species of bacterium was streaked over the agar surface and the plates incubated for 24 hours at $37^{o}C$.

Where inhibition of bacterial growth occurs a clear zone will be present around the well. The sea water is a control. 5 species of bacteria were used; 3 terrestrial, streaked onto YEP agar, Micrococcus, Bacterium megaterium and E. coli, and 2 marine species, streaked onto ZoBell, Serratia and 'A' (see Section 2.vii.). Each fluid was tested against each of the 5 bacterium species.

Results

The results were calculated as the mean number of bacteria per mm^3 for the 2 dilutions for each test culture.

The results for 'A' are not included as its growth in haemolymph and sea water was so rapid that after 12 hours incubation the cultures were too concentrated for colony counts to be made.

In Table 4.iii.1. the mean numbers of bacteria are calculated from the results of all the trials at each time. These results are plotted as logs on the graphs (4.iii.1-3.).

Two different concentrations of bacteria were used in order to see if numbers had any effect on their growth patterns. Table 4.iii.2. shows the rate of multiplication of the bacteria calculated as per cent change over the time for the 2 concentrations.

Fig. 4.iii.4. shows the growth of E. coli over 24 hours, with more frequent sampling times.

No inhibition of the streaked bacteria was observed after

Table 4.iii.1. LEGEND

The survival of 3 species of bacteria incubated over 24 hours in fresh, cell-free haemolymph of individual animals (F), cell-free haemolymph which had been heated to $56^{o}C$ for 20 minutes (H), and sterile sea water (SW). 2 dilutions of overnight broths of each bacterial species were used ($\times 10^{-4}$ and $\times 10^{-5}$). Results recorded are the mean number of surviving bacteria mm^{-3} in all the trials for each dilution of the bacterial broth.

** = standard error

* = number of trials

TABLE 4.iii.1.

Bacterial species	Culture medium & broth dil.	Sampling time (hours) no. bacteria mm^{-3} ± s.e.**							
		n*	0 hrs	n	12 hrs	n	18 hrs	n	24 hrs
E. coli	F - 10^{-5}	4	4.600 (± 4.47)	3	2.015 (± 2.16)	-	-	4	0.461 (± 0.44)
	F - 10^{-4}	4	32.250 (±26.64)	3	13.378 (±11.72)	-	-	4	6.105 (± 4.40)
	H - 10^{-5}	5	3.254 (± 2.96)	4	1.172 (± 1.06)	-	-	5	0.538 (± 0.49)
	H - 10^{-4}	4	28.200 (±22.72)	3	11.482 (± 8.22)	-	-	4	2.728 (± 1.19)
	SW - 10^{-5}	5	3.400 (± 3.09)	4	1.222 (± 0.94)	-	-	5	0.346 (± 0.24)
	SW - 10^{-4}	5	18.933 (±14.06)	4	12.646 (±10.41)	-	-	5	7.271 (± 2.23)
Serratia marino-rubra	F - 10^{-5}	2	0.633 (± 0.50)	1	0.867	1	1.933	-	-
	F - 10^{-4}	2	6.133 (± 2.40)	1	7.467	1	61.333	-	-
	H - 10^{-5}	3	0.689 (± 0.42)	3	1.733 (± 1.17)	1	7.800	2	3.566 (± 2.437)
	H - 10^{-4}	3	12.244 (± 5.78)	3	24.648 (±15.11)	1	19.400	-	-
	SW - 10^{-5}	3	0.756 (± 0.38)	3	0.689 (± 0.28)	2	2.500 (±0.83)	2	1.866 (± 1.45)
	SW - 10^{-4}	3	10.244 (± 2.90)	3	13.089 (± 3.39)	1	14.133	1	38.400
Micro-coccus	F - 10^{-5}	2	4.500 (± 4.17)	2	1.667 (± 1.60)	1	0	2	0
	F - 10^{-4}	1	12.333	1	2.533	-	-	1	15.867
	H - 10^{-5}	2	5.833 (± 5.58)	2	0.300 (± 0.17)	1	0.267	2	1.200 (± 0.87)
	H - 10^{-4}	2	11.700 (±11.43)	2	3.533 (± 3.47)	1	0.467	1	0.067
	SW - 10^{-5}	2	4.533 (± 4.40)	2	0.033 (± 0.034)	1	1.467	2	0.767 (± 0.70)
	SW - 10^{-4}	2	7.866 (± 7.70)	2	4.233 (± 4.23)	1	0	2	2.766 (± 2.77)

Fig. 4.iii.1. LEGEND

(from Table 4.iii.1.) The mean change in number of 2 dilutions of a suspension of Escherichia coli incubated in cell-free haemolymph (+), haemolymph which had been heated (o), and sterile sea water (x).

Fig. 4.iii.2. LEGEND

(from Table 4.iii.1.) The change in number of 2 dilutions of a suspension of Serratia marinorubra incubated in cell- free haemolymph (+), haemolymph which had been heated (o) and sterile sea water (x).

Fig. 4.iii.3. LEGEND

(from Table 4.iii.1.) The change in number of 2 dilutions of a suspension of Micrococcus luteus incubated in cell-free haemolymph (+), haemolymph which had been heated (o) and sterile sea water (x).

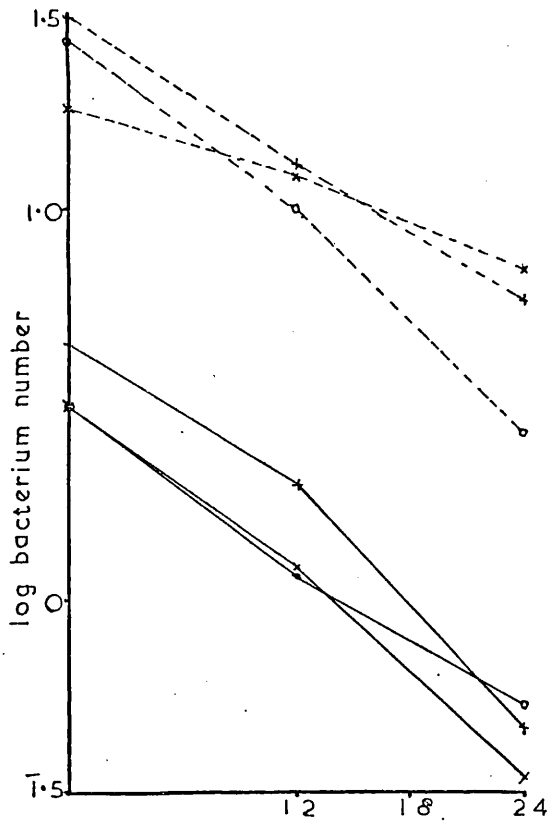


Fig. 4.iii.1.

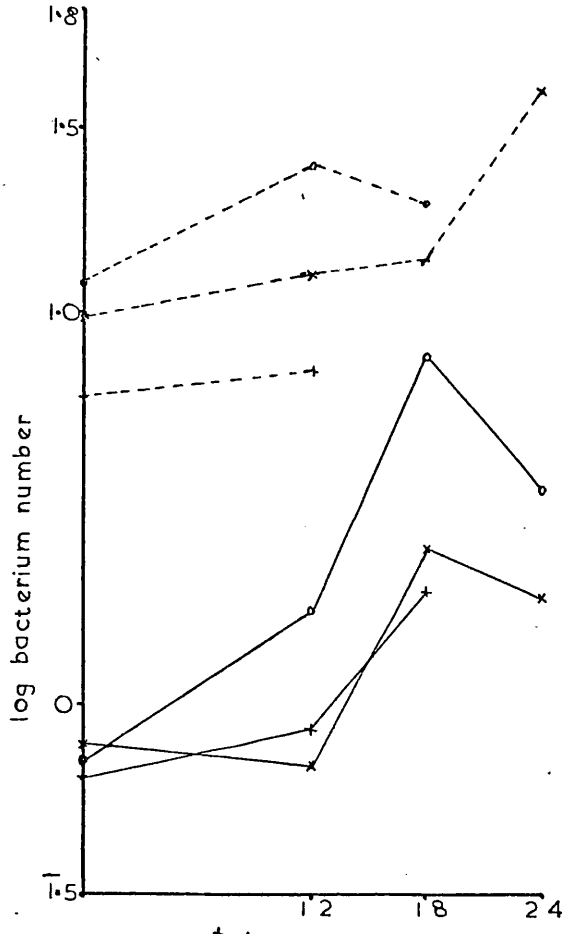


Fig. 4.iii.2.

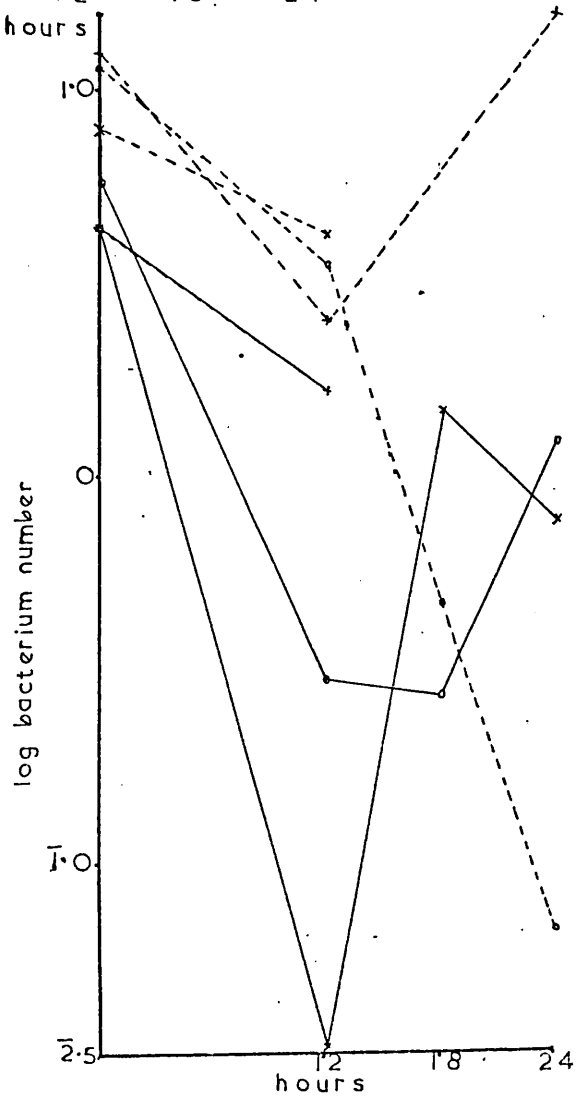


Fig. 4.iii.3.

Table 4.iii.2. LEGEND

The proportion of 3 species of bacteria surviving after incubation in fresh, cell-free haemolymph (F), haemolymph which had been heated to 56^{o}C for 20 minutes (H), and sterile sea water (SW). A 10^{-4} and 10^{-5} dilution of an overnight broth was used for each species. The results are those from Table 4.iii.1., the mean number of surviving bacteria is expressed as a percentage of the number present in the initial sample (time '0').

TABLE 4.iii.2.

Bacterium species	Incubation medium and dilution	Sample Time 12 hrs. % age	18 hrs. % age	24 hrs. % age
E. coli	F - 10^{-5}	43.80	-	10.02
	F - 10^{-4}	41.48	-	18.93
	H - 10^{-5}	36.02	-	16.53
	H - 10^{-4}	40.72	-	9.67
	SW - 10^{-5}	35.94	-	10.18
	SW - 10^{-4}	66.79	-	38.40
Serratia marino-rubra	F - 10^{-5}	136.67	305.37	-
	F - 10^{-4}	121.75	1000.05	-
	H - 10^{-5}	251.52	1135.07	517.56
	H - 10^{-4}	201.28	158.44	-
	SW - 10^{-5}	91.14	300.69	246.82
	SW - 10^{-4}	127.77	137.96	-
Micro-coccus	F - 10^{-5}	37.04	0.00	0.00
	F - 10^{-4}	20.54	-	128.65
	H - 10^{-5}	5.14	4.58	20.57
	H - 10^{-4}	30.20	3.99	0.57
	SW - 10^{-5}	0.73	32.36	16.92
	SW - 10^{-4}	53.81	0.00	35.16

Table 4.iii.3. LEGEND

The survival of a x 10^{-5} and a x 10^{-4} dilution of an overnight broth of E. coli incubated in fresh, cell-free haemolymph (F), cell-free haemolymph which had been heated to $56^{o}C$ for 20 minutes (H) and sterile sea water (SW). Results are recorded as the mean number of surviving bacteria mm^{-3} from 9 replicate drop plates for each culture.

TABLE 4.iii.3.

culture medium and broth dilution	Sampling Time 0	1 hr.	4 hrs.	6 hrs.	9 hrs.	12 hrs.	24 hrs.
F - 10^{-5}	*18.000 **(± 6.145)	9.956 (± 1.618)	5.733 (± 1.342)	5.733 (± 1.685)	5.378 (± 1.601)	5.911 (± 1.572)	1.778 (± 0.570)
F - 10^{-5}	*112.133 (±24.082)	79.467 (± 9.658)	62.089 (± 7.113)	51.378 (± 6.882)	44.578 (± 7.054)	36.800 (± 4.991)	19.289 (± 1.998)
H - 10^{-5}	*15.067 (± 2.201)	12.311 (± 2.636)	9.556 (± 1.915)	8.889 (± 2.621)	7.111 (± 1.277)	4.356 (± 0.811)	2.489 (± 0.819)
H - 10^{-4}	*96.333 (±12.771)	64.800 (±10.626)	°55.150 (± 5.849)	46.533 (± 3.538)	42.044 (± 3.891)	27.911 (± 4.629)	5.778 (± 2.210)
SW - 10^{-5}	*15.733 (± 0.969)	13.378 (± 3.275)	9.067 (± 2.218)	5.556 (± 1.838)	5.022 (± 1.823)	°3.956 (± 1.555)	0.622 (± 0.533)
SW - 10^{-4}	*75.133 (± 6.266)	62.178 (± 4.530)	57.022 (± 8.697)	29.289 (± 2.621)	51.911 (± 6.913)	43.850 (± 5.906)	12.889 (± 3.615)

** = ± standard error of mean

* = n = 6

° = n = 8

Fig. 4.iii.4. LEGEND

(from Table 4.iii.3.) The change in number of 2 dilutions of a suspension of E. coli incubated in cell-free haemolymph (+), haemolymph which had been heated (o) and sterile sea water (x).

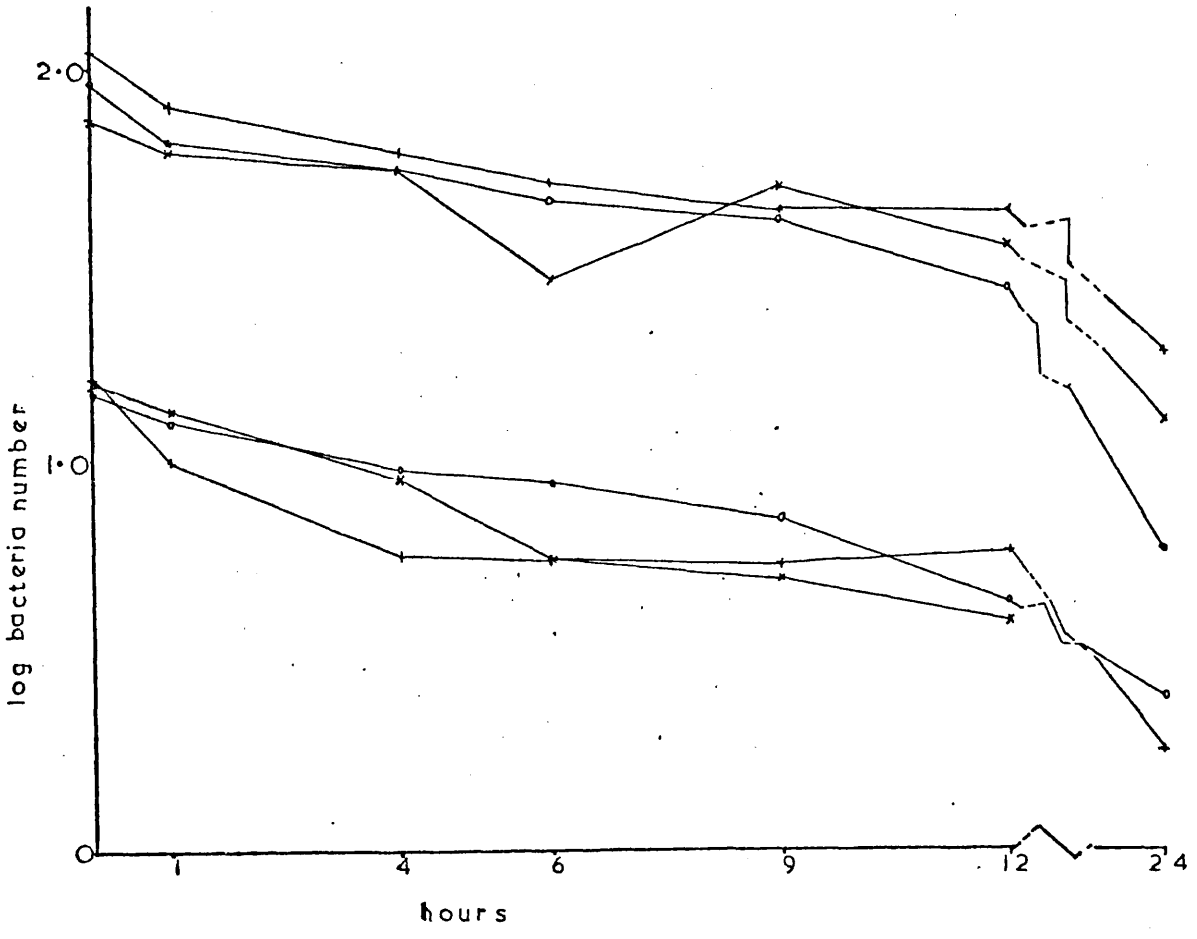


Fig. 4.iii.4.

Fig. 4.iii.5. LEGEND

(From Table 4.iii.2.) The percentage of the initial number of E. coli which remained at each sampling time after incubation in cell-free haemolymph (+), haemolymph which had been heated (o) and sterile sea water (x).

Fig. 4.iii.6. LEGEND

(from Table 4.iii.2.) The percentage of the initial number of Micrococcus which remained at each sampling time after incubation in cell-free haemolymph (+), haemolymph which had been heated (o) and sterile sea water (x).

Fig. 4.iii.7. LEGEND

(from Table 4.iii.2.) The percentage of the initial number of Serratia which remained at each sampling time after incubation in cell-free haemolymph (+), haemolymph which had been heated (o) and sterile sea water (x).

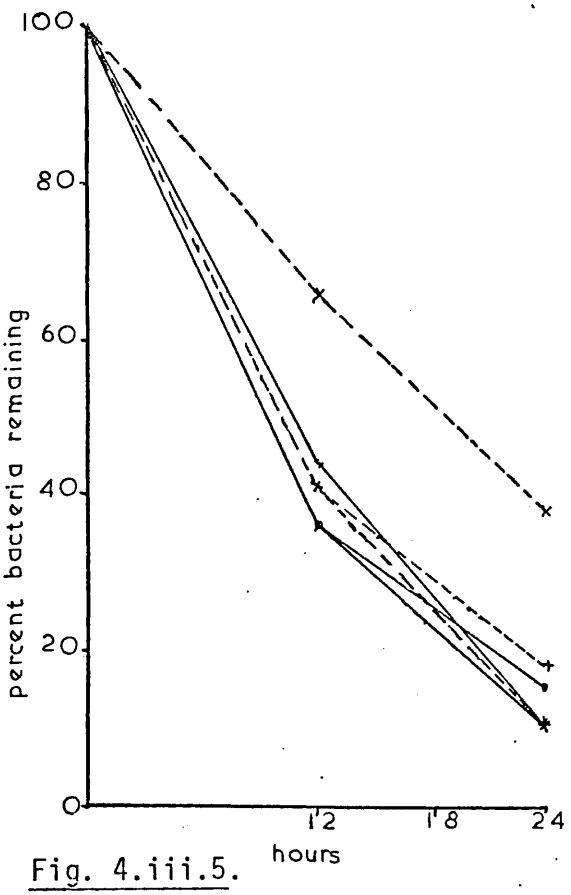


Fig. 4.iii.5.

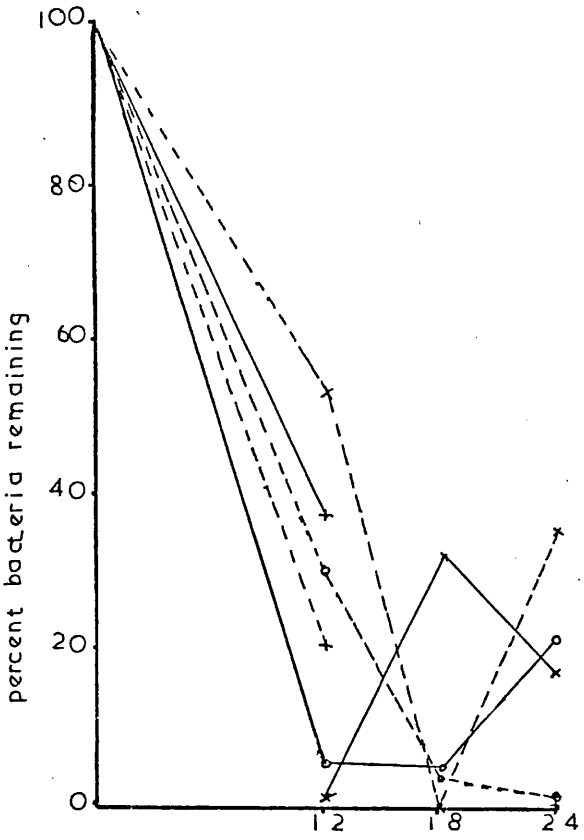


Fig. 4.iii.6.

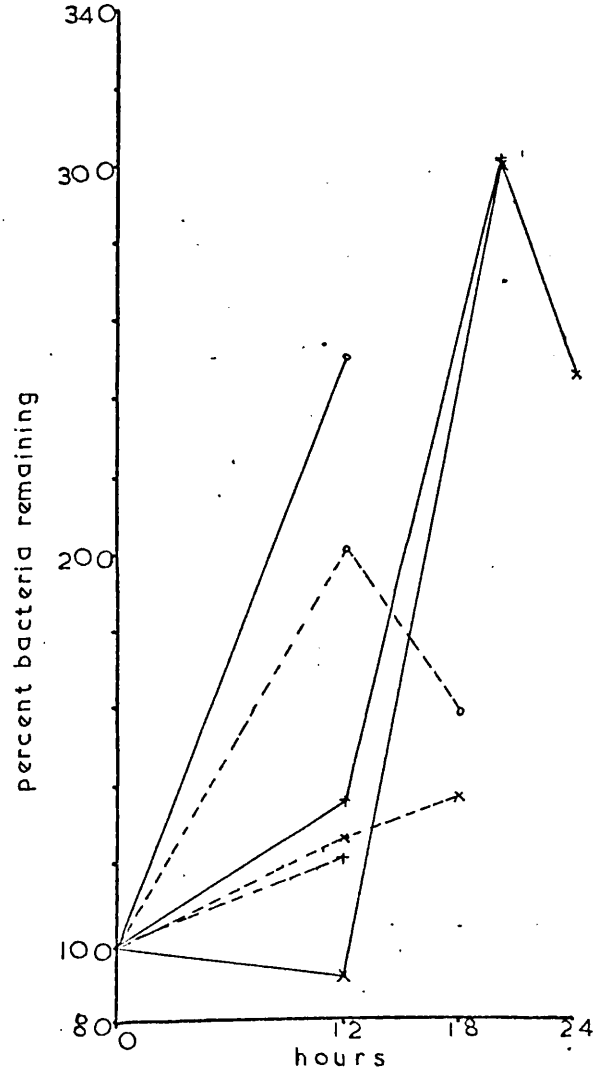


Fig. 4.iii.7.

24 hours of incubation. Five animals were tested at each post-injection sampling time. The haemolymph of 15 naive animals was also tested.

Inhibition will result only where an effective bactericide is present in the fluid. The presence of a bacteristatic factor would be evident as relatively poorer growth of the lawns where the factor had diffused into the agar.

Conclusions

The bacteria used were no more inhibited from growing by the haemolymph than by sea water. E. coli shows a steady decline over the time, but there is no significant difference in the decline between the 3 test media (Fig. 4.iii.1). Serratia seems to grow rather better in haemolymph as it grew to excess for counting in 18 hours in haemolymph but was still countable after 24 hours in sea water. Micrococcus shows a less clear cut result, probably partially caused by there being fewer results. An initial fall in numbers seems to be followed by a net rise.

The initial number of bacteria does not seem to affect the subsequent rate of decrease of E. coli and Micrococcus. When expressed as a proportion of the initial dose, the remaining numbers of both dilutions of bacteria follow very much the same course (Figs. 4.iii. 5 and 6) over the first 12 hours.

That growth of the terrestrial species is not enhanced in heated fluid probably indicates that the conditions were not optimal, which is not surprising as the haemolymph is close to sea water in

Fig. 4.iii.8. LEGEND

The percentage of the initial number of E. coli which remained at each sampling time after incubation in cell-free haemolymph (+), haemolymph which had been heated (o) and sterile sea water (x).

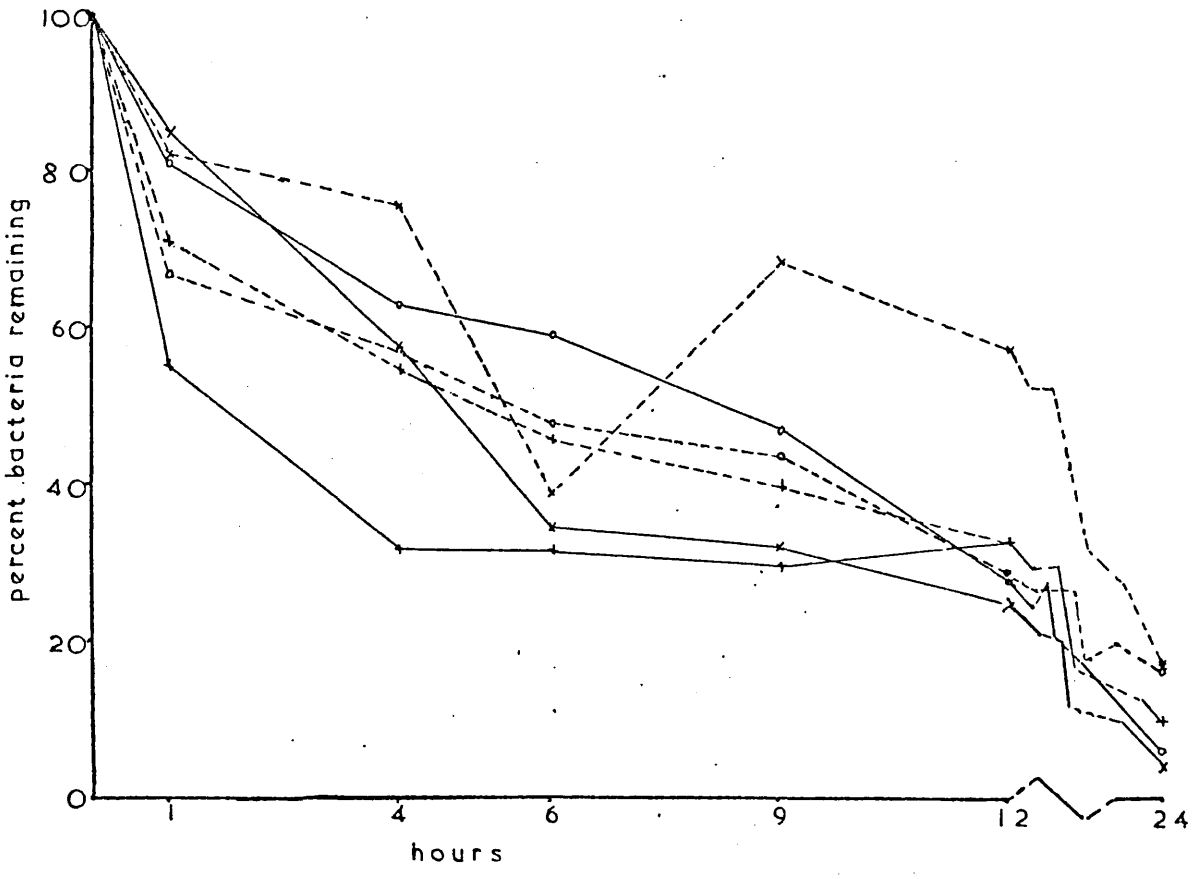


Fig. 4.iii.8.

TABLE 4.iii.4.

culture medium & broth dilution	Sampling Time 0 %	1 hr %	4 hrs %	6 hrs %	9 hrs %	12 hrs %	24 hrs %
F - 10^{-5}	100	55.31	31.85	31.85	29.88	32.84	9.88
F - 10^{-4}	100	70.87	55.37	45.82	39.75	32.82	17.20
H - 10^{-5}	100	81.71	63.42	59.00	47.20	28.91	16.52
H - 10^{-4}	100	67.27	57.25	48.30	43.64	28.97	6.00
SW - 10^{-5}	100	85.03	57.63	35.31	31.92	25.14	3.95
SW - 10^{-4}	100	82.76	75.89	38.98	69.09	58.36	17.15

LEGEND The proportion of a 10^{-5} and a 10^{-4} dilution of an overnight broth of E. coli surviving after incubation in fresh, cell-free haemolymph (F), haemolymph which had been heated to $56^{o}C$ for 20 minutes (H) and sterile sea water (SW). From the results in Table 4.iii.3., the mean number of surviving bacteria is expressed as a percentage of the number present in the initial sample (time '0').

ionic composition and osmolarity and the incubating temperature was rather low. But if any antibacterial factors were present one would expect to observe a marked difference in growth rates between the fresh haemolymph and the heated, and sea water controls.

The rate of increase of Serratia was also unaffected by the initial dose of bacteria. It did, however, seem to multiply more rap idly in the heated haemolymph than in the fresh, or than in sea water, (but this difference was not significant - $t = 1.08$; $p \geq 0.1$ for rate of multiplication). Over the first 12 hours the rate of increase is about the same in the latter two (Fig. 4.iii.7.). This suggests that the haemolymph is a good growth medium for Serratia, but that it could contain a factor which is very slightly inhibitory and is destroyed by heating.

The first 12 hours of this experiment are likely to be the more significant and to give a better notion of the capabilities of the haemolymph. Fig. 4.iii.8. shows that the sharpest decrease in numbers occurs in the first 4 hours of incubation. The latter 12 hours of the experiment cannot really give a true picture of what might occur in vivo. It seems that the proportional decline in numbers is more marked in the first 12 hours of the experiment with both E. coli and Micrococcus (Figs. 4.iii.5 and 6). With Serratia growth is more restrained during this period. As a result of the metabolic processes of the bacteria over 24 hours changes in the haemolymph would be expected. The haemolymph seems naturally to be poorly buffered (see Section 2.ii.) so changes in pH could occur. Such changes might well adversely affect any factors present in the haemolymph which were capable of slightly retarding bacterial

multiplication. After 24 hours conditions in the test cultures would be unlikely to give an accurate reflection of those which would obtain in vivo.

The results of incubating bacteria in haemolymph and of streaking bacteria over haemolymph impregnated agar indicate that Patella has no effective native bacteristatic agents in the haemolymph.

That the haemolymph of none of the pre-injected animals caused any inhibition of bacterial growth suggests that Patella has no inducible bactericidal factors. The inducible bactericidin of Haliotis reached a peak of activity between one and two days (Cushing, Evans and Evans, 1971) whereas that of the sipunculid, Dendrostomum zostericolum, reached its peak after only 90 minutes (Evans, Cushing and Evans, 1973). The sampling times post-injection used for Patella were spaced over sufficient time, that if any inducible factor were present, either a short term or a longer term response should have been apparent.

iv. Haemagglutination inhibition.

Method

Double dilutions of the haemolymph of individual animals were made in microtiter plates as before (see Section 4.iii.) using MBB as the diluent. 25 μl of a 1%, v:v, suspension of RBC, sheep or human, in MBB was then added to each well. Haemolymph was used fresh and after heating for 20 minutes at 56^{o}C. The control well had no haemolymph.

The titer plates were incubated for 1 hour at room temperature, then the appropriate antiserum was added to each well, the plates covered and left to stand overnight at room temperature.

For sheep RBC the antiserum used was rabbit anti sheep red blood cell (Flow Laboratories) and for human A and B, human antiserum (see Section 2.ix).

The results were recorded as the highest dilution of haemolymph which caused a reduction of agglutination over the control. Only cases which showed an agglutinated control could be recorded.

In the above experiment the haemolymph remains in the system. To see if the factor causing inhibition of agglutination blocks the antigenic sites by interaction with the red blood cell membrane, or blocks the recognition sites of the antibody, HRBC were pre-incubated in fresh haemolymph and washed prior to use.

The HRBC were allowed to stand for one hour at room temperature as a 10% suspension in cell free haemolymph. Aliquots of the same haemolymph were used to incubate both A and B cells. The HRBC were washed twice in PBS after incubation, and made up to a 1%, v:v, in MBB. Control HRBC were incubated in sterile sea water and in PBS.

Double dilutions of antiserum were made in microtiter plates with MBB, and 25 μl of one of the HRBC suspensions added to each. The plates were covered and left at room temperature overnight.

Results were recorded as the lowest dilution of antiserum

which showed no agglutination, and was recorded as negative if this was the same, or higher than that of the PBS control.

Results

The agglutination of SRBC by rabbit anti-sheep antiserum was not inhibited by the presence of Patella haemolymph in the titer wells, when compared to the controls which had no haemolymph in them. The experiment was done using the haemolymph of 16 animals separately.

HRBC agglutination by human antisera was inhibited by Patella haemolymph (see Table 4.iv.1.). The results are expressed as the log of the reciprocal of the titer as this gives a more normal distribution of the endpoints (Lutz, 1973, in Wier)

The haemagglutination-inhibiting factor appears to be heat stable since the results given in both Tables 4.iv.1 and 4.iv.2. indicate that heating did not significantly alter the mean titer (Table 4.iv.1.; $p = > 0.1$ for fresh and heated haemolymph against both A and B HRBC), or the number of trials giving a positive result at 1/16 dilution (A HRBC - $p = > 0.05$; B HRBC - $p = > 0.1$).

Examining the results, it was evident that the endpoints of titers done in summer (June - October) were rather higher than those done in winter (November - January). The results are tabled separately in Table 4.iv.3. (at 1/16 dilution for A $p = < 0.02$, therefore significant difference; B $p = > 0.05$, therefore not significant).

When the HRBC were incubated in Patella haemolymph and washed before use, the inhibition of agglutination was very much less than

TABLE 4.iv.1.

reciprocal of titre	log recip. titre	HRBC A haemolymph fresh	A haemolymph heated	HRBC B haemolymph fresh	B haemolymph heated	HRBC AB haemolymph fresh	AB haemolymph heated
no inhibition	-	3	6	0	0	1	0
1	0	7	4	9	1	0	0
2	0.301	2	1	1	0	0	0
4	0.602	2	0	0	2	1	0
8	0.903	2	0	3	3	2	3
16	1.204	5	1	4	4	1	1
32	1.505	8	1	6	4	0	0
64	1.806	0	0	1	2	0	0
128	2.107	2	0	0	0	-	-
256	2.408	1	0	0	0		
512	2.709	0	0	1	0		
1024	3.010	0	1	0	1		
	number of trials	32	14	25	17	5	4

LEGEND Aliquots of A, B or AB HRBC, suspended in sterile sea water were added to serial double dilutions of fresh cell-free haemolymph and cell-free haemolymph which had been heated to 56°C for 20 minutes in a titer plate when human anti-serum was added to the wells haem-agglutination was observed to be inhibited. The number of trials which gave a haemagglutination-inhibition end-point at each dilution of haemolymph was recorded. The haemagglutination-inhibition end-point is the highest dilution of haemolymph which prevents the agglutination of the HRBC by the anti-serum.

Fig. 4.iv.1. LEGEND

(from Table 4.iv.1.) The number of trials which gave haemagglutination-inhibition end-points at each haemolymph dilution when titrated against A HRBC (x), B HRBC (o) and AB HRBC (+---).

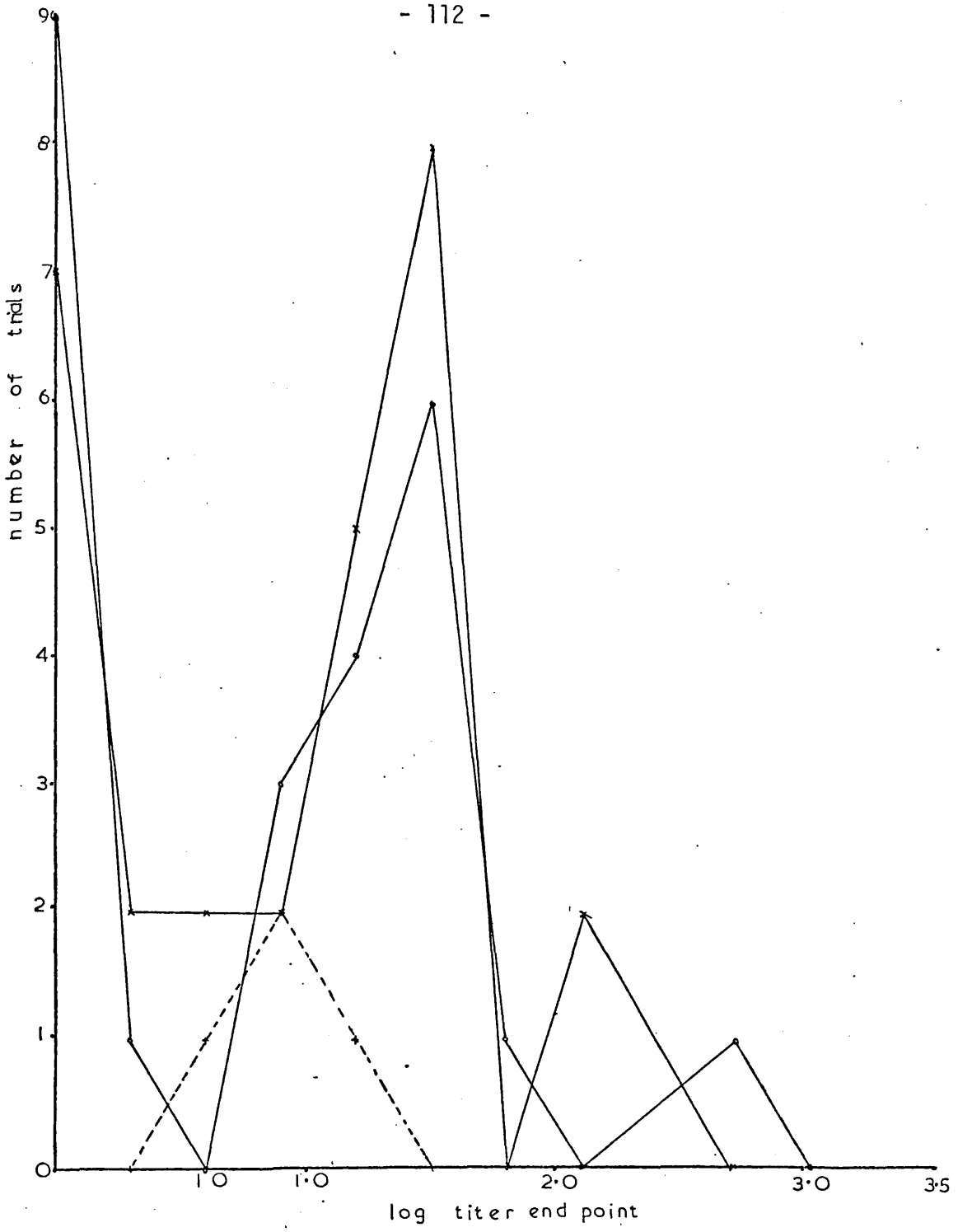

number of trials
0
1
2
3
4
5
6
7
8
9
1·0
1·0
2·0
3·0
3·5
log titer end point

Fig. 4.iv.1.

TABLE 4.iv.2.

log recip. titre	HRBC A Haemolymph fresh % age	A heated % age	B Haemolymph fresh % age	B heated % age	AB Haemolymph fresh % age	AB heated % age
no inhibition	9.37	42.86	0.00	0.00	20.00	0.00
0	21.87	28.57	36.00	5.88	0.00	0.00
0.301	6.25	3.12	4.00	0.00	0.00	0.00
0.602	6.25	0.00	0.00	11.76	20.00	0.00
0.903	6.25	0.00	12.00	17.65	40.00	75.00
1.204	15.62	3.12	16.00	23.53	20.00	25.00
1.505	25.00	3.12	24.00	23.53	0.00	0.00
1.806	0.00	0.00	4.00	11.76	0.00	0.00
2.107	6.25	0.00	0.00	0.00	-	-
2.408	3.12	0.00	0.00	0.00		
2.709	0.00	0.00	4.00	0.00		
3.010	0.00	3.12	0.00	5.88		
number of trials	32	14	25	17	5	4

LEGEND The results in Table 4.iv.1. expressed as the percentage of the total number of trials for A, B or AB HRBC which gave an end-point at each haemolymph dilution.

TABLE 4.iv.3.

log recip. titer	HRBC A S Fresh % age	A S Heated % age	A W Fresh % age	A W Heated % age	B S Fresh % age	B S Heated % age	B W Fresh % age	B W Heated % age
0	93 *(n=29)	68 (n=28)	70 (n=27)	36 (n=22)	100 (n=23)	100 (n=17)	69 (n=26)	23 (n=22)
0.301	93 (n=29)	68 (n=28)	59 (n=27)	27 (n=22)	74 (n=23)	100 (n=17)	58 (n=26)	23 (n=22)
0.602	93 (n=29)	68 (n=28)	52 (n=27)	23 (n=22)	74 (n=23)	100 (n=17)	54 (n=26)	23 (n=22)
0.903	87 (n=29)	68 (n=28)	44 (n=27)	23 (n=22)	74 (n=23)	94 (n=17)	54 (n=26)	23 (n=22)
1.204	87 (n=29)	68 (n=28)	37 (n=27)	23 (n=22)	70 (n=23)	94 (n=17)	46 (n=26)	17 (n=22)
1.505	78 (n=23)	59 (n=17)	22 (n=27)	9 (n=22)	61 (n=23)	82 (n=17)	38 (n=26)	17 (n=22)
1.806	58 (n=12)	0 (n= 6)	11 (n=27)	9 (n=22)	8 (n=12)	50 (n= 6)	27 (n=26)	17 (n=22)
2.107	33 (n= 6)	-	0 (n=14)	9 (n=11)	0 (n= 6)	-	0 (n=14)	0 (n=11)
2.408	17 (n= 6)	-	0 (n=14)	9 (n=11)	0 (n= 6)	-	0 (n=14)	0 (n=11)
2.709	0 (n= 6)	-	0 (n=14)	9 (n=11)	0 (n= 6)	-	0 (n=14)	0 (n=11)
3.010	0 (n= 6)	-	0 (n=14)	9 (n=11)	0 (n= 6)		0 (n=14)	0 (n=11)

LEGEND The percentage of trials which showed haemagglutination-inhibition of A and B HRBC by human anti-sera, at each dilution of fresh cell-free haemolymph or of cell-free haemolymph which had been heated to 56°C for 20 minutes. The results are tabulated to show trials done during summer (S), June-October and winter (W), November-January, separately.

* n = number of trials

TABLE 4.iv.4.

log. recip. titer	HRBC A haemolymph Fresh % age	HRBC A haemolymph Heated % age	HRBC B haemolymph Fresh % age	HRBC B haemolymph Heated % age
0.	82 *(n=56)	53 (n=47)	84 (n=49)	56 (n=39)
0.301	77 (n=56)	49 (n=47)	65 (n=49)	56 (n=39)
0.602	73 (n=56)	47 (n=47)	63 (n=49)	56 (n=39)
0.903	68 (n=56)	47 (n=47)	63 (n=49)	51 (n=39)
1.204	64 (n=56)	47 (n=47)	57 (n=49)	46 (n=39)
1.505	48 (n=50)	31 (n=39)	49 (n=49)	11 (n=28)
1.806	26 (n=39)	7 (n=28)	21 (n=38)	0 (n=11)
2.107	10 (n=20)	9 (n=11)	0 (n=20)	0 (n=11)
2.408	5 (n=20)	9 (n=11)	0 (n=20)	0 (n=11)
2.709	0 (n=20)	9 (n=11)	5 (n=20)	0 (n=11)
3.010	0 (n=20)	9 (n=11)	0 (n=20)	0 (n=11)

LEGEND The results from Table 4.iv.3. added to give the total percentage of trials which showed inhibition of agglutination of A and B HRBC at each dilution of fresh cell-free haemolymph and of cell-free haemolymph which had been heated to 56^{o}C for 20 minutes.

* n = number of trials

Fig. 4.iv.2. LEGEND

(from Table 4.iv.3.) The percentage of the trials carried out in summer (x) and in winter (o) which inhibited the agglutination of A HRBC.

Fig. 4.iii.3. LEGEND

(from Table 4.iv.3.) The percentage of the trials carried out in summer (x) and in winter (o) which inhibited the agglutination of B HRBC.

Fig. 4.iii.4. LEGEND

(from Table 4.iv.4.) The percentage of the trials of fresh cell-free haemolymph which caused haemagglutination-inhibition of A HRBC (x) and of B HRBC (o) and the percentage of the trials of haemolymph which had been heated which caused haemagglutination-inhibition of A HRBC (+) and of B HRBC (△).

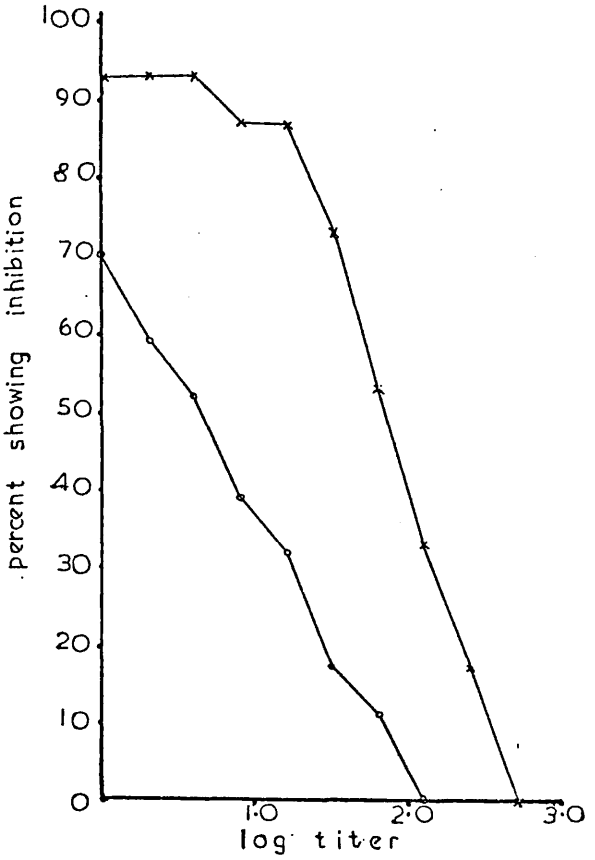


Fig.4iv.2.

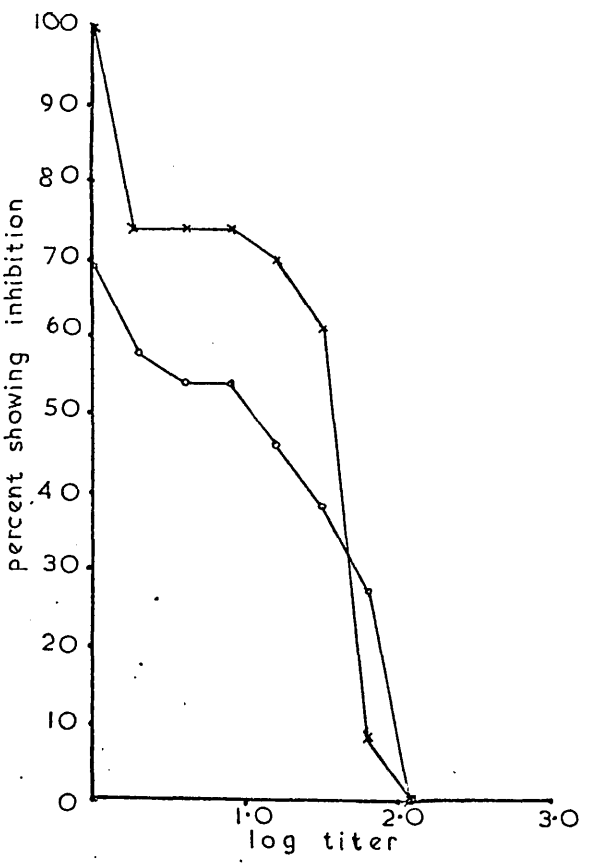


Fig. 4.iii.3.

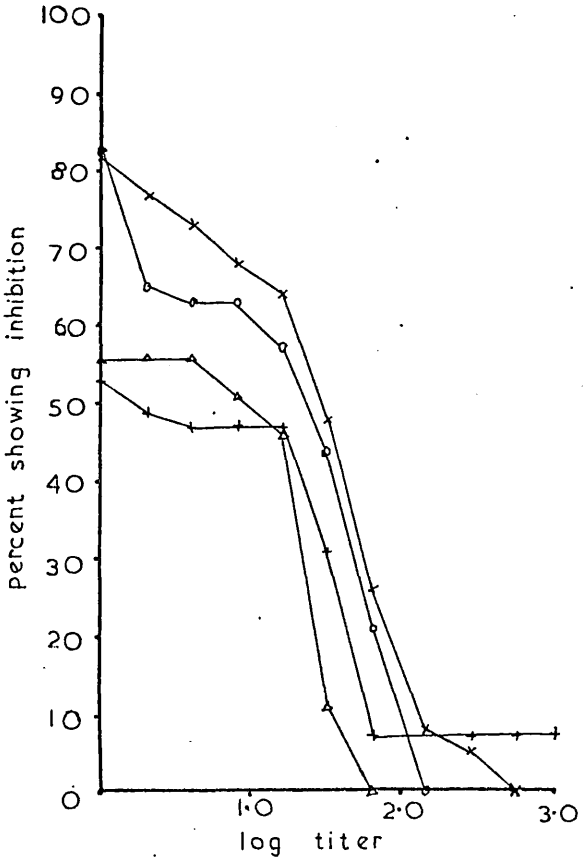


Fig. 4.iii.4.

when the haemolymph remained in the system.

Since the antisera were diluted in this case, inhibition was recorded as the increase in the minimum agglutinating titer of the antiserum with the HRBC pre-incubated in haemolymph, compared with the control HRBC pre-incubated in PBS. HRBC incubated in sea water were also used as a control; their agglutination was not inhibited. The endpoint for PBS and sea water incubated HRBC were the same.

The experiment was repeated 10 times. Of these, 5 showed no inhibition of A or B HRBC; 3 caused inhibition of B but not of A; and 2 caused inhibition of both A and B (see Table 4.iv.5.). In only 2 cases is the inhibition more than one dilution less than the control.

In 4 cases haemolymph which had previously been adsorbed with A was used for pre-incubating B HRBC, and vice versa. No inhibition of agglutination was observed.

Preliminary trials were made using haemolymph previously adsorbed with the heterologous HRBC. The technique, as above, where the haemolymph remains in the test system was used. A positive result is one where the expected inhibition of agglutination fails to occur, so is recorded as negative inhibition (see Table 4.iv.6.).

Conclusions

There is evidently a factor in the haemolymph which inhibits the agglutination of A and B HRBC by human antisera, but does not affect that of SRBC by rabbit antiserum. This suggests that the factor is interacting with an antigenic site which is not common

TABLE 4.iv.5.

	HRBC			
	A		B	
trial	expt'al endpoint	control endpoint	expt'al endpoint	control endpoint
1	1/64	1/128	1/32	1/256
2	*NI	1/128	NI	1/32
3	NI	1/128	1/64	1/128
4	NI	1/256	NI	1/128
5	1/16	1/32	1/8	1/32
6	NI	1/32	1/8	1/32
7	NI	1/32	1/16	1/32
8	NI	1/32	NI	1/32
9	NI	1/32	NI	1/32
10	NI	1/32	NI	1/32

LEGEND The inhibition of agglutination of A and B HRBC suspended in sterile sea water, after prior incubation in fresh, cell-free haemolymph, added to serial double dilutions of human anti-sera. Control HRBC were pre-incubated in PBS.

*NI = no inhibition

TABLE 4.iv.6.

HRBC used for pre-adsorption	expt'al HRBC	no trials	Inhibition positive	Inhibition negative
A	A	3	2	1
	B	3	3	0
B	A	2	0	2
	B	4	2	2
O	A	2	0	2
	B	2	2	0

LEGEND The number of trials of fresh cell-free haemolymph which caused the inhibition of agglutination of A and B HRBC by human anti-sera after prior adsorption with A, B or O HRBC.

to sheep and human RBC, but since it is able to inhibit the agglutination of both A and B HRBC it could bear some similarity to the human blood group determinant.

The peaks of the titer distributions for A, B and AB all occur at the same dilution which suggests that a single factor is responsible for the inhibition of the agglutination of all three, (see Fig. 4.iv.1.). If more than one factor were present, the factors should have occurred in exactly equal quantities to give titer end-points at the same dilutions. Heating to 56^{o}C for 20 minutes usually caused proteins to denature, the haemolymph was observed to contain flocculent precipitate after heating. Some of the blood group-like substances found in the haemolymph of Biomphalaria have been identified as glycoproteins (Stanislawski, Renwrantz and Becker, 1976), as have those of Sepia and Loligo (Renwrantz and Uhlenbruck, 1974). The heat stability of the factor in Patella haemolymph suggests that it is not purely a protein, results from other molluscs indicate that a glycoprotein factor is a possibility.

From Figs. 4.iv.2. and 4.iv.3. it is apparent that the haem-agglutination-inhibition titer drops in winter. This drop in activity might be an indirect result of decreasing day length. Many animals are known to undergo physiological changes in response to this. But in this case it is more probably the result of temperature drop. Crassostrea virginica is known to stop feeding in winter, and the acid and alkaline phosphatases and a non-specific esterase in its haemocytes have been found to show a regression at this time (Eble, 1966).

Since haemagglutination-inhibition is so much reduced when the haemolymph was washed off the HRBC it seems probable that the factor is competing with the antigenic sites of the HRBC for the antibody and possibly blocking the antigen combining sites, rather than masking the HRBC surface antigens. Some interaction with the HRBC membrane probably does occur since haemagglutination-inhibition activity was not completely removed when the HRBC were pre-incubated in haemolymph.

From Table 4.iv.5. it seems that the haemagglutination-inhibition factor has a stronger affinity for B HRBC than for A HRBC, the results in Table 4.iv.6. also suggest this. If inhibition of A and B HRBC was unaffected by prior adsorption with the heterologous HRBC this could indicate the involvement of more than one factor in the haemagglutination-inhibition activity of the haemolymph. The results in Table 4.iv.6. are not clear cut, but they appear to substantiate the results recorded in Table 4.iv.5.; that B HRBC are more effective in removing the haemagglutination-inhibition activity from the haemolymph than are A or O HRBC. These results do not suggest the presence of multiple blood group like factors.

The stronger affinity for B HRBC could account for the higher titer observed against A HRBC when the haemolymph remained in the system. If the factor were being removed by attaching to the B HRBC membrane, then its concentration would be effectively reduced and less would be available to block the agglutinating antibody.

v. Ouchterlony immunodiffusion.

Method

Petri dishes were prepared using purified agar (Difco) made up to a 1%, w:v solution, in a 1%, w:v, sodium azide. 20 ml were pipetted into each of 7 plates. When cool, 2 patterns of 5 wells each were cut out of every plate. The patterns were cut with a 0.5 cm cork borer, and consisted of 1 central and 4 peripheral wells.

1 central well on each plate was filled with the undiluted, cell free haemolymph of 1 animal. The same haemolymph, diluted by a half with sterile sea water, was used to fill the central well of the other pattern. Half and quarter dilutions of human anti-A and anti-B sera in 0.9% saline were used to fill the 4 surrounding wells of each pattern. The plates were left at room temperature for diffusion to occur (method after Cox, 1975, in Hall and Hawkins, 1975).

Results and conclusions

Over 7 days no lines of precipitation were observed. The lack of precipitate may have been a result of an excess of antiserum, so presence of a soluble blood group reactive substance in the haemolymph of Patella has not been precluded. The human antisera should probably have been further diluted as they had quite high agglutinating titers, but further dilutions could not be made as no more anti-serum was available.

vi. Discussion

From these experiments using only cell-free haemolymph it

seems evident that in Patella the haemolymph contributes very little, directly to internal defence mechanisms.

The agglutinins found in the haemolymph of many molluscs are thought to act as opsonins, but, as stated above, their absence does not preclude the presence of opsonic factors (Anderson and Good, 1976; Scott, 1968). To act as an opsonin some interaction of soluble factors and the surface of the foreign particle must occur, so that it is labelled and, in some way, made more liable to phagocytosis. From the haemagglutination-inhibition experiments it seems that there is very little in the haemolymph capable of attaching to the surface of HRBC. So only a limited opsonic capacity might be expected.

The results of the haemagglutination-inhibition experiments (Section 4.iv.) agree with those of Pemberton (1970), in that A HRBC agglutination was the more inhibited and that the factor was fairly heat stable, inhibition being only slightly diminished by heating. Using an aqueous extract of the whole animal, Pemberton (1970) found a weak haemolysin, active to a titer of 4. As no haemolytic activity was noted when cell-free haemolymph was used (Section 4.ii.) it seems more probable that the haemolytic activity was due to lytic enzymes released from the digestive gland, since the gland is known to be very rich in such enzymes (Levvy, Hay and Marsh, 1957).

It is probable that the haemolymph of Patella does contain at least one soluble blood group-like substance. Blood group specific agglutinins are quite common among bacteria and viruses, so the blood group-like substances could have a role in defence. Renwarantz and Uhlenbruck (1974) suggest that these substances might act as

receptors for neutralising bacterial or viral agglutinins, or as inhibitors to bacterial proteases.

The haemolymph shows no inhibitory activity against 2 species of marine bacteria, 'A' and Serratia, both of which grew extremely well when incubated in it. Patella is likely to come into contact with large numbers of bacteria on the rock surface. It is able to live quite satisfactorily in areas close to sewage outfalls and, as a surface grazer probably ingests both natural marine and polluting terrestrial species of bacteria.

To maintain the sterility of its internal environment some effective method of isolating internal from external environment must exist, or else there must be an efficient method of killing invading organisms.

The haemolymph of Mya arenaria (Cheng and Yoshino, 1976), and of Mercenaria mercenaria and Crassostrea virginica (Cheng and Rodric, 1975) are known to contain lytic enzymes. Crassostrea haemolymph is capable of inhibiting the growth of bacteria incubated in it in vitro McDade and Tripp, 1967b). But the haemolymph of most molluscs does not inhibit bacterial growth. Bacteria grew well in vitro in the haemolymph of Aplysia californica (Johnson and Chapman, 1970), as they did in the presence of that of Helix pomatia (Bayne and Kime, 1970) and of Octopus dofleini (Bayne, 1973a). In the general lack of activity shown by its haemolymph, Patella is not dissimilar from many other gastropods, the lamellibranchs, on the whole, seem to have more substances of possible significance in internal defence in solution in their body fluids.

It is generally considered that molluscan internal defence relies principally on the haemocytes and on the fixed phagocytes, which line many of the haemocoelic spaces (Bayne, 1974; Cheng, 1975; Cheng and Auld, 1977). Certainly these results from Patella seem to substantiate this opinion.

SECTION 5

THE PHAGOCYTIC REACTIONS OF THE HAEMOCYTES

i. Introduction.

Internal defence in the molluscs is generally considered to depend largely on phagocytosis by haemocytes and fixed phagocytes (Tripp, 1960; Bayne, 1973b; Lafferty and Crichton, 1973; Cheng, 1975; Reade and Reade, 1976; Cheng and Auld, 1977). Although Bayne (1974) found that Helix pomatia cleared large numbers of bacteria from the circulation so rapidly that it seemed unlikely that phagocytosis alone could account for their disappearance, an assessment of the phagocytic capacity of the haemocytes is important in any attempt to understand the mechanisms of internal defence of an organism.

So that the environment of the haemocytes may be closely controlled and varied at will, in vitro systems are frequently used for quantifying phagocytosis, though conditions may not be optimal for the cells.

Through the use of such haemocyte cultures, it has been found that the haemo-lymph of many molluscs contains factors which enhance phagocytosis. Particles pre-soaked in haemolymph, then washed, are more readily taken up by haemocytes in an artificial culture medium than particles not so treated.

Some of the agglutinins found in the molluscan haemolymphs are thought to be opsonic factors also. The haemolymphs of Crassostrea

virginica (Tripp and Kent, 1967); Velesunio ambuus (McKay, Jenkin and Rowley, 1969); Biomphalaria glabrata (Stanislawski, Renwrantz and Becker, 1976) and Aplysia californica (Pauley, Krassner and Chapman, 1971) have both opsonic and agglutinating properties, whereas those of Otala lactea (Anderson and Good, 1976); Helix aspersa (Prowse and Tait, 1969) and Eledone cirrosa (Stuart, 1968) have opsonic properties, but no agglutinins.

The opsonins adsorb to the particle surface and after thorough washing, particles which have been opsonised are more readily taken up by the haemocytes. In the case of Helix aspersa and Eledone cirrosa no phagocytosis occurred unless particles were first opsonised in the respective haemolymphs. With Aplysia and Crassostrea the opsonisation enhanced uptake generally, whereas the opsonin from Otala haemolymph was found only to increase the uptake of formalised yeast. When fresh yeast, sheep red blood cells (SRBC) or formalised SRBC were used opsonising had no effect on the extent of phagocytosis. Helix aspersa haemolymph appeared, from cross adsorption experiments (Prowse and Tait, 1969), to contain opsonins specific for formalised yeast and formalised SRBC.

From these results it is evident that a wide range of opsonic involvement in phagocytosis occurs among the molluscs. Though some of these haemocytes will take up particles in the absence of haemolymph factors, in general in vitro levels of phagocytosis are not very high. About 24% of Crassostrea haemocytes were found to have taken up bacteria after 2 hours (Tripp and Kent, 1967) and 29% of Aplysia haemocytes showed phagocytosis of chicken RBC (Pauley, Krassner and Chapman, 1971). Otala haemocytes took up fresh yeast

and SRBC to the same extent, about 10% of the cells were involved, whether the particles were opsonised or not, but opsonising with an extract of the albumen gland increased the proportion of cells taking up particles to a maximum of 62% (Anderson and Good, 1976).

Using Homarus americanus haemocytes, Patterson and Stewart (1974) found only 2% of the cells had taken up SRBC, while about 10% of Parachaeraps bicarinatus haemocytes took them up after opsonising (McKay and Jenkin, 1970a). But if animals were previously vaccinated, the cells seemed to be activated. Injections of Pseudomonas petrolens endotoxin administered weekly for 4 weeks resulted in an increase in uptake of opsonised SRBC from 1.6% to 4.4% for the vaccinated animals (Patterson, Stewart and Zwicker, 1976), and from 0.02% to 1.6% respectively for unopsonised particles, which demonstrated the cellular nature of the response. McKay and Jenkin (1970a, b and c) found a similar reaction to vaccination in Parachaeraps. Though the difference in phagocytosis was rather more marked when opsonised SRBC were used, the uptake of unopsonised particles was also significantly increased.

Helix pomatia was found to be able to remove most of a dose of 8×10^4 injected bacteria over one hour, (Bayne and Kime, 1970), and Octopus dofleini could completely clear 10^6 bacteria in 6 hours (Bayne, 1973a). Aplysia, injected with 2×10^9 Micrococcus aquivivus, cleared its circulation in 8 hours, when maintained at $20^{\circ}C$, this represents about 2.5 bacteria being removed by each haemocyte. These rates of phagocytosis are rather higher than generally observed in in vitro haemocyte cultures. It is often suggested that the low

levels of phagocytosis are a result of suboptimal in vitro conditions.

A comparison of in vitro and in vivo levels of phagocytosis is probably necessary in order to get a more accurate idea of possible internal defence mechanisms. Comparison of in vitro and in vivo rates of particle removal should also elucidate the extent of the contribution of the haemocytes to the clearance of the internal environment. In Helix pomatia (Bayne, 1974); Aplysia californica (Pauley and Krassner, 1972); Liolophura gaimardi (Crichton, Kilby and Lafferty, 1973) and Eledone cirrosa (Stuart, 1968) the fixed phagocytes, which line many of the haemocoelic spaces, have been found to play an important part in the removal of foreign particles from the circulation. In the lamellibranchs, however, it seems that only the circulating haemocytes are active in this capacity (Reade and Reade, 1976).

The haemocytes of Patella were challenged in vitro with human RBC and with heat-killed 'A'. HRBC make convenient test particles as their surface structure is fairly well known. 'A' is a marine species and so might be one Patella would encounter, also being a rod it is quite distinctive in the fixed and stained preparations and not easily mistaken for other particles or granules which might be present in the cytoplasm of the haemocytes (see Plate 3.iii.7. and 3.iii.8.).

In vitro experiments were conducted over 2 hours at room temperature, as in these conditions the haemocytes remained viable throughout (Section 2.iv.).

In vitro the haemocytes of Patella form rosettes with HRBC (Section 3.iv.). Few cells take up HRBC, but after about 20 minutes the attached HRBC begin to lyse. Phagocytosis is generally thought to be a two-stage process (Carr, 1973). Attachment of the target particle to the phagocyte is the first stage, and engulfment the second stage. The association between HRBC and the haemocytes could give a measure of phagocytic potential. In these circumstances, visual scoring of attachment was not practicable, since once an HRBC had lysed its ghost was not readily apparent and another HRBC might become attached to the haemocyte. The method employed was a spectrophotometric quantification of the haemoglobin released into the culture medium by the lytic action of the haemocytes. The *in vitro* phagocytosis of 'A' was quantified by visual scoring of the fixed and stained cell monolayers.

The possible presence of opsonins in the haemolymph of *Patella* and enhancement of opsonic activity and phagocytosis as a result of prior vaccination were also investigated. The haemocytes of animals previously vaccinated with 'A' might already contain bacteria, so to use these cells to quantify the phagocytosis of 'A' would not give a true result. Haemocyte activation by pre-vaccination could therefore only be tested when HRBC were the test particles.

In order that *in vitro* and *in vivo* rates of phagocytosis could be compared the haemocytes from previously injected animals were visually scored for the presence of bacteria. This enabled an assessment of the *in vitro* conditions to be made, since if the level of phagocytosis were much higher than that of the *in vitro* cultured

cells, then the conditions of the culture would not be optimal. Animals were injected with live bacteria and bled at intervals, the number of colonies grown from the plated haemolymph gave an indication of the change in numbers of bacteria in the circulation. From the results of these three methods of quantifying phagocytosis, comparison of the cells' capacity to clear the circulation of the animal of foreign particles and the probable contribution of the haemocytes to this process can be made.

ii. Phagocytosis of human red blood cells in vitro.

Method

Human red blood cells, A, B, and O, were washed (see Section 2.vii) and a 10% v:v suspension of each was made up in sterile sea water and in cell-free haemolymph. These suspensions were left for one hour at room temperature to allow opsonisation to occur. They were then washed by centrifugation and resuspension until the supernatant was clear, then made up to 1% v:v in sterile sea water. The suspensions were standardised by lysing 500µl in 5 ml of distilled water and reading the optical density at 530 nm in a spectrophotometer (Beckman DB-G). The 6 suspensions were adjusted to read within 0.01% absorbance of each other, for each experiment, (method after Campbell, Garvey, Cremer and Sussdorf, 1963).

Haemolymph was taken from about 3 animals and pooled. The container was kept on ice throughout. When sufficient haemolymph had been collected a cell count was made in a haemocytometer (Improved Neubauer).

Equal volumes of the pooled, whole haemolymph were then put into six 5 cm plastic Petri dishes and the cells left to settle out for 20 minutes at room temperature. The haemolymph was then pipetted off, leaving only the attached cells. 5 ml of one of the HRBC suspensions was added to each dish and as a control, an equal volume was added to a clean dish, with no cells in it.

The 12 Petri dishes were agitated throughout the experiment on a Gallenkamp oscillating table, to allow maximum exposure of HRBC to haemocytes and to prevent the establishment of concentration gradients of dissolved gas es or products of metabolism in the medium. Incubation was at room temperature. $15^{o}C$ was tried initially, as closer to the natural environmental temperature of Patella but reactions were found to be very slow.

A time 0 sample of the HRBC suspension was taken immediately from each of the dishes. The dish was agitated until the HRBC seemed to be fairly well in suspension, then 500µl was removed with a fixed delivery micrometer pipette (Excalibur). The sample was then added to 2 ml of 0.9% saline to prevent any further lysis of the HRBC. The samples were spun at about 600 x g for 5 minutes to remove HRBC from suspension. The haemoglobin in the supernatant was then read at 530 nm in the spectrophotometer. Subsequent samples were taken at 1 and 2 hours after the start of the experiment.

To ascertain the number of HRBC that the spectrophotometer readings represented, counts of the HRBC in suspensions of known absorbance were made in a haemocytometer. These results were then used to plot a standard line. A similar procedure was used to

make a standard plot of HRBC number represented by the experimental results.

At each sampling time the control result gives the natural level of lysis of the HRBC, so this subtracted from the experimental reading gives the lysis due to the presence of the haemocytes in the dishes. The number of HRBC given by this result was then read off from the standard plot and as the number of cells in each dish was known, the mean number of HRBC lysed per haemocyte could be calculated.

Using only O HRBC, similar experiments were conducted using the haemocytes of animals which had been injected with washed O HRBC 18 hours previously. The haemolymph of these animals was used to opsonise O HRBC, so both naive and experienced haemocytes were tested with O HRBC which had been opsonised in sea water, naive haemolymph or the haemolymph of experienced animals.

The spectrophotometer readings give the number of HRBC lysed per unit volume in each dish. The mean number of HRBC lysed by each haemocyte cannot be calculated directly from these readings as the volume of sea water in the dishes was not constant from one sampling time to the next. To give the mean lysis, the observed number of HRBC lysed in the whole dish is divided by the number of haemocytes present. The actual number of HRBC lysed during the first hour of the experiment is calculated by subtracting the number lysed in the initial sample from that lysed after 1 hour of incubation.

The lysis by each haemocyte is similarly calculated for the

second hour. Addition of these results gives the total number of HRBC lysed by each haemocyte over the 2 hours of the experiment.

As the haemocytes tested against each of the 6 HRBC suspensions were all from the same source, variation in the extent of lysis of the HRBC should be due only to differences caused by their differing treatments.

Results

The mean number of HRBC lysed by each haemocyte and the total for the 2 hours are tabulated separately for the A, B and O HRBC, and for the opsonised and unopsonised HRBC in Tables 5.ii.1-4. From the graphs, Figs. 5.ii.1-3, it can be seen that there is very little difference in amount of lysis whether the HRBC were opsonised or not (in all cases $t = < 1.00$; $p = > 0.1$).

The results of the trials using both experienced and naive haemocytes are shown in Table 5.ii.5. When the extent of lysis of the HRBC opsonised in the experienced haemolymph is compared with that of HRBC opsonised in naive haemolymph, disregarding the source of the haemocytes, the difference is found not to be significant, (Table 5.ii.6., Fig. 5.ii.5. - $p = > 0.1$). But if the treatment of the HRBC is ignored and the mean lysis by experienced haemocytes is compared with that by the naive haemocytes, the difference is found to be significant, (Table 5.ii.6., Fig. 5.ii.4.)(mean lysis per haemocyte after 2 hours, experienced and naive, $t = 2.244$; $p = < 0.05$).

Since the concentration of HRBC is known at the beginning of the experiment and could be calculated for the end after lysis had

Table 5.ii.1. LEGEND

The mean number of HRBC lysed by each haemocyte after 1 and 2 hours of *in vitro* culture in sterile sea water with A HRBC which had been previously either incubated in sea water or opsonised in fresh cell-free haemolymph for 1 hour.

*n = number of trials
$\bar{x}$ = mean
SE = standard error of mean

TABLE 5.ii.1.

A HRBC - sea water incubated

RBC/ dish x 10^4	h'cytes per dish x 10^4	RBC per h'cyte	0 hrs RBC lysed x 10^4	0 hrs lysis/ h'cyte	1 hr RBC lysed x 10^4	1 hr lysis/ h'cyte	2 hrs RBC lysed x 10^4	2 hrs lysis/ h'cyte	total lysis/ h'cyte (0+1+2)
33500	471	71	0	0	945	2.01	0	0	2.01
35700	402	89	0	0	560	1.39	-	-	-
22750	337	68	0	0	665	1.97	705	2.09	4.06
28400	230	123	900	3.91	315	1.37	780	3.39	8.67
22750	419	54	500	1.19	450	1.07	480	1.15	3.41
21000	364	58	450	1.24	922	2.53	100	0.27	4.04
27500	402	68	0	0	1440	3.58	760	1.89	5.47
28000	451	62	0	0	1050	2.33	465	1.03	3.36
n* 8				8		8		7	7
$\bar{x}$ 27456				0.79		2.03		1.40	4.43
SE 1854				0.49		0.28		0.44	0.81

A HRBC - opsonised

33500	471	71	0	0	1767	3.75	105	0.22	3.97
28500	402	71	500	1.24	420	1.04	360	0.90	3.18
31500	337	93	0	0	350	1.04	285	0.85	1.89
27000	230	117	450	1.96	0	0	1240	5.39	7.35
26000	419	62	400	0.95	990	2.36	780	1.86	5.17
20750	364	57	0	0	1440	3.96	0	0	3.96
28400	402	71	0	0	630	1.57	1440	3.58	5.15
28000	451	62	0	0	385	0.85	2805	6.22	7.07
n 8				8		8		8	8
$\bar{x}$ 27956				0.52		1.83		2.38	4.72
SE 1339				0.27		0.51		0.85	0.66

Table 5.ii.2. LEGEND

The mean number of HRBC lysed by each haemocyte after 1 and 2 hours of *in vitro* culture in sterile sea water with B HRBC which had been previously either incubated in sea water or opsonised in fresh cell-free haemolymph for 1 hour.

*n = number of trials
$\bar{x}$ = mean
SE = standard error of mean

TABLE 5.ii.2.

B HRBC - sea water incubated

	RBC/ dish x 10^4	h'cytes per dish x 10^4	RBC per h'cyte	0 hrs RBC lysed x 10^4	lysis/ h'cyte	1 hr RBC lysed x 10^4	lysis/ h'cyte	2 hrs RBC lysed x 10^4	lysis/ h'cyte	total lysis/ h'cyte (0+1+2)
	32750	402	81	0	0	997	2.48	1155	2.87	5.35
	25000	337	74	0	0	805	2.39	645	1.91	4.30
	21250	230	92	0	0	855	3.72	0	0	3.72
	25000	419	60	0	0	900	2.15	520	1.24	3.39
	27000	364	74	0	0	1215	3.34	240	0.66	4.00
	33250	402	83	0	0	945	2.35	240	0.60	2.90
*n	6				6		6		6	6
$\bar{x}$	27375				0		2.74		1.21	3.94
SE	1935				0		0.26		0.42	0.32

B HRBC - opsonised

	33250	402	83	0	0	875	2.18	900	2.24	4.42
	31250	337	93	450	1.33	87	0.26	1245	3.69	5.28
	25000	230	109	925	4.02	472	2.05	0	0	6.07
	25500	419	61	0	0	990	2.36	400	0.95	3.31
	21250	364	58	0	0	1260	3.46	600	1.65	5.11
	29750	402	74	0	0	1620	4.03	0	0	4.03
n	6				6		6		6	6
$\bar{x}$	27667				0.89		2.39		1.42	4.70
SE	1838				0.66		0.53		0.58	0.40

Table 5.ii.3. LEGEND

The mean number of HRBC lysed by each haemocyte after 1 and 2 hours of *in vitro* culture in sterile sea water with 0 HRBC which had been previously either incubated in sea water or opsonised in fresh, cell-free haemolymph for 1 hour.

*n = number of trials
$\bar{x}$ = mean
SE = standard error of mean

TABLE 5.ii.3.

0 HRBC - sea water incubated

RBC/ dish x 10^4	h'cytes per dish x 10^4	RBC per h'cyte	0 hrs RBC lysed x 10^4	lysis/ h'cyte	1 hr RBC lysed x 10^4	lysis/ h'cyte	2 hrs RBC lysed x 10^4	lysis/ h'cyte	total lysis h'cyte (0+1+2)
31000	471	66	0	0	1120	2.38	465	0.99	3.37
32750	402	81	500	1.24	245	0.61	150	0.37	2.22
22750	337	67	0	0	1120	3.32	780	2.31	5.63
27000	230	117	0	0	1350	5.87	120	0.52	6.39
28400	419	68	600	1.43	540	1.29	340	0.81	3.53
25000	364	69	0	0	855	2.35	540	1.48	3.83
22200	402	55	0	0	1035	2.57	220	0.55	3.12
*n 7				7		7		7	7
$\bar{x}$ 27014				0.38		2.63		1.00	4.01
SE 1513				0.25		0.63		0.26	0.56

0 HRBC - opsonised

33500	471	71	400	0.85	560	1.19	540	1.15	3.19
29400	402	73	0	0	1225	3.05	540	1.34	4.39
33750	337	100	0	0	1050	3.12	840	2.49	5.61
22200	230	96	550	2.39	855	3.72	1160	5.04	11.51
24000	419	57	500	1.19	855	2.04	840	2.00	5.23
16000	364	44	0	0	945	2.60	860	2.36	4.96
30000	402	75	0	0	1485	3.69	0	0	3.69
n 7				7		7		7	7
$\bar{x}$ 26979				0.63		2.77		2.05	5.46
SE 2467				0.35		0.34		0.59	1.00

TABLE 5.ii.4.

HRBC treatment	n*	mean no. HRBC lysed ±SE** 0	1 (0+1)	2 (0+1+2)
A - SW	8	0.79 (± 0.48)	2.84 (± 0.45)	4.43° (± 0.81)
A - OP	8	0.52 (± 0.27)	2.34 (± 0.43)	4.72 (± 0.66)
B - SW	6	0	2.74 (± 0.26)	3.94 (± 0.34)
B - OP	6	0.89 (± 0.66)	3.28 (± 0.66)	4.70 (± 0.40)
O - SW	7	0.38 (± 0.25)	3.01 (± 0.51)	4.01 (± 0.56)
O - OP	7	0.63 (± 0.35)	3.41 (± 0.49)	5.46 (± 1.00)

* n - number of trials

** SE - standard error of mean

° n = 7

LEGEND The mean number of HRBC lysed by each haemocyte after 1 and 2 hours of *in vitro* culture in sterile sea water with A, B and O HRBC which had previously either been incubated in sea water (SW) or opsonised in fresh, cell-free haemolymph (OP) for 1 hour (from Tables 5.ii.1-3).

Fig. 5.ii.1. LEGEND

(from Table 5.ii.4.) The mean number of A HRBC lysed by haemocytes after opsonisation in haemolymph (x) and after incubation in sea water (o).

Fig. 5.ii.2. LEGEND

(from Table 5.ii.4.) The mean number of B HRBC lysed by haemocytes *in vitro* after opsonisation in haemolymph (x) and after incubation in sea water (o).

Fig. 5.ii.3. LEGEND

(from Table 5.ii.4.) The mean number of O HRBC lysed by haemocytes *in vitro* after opsonisation in haemolymph (x) and after incubation in sea water (o).

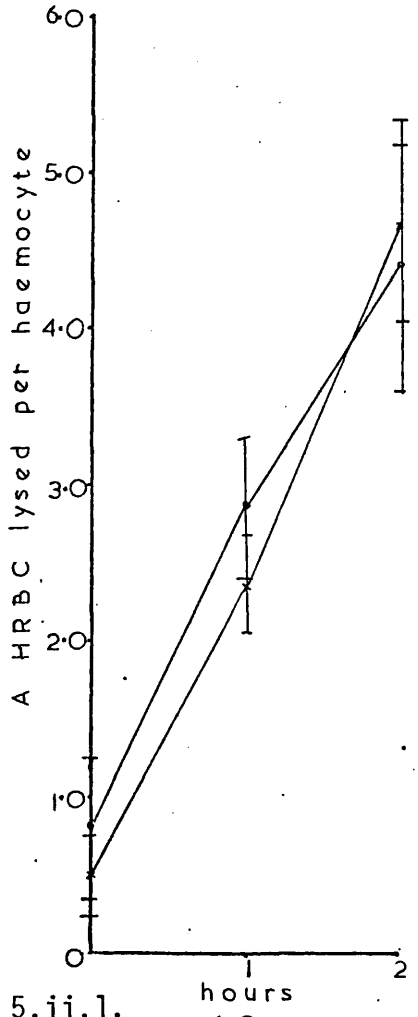


Fig. 5.ii.1.

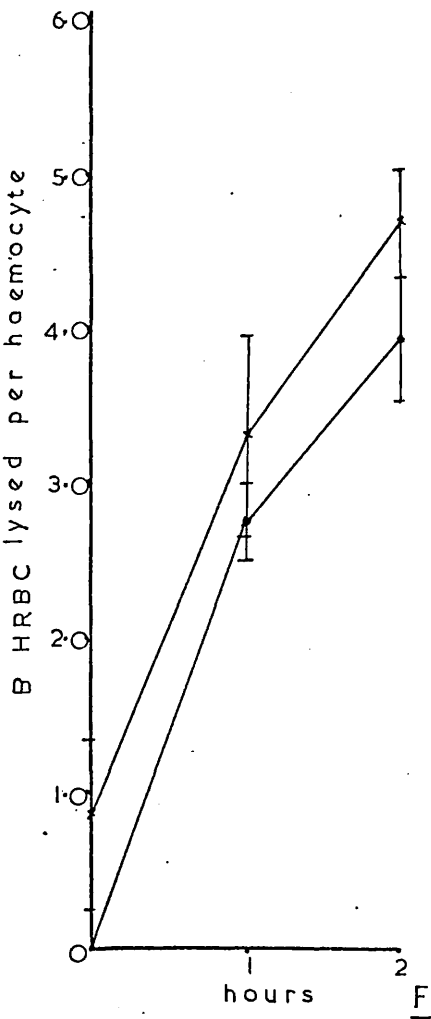


Fig. 5.ii.2.

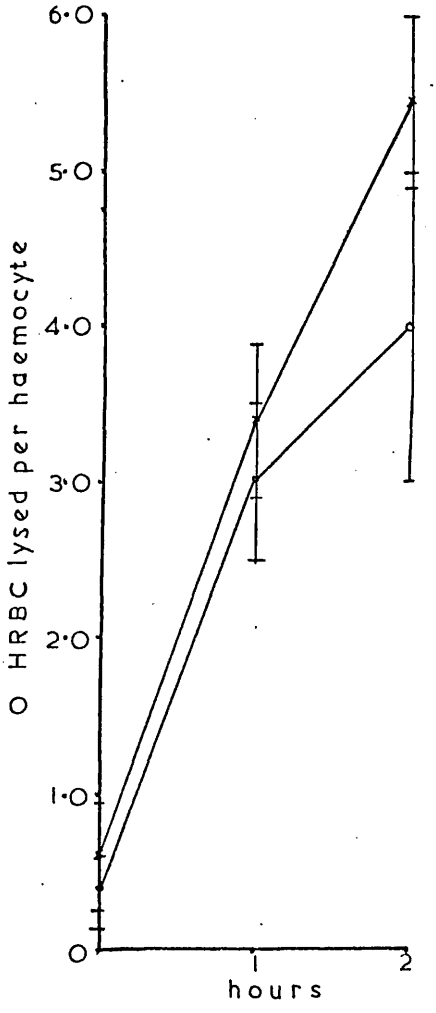


Fig. 5.ii.3.

occurred, a 'phagocytic index' for the haemocytes can be estimated. This will give a comparable measure of the avidity of the haemocytes for the different HRBCs after their different treatments. Nelstrop, Taylor and Collard (1968a) give a formula from which the phagocytic index, or rate of elimination, can be calculated:-

$$K = \frac{\log \text{conc. a} - \log \text{conc. b}}{tb - ta}$$

where

ta = time of conc. a

tb = time of conc. b (hours)

K values were calculated for both treatments of each type of HRBC and for the naive and experienced haemocytes (Table 5,ii,7.).

Conclusions

It is evident from the results that A, B and O HRBC all undergo lysis to a similar extent (Table 5,ii,4.). In no case are the different extents of lysis observed between sea water and haemolymph treated HRBC significant. It seems probable that factors capable of opsonising HRBC are not naturally present in the haemolymph.

The results of the haemagglutination-inhibition tests indicated that blood group reactive substances in the haemolymph did not readily attach to the HRBC surface as haemagglutination was only slightly inhibited when the HRBC were pre-incubated in cell-free haemolymph (Section 4,iv.). The lack of opsonic factors observed in this case is consistent with the results reported in Section 4.iv., since the HRBC underwent the same treatment in both experiments. In this experiment the opsonic effect of the haemolymph seems to be slightly greater on O HRBC than that on A or B

Table 5.ii.5. LEGEND

The mean number of HRBC lysed by haemocytes from animals which had been vaccinated with 0 HRBC and by haemocytes from naive animals after 1 and 2 hours of *in vitro* culture with 0 HRBC which had been either opsonised in fresh cell-free haemolymph from vaccinated animals (E op) or in fresh, cell-free haemolymph from naive (N op) or incubated in sea water (SW) for 1 hour.

*n = number of trials

$\bar{x}$ = mean

SE = standard error of mean

TABLE 5.ii.5.

Naive haemocytes

RBC treatment	RBC/dish x 10^4	h'cytes per dish x 10^4	RBC per h'cyte	0 hrs RBC lysed x 10^4	0 hrs lysis/ h'cyte	1 hr RBC lysed x 10^4	1 hr lysis/ h'cyte	2 hrs RBC lysed x 10^4	2 hrs lysis/ h'cyte	total lysis/ h'cyte (0+1+2)
N op	26250	751	35	0	0	1080	1.44	160	1.54	2.98
N op	20350	633	31	0	0	1485	2.05	1080	1.71	3.76
E op	25000	751	33	0	0	1170	1.56	1380	1.84	3.40
E op	22200	633	35	0	0	1395	2.20	780	1.23	3.43
SW	28000	751	37	0	0	1350	1.80	1640	2.18	3.98
SW	21750	633	34	0	0	900	1.42	1280	2.02	3.44
*n	6				6		6		6	6
$\bar{x}$	23925				0		1.74		1.75	3.50
SE	1206				0		0.13		0.14	0.14

Experienced haemocytes

N op	26250	743	35	0	0	3195	4.30	2000	2.69	6.99
N op	20350	725	28	0	0	1327	1.83	900	1.24	3.07
E op	25000	743	34	2250	3.03	1350	1.82	2040	2.75	7.60
E op	22200	725	31	0	0	1867	2.58	40	0.05	2.63
SW	28000	743	38	0	0	2655	3.57	2500	3.36	6.93
SW	21750	725	30	0	0	450	0.62	1840	2.54	3.16
n	6				6		6		6	6
$\bar{x}$	23925				0.51		2.45		2.11	5.06
SE	1206				0.50		0.54		0.50	0.95

Table 5.ii.6. LEGEND

The mean number of HRBC lysed by haemocytes from animals which had been previously vaccinated with O HRBC and haemocytes from naive animals after 1 and 2 hours of *in vitro* culture with O HRBC. The results are tabulated to compare the 2 haemocyte types, irrespective of HRBC prior treatment, and the 3 types of HRBC treatment irrespective of haemocyte source (results from Table 5.ii.5.).

TABLE 5.ii.6.

Haemocytes	n	mean no. HRBC lysed 0 hr	1 hr (0+1)	2 hrs (0+1+2)
Naive (RBC treatment ignored)	6	0	1.74 *(± 0.13)	3.50 (± 0.12)
Experienced (RBC treatment ignored)	6	0.51 (± 0.50)	2.96 (± 0.65)	5.06 (± 0.95)

RBC treatment	n	$\bar{x}$ 0 hr	$\bar{x}$ 1 hr (0+1)	$\bar{x}$ 2 hrs (0+1+2)
N op (haemocyte source ignored)	4	0	2.41 (± 0.64)	4.20 (± 0.95)
E op	4	0.76 (± 0.76)	2.80 (± 0.72)	4.27 (± 1.13)
SW	4	0	1.86 (± 0.62)	4.38 (± 0.87)

* ± : SE = standard error of mean

Fig. 5.ii.4. LEGEND

(from Table 5.ii.6.) The mean number of O HRBC lysed by naive haemocytes (o) and haemocytes from pre-injected animals (x), ignoring the pre-treatment of the HRBC.

Fig. 5.ii.5. LEGEND

(from Table 5.ii.6.) The mean number of O HRBC lysed after opsonisation in haemolymph from pre-injected animals (x), from naive animals (o) and incubated in sea water (+) by haemocytes *in vitro*, ignoring the source of the haemocytes.

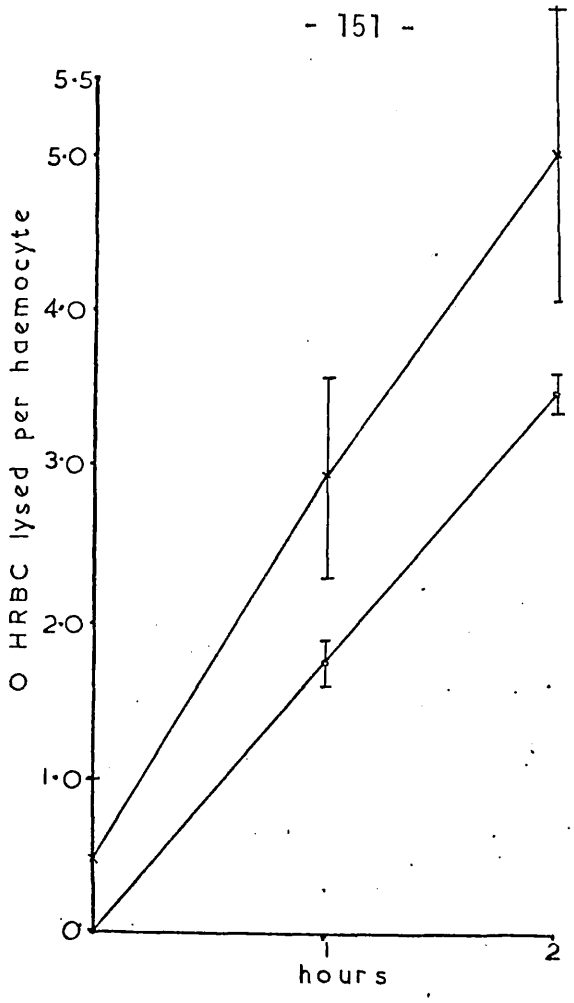


Fig. 5.ii.4.

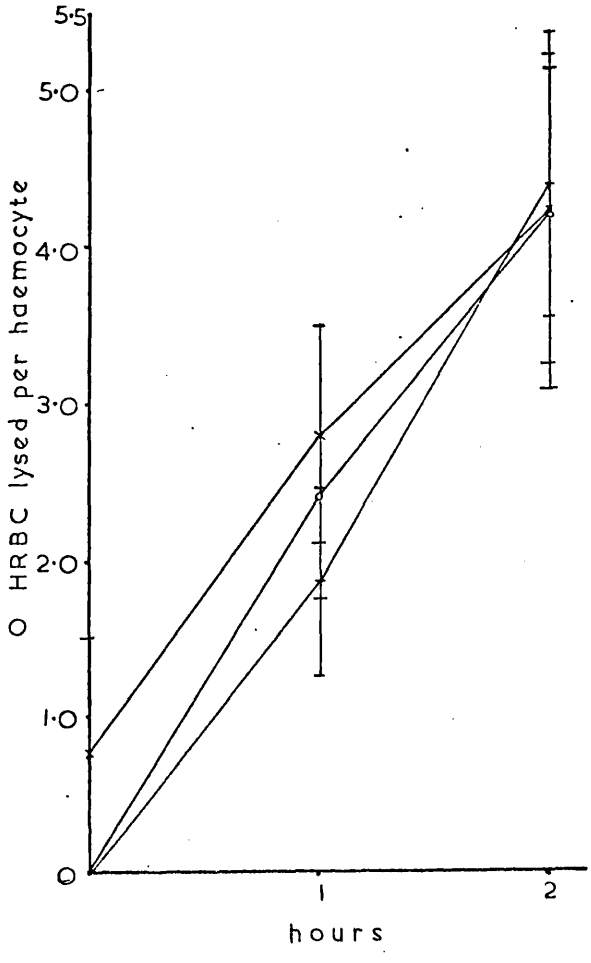


Fig. 5.ii.5.

HRBC. It seemed that A HRBC were the least effective in removing the factor (Section 4.iii.) and from Figs. 5.ii.1-3. it appears that the opsonising effect on A was less than on B or O HRBC. The K values calculated from the formula given (Table 5.ii.7.) also indicate an absence of opsonin as the K values for sea water and haemolymph treated HRBC are very little different. Though the differences are not significant, both these results and those of the haemagglutination-inhibition experiments (Section 4.iv.) suggest that A HRBC have the lowest affinity for the haemagglutination-inhibition factor.

The use of haemolymph from previously vaccinated animals to opsonise the HRBC did not seem to enhance lysis either (Fig. 5.ii.5.), which suggests that prior experience of O HRBC does not cause the induction of any opsonic factors not present in the naive animal.

O HRBC were used as they showed the most enhanced lysis after opsonising with the naive haemolymph. Since it seems that O HRBC are more effective in removing the factor from the haemolymph, it is probable that if there were an inducible opsonin, O HRBC would be most likely to enable its presence to be detected.

The results do, however, suggest that prior vaccination activates the haemocytes, since the lysis caused by the experienced cells is significantly more than that caused by the naive cells (Table 5.ii.6.). The K values also reflect this difference (Table 5.ii.7.).

It appears probable that the haemolymph of Patella takes

TABLE 5.ii.7.

RBC & treatment	n*	mean RBC mm^{-3} remaining: time 0	1 hr	2 hrs	K_1	K_2
A - SW*	8	5491 (± 371)	5243 (± 375)	4862° (± 331)	0.020	0.026
A - op*	8	5591 (± 268)	5374 (± 265)	5124 (± 270)	0.017	0.019
B - SW	6	5714 (± 438)	5506 (± 447)	5361 (± 423)	0.016	0.014
B - op	6	5700 (± 381)	5484 (± 409)	5324 (± 381)	0.017	0.015
O - SW	7	5403 (± 302)	5136 (± 315)	5026 (± 327)	0.022	0.016
O - op	7	5389 (± 492)	5100 (± 484)	4902 (± 492)	0.024	0.021
O - naive h'cytes	6	4785 (± 223)	4512 (± 226)	4207 (± 209)	0.025	0.028
O - experienced h'cytes	6	4785 (± 223)	4296 (± 166)	3907 (± 109)	0.047	0.044

LEGEND

The results in Tables 5.ii.1, 2, 3 and 5 calculated as the mean number of A, B and O HRBC lysed mm^{-3} (± SE) after *in vitro* incubation with haemocytes, from which a rate of clearance - K - was calculated for 1 (K_1) and 2 (K_2) hours of incubation.

* n = number of trials
SW = HRBC pre-incubated in sea water
op = HRBC opsonised in fresh, cell-free haemolymph

° n = 7

very little part in internal defence, so the cellular aspects are likely to be the more important. The activation of haemocytes could be a significant aspect of the animal's defence.

iii. Phagocytosis of bacteria in vitro and in vivo.

Method

Haemolymph was collected and a cell count made as before (Section 5.ii.).

Four halved, glass coverslips were placed in each of three 5 cm plastic Petri dishes. 1.5 ml of whole haemolymph was added to each dish and the haemocytes left to settle out for 20 minutes at room temperature. The haemolymph was then removed. 100 µl of a suspension of washed, heat-killed 'A', standardized to contain 3×10^9 bacteria per ml, was delivered into each dish using a fixed delivery pipette (Excalibur). 2 ml of sterile sea water were then added.

At 30, 60, 90 and 120 minutes after the bacteria had been added one half coverslip was removed from each dish. The coverslips were rinsed in sterile sea water, to remove any non-adherent bacteria, and fixed in Baker's formol-calcium (Section 2.vi.), serially dehydrated in alcohol, and with the cells uppermost the coverslips were stuck with DPX onto slides. The whole slide was then stained with Leishman's stain (see Section 3.iii.).

Using oil immersion on a Zeiss Photomicroscope II, 200 cells on each half coverslip were scored for uptake of bacteria and the number taken up by each cell recorded.

The experiment was conducted at room temperature and the Petri dishes were agitated on an oscillating table (Gallenkamp) throughout to maximise the chance of contact between cells and bacteria and to prevent concentration gradients of dissolved gases or metabolites building up.

To opsonise the bacteria they were incubated for 1 hour at room temperature in the cell-free haemolymph of naive animals or of animals vaccinated with the same bacterial suspension about 18 hours previously. After opsonising the bacteria were rinsed twice in sterile sea water and resuspended in sea water.

The same bacterial suspension was used in all the experiments. After killing, the bacteria were washed and made up to a standard density in sterile sea water (Section 2.vii.). Since the cells from the same source were used in each dish, variation in the extent of uptake of bacteria should be due only to their pre-treatment; opsonising in naive or experienced haemolymph or soaking in sea water.

It has been suggested (Scott, 1971; Tripp and Kent, 1967) that low rates of phagocytosis observed in vitro might be due to the conditions not being optimal for the haemocytes. As a comparison to the in vitro phagocytic rate 4 animals were chosen as close in size and weight as possible. The haemocoelic volume of one was estimated by removing as much haemolymph as possible from it and measuring the volume. It was then assumed that the other 3 animals would have about the same internal volume.

The volume of haemolymph of the animals was assumed to be

about 2 ml. They were injected through the foot with 100 µl of heat-killed 'A' at the same concentration as had been used to add to the *in vitro* systems. So the dilution factor of the bacteria was comparable in both cases.

The animals were kept at room temperature for 1, 2 or 3 hours before being bled. A cell count was made of each sample and drops of the whole haemolymph were left on slides to allow the cells to settle out for 20 minutes, at room temperature.

The slides were then rinsed in sterile sea water, to remove any non-adherent bacteria, then the cells were fixed and stained in Leishman's stain (Section 3.iii.). 200 cells on each slide were scored as before.

Results

Some bacteria appeared to be within vacuoles in the cytoplasm of the cells, but frequently no vacuole was visible. Bacteria in the same plane of focus as the cell cytoplasm were judged to be intracellular.

The number of bacteria taken up by 200 cells in each trial and the mean number of bacteria per cell for each time after each treatment are shown in Table 5.iii.1. After 30 minutes incubation, bacteria opsonised in naive haemolymph were taken up significantly less than those treated with sea water or experienced haemolymph ($t = 2.439$ - $p = < 0.02$). There was no significant difference in the uptake of bacteria treated with sea water or the haemolymph of previously vaccinated animals ($t = 0.585$ - $p = > 0.10$). After

Table 5.iii.1. LEGEND

The mean number of bacteria taken up by each haemocyte when cultured in vitro with heat-killed 'A' which had been previously either incubated in sterile sea water (SW), opsonised in the fresh, cell-free haemolymph of naive animals (N op) or opsonised in the fresh cell-free haemolymph of animals which had been vaccinated with the same bacterium (E op), for one hour.

*n = number of haemocytes scored

$\bar{x}$ = mean number of bacteria per haemocyte

SE = standard error of mean

TABLE 5.iii.1.

Bacterium treatment	Expt	Bacteria per 200 haemocytes 0.5 hr	1 hr	1.5 hr	2.0 hrs
SW	1	51	39	48	50
	2	32	34	91	91
	3	122	129	97	225
	4	16	134	192	136
	5	88	191	202	181
	6	84	161	164	119
	*n	1199	1200	1199	1200
	$\bar{x}$	0.411	0.573	0.662	0.665
	SE	0.024		0.030	
N op	1	15	35	30	45
	2	24	40	78	52
	3	75	67	74	104
	4	78	87	136	121
	5	101	-	153	161
	6	117	146	162	131
	n	1199	1000	1198	1200
	$\bar{x}$	0.340	0.395	0.535	0.572
	SE	0.017		0.029	
E op	2	46	38	117	69
	3	130	99	103	145
	4	90	124	136	-
	5	88	-	174	175
	6	80	118	173	149
	n	998	800	998	800
	$\bar{x}$	0.435	0.474	0.704	0.672
	SE	0.031		0.039	

Fig. 5.iii.1. LEGEND

(from Tables 5.iii.1. and 5.iii.5.) The mean number of bacteria opsonised in the haemolymph of pre-injected animals (o), in the haemolymph of naive animals (x) and incubated in sea water (△) taken up by haemocytes in vitro and the mean number of bacteria found in the haemocytes of animals injected with bacteria 2 hours previously (+).

Fig. 5.iii.2. LEGEND

(from Tables 5.iii.2. and 5.iii.5.) The percentage of haemocytes found to have taken up bacteria which had been opsonised in the haemolymph of pre-injected animals (o), in the haemolymph of naive animals (x) and incubated in sea water (△) and the percentage of haemocytes from animals injected with bacteria 2 hours previously found to contain bacteria (+).

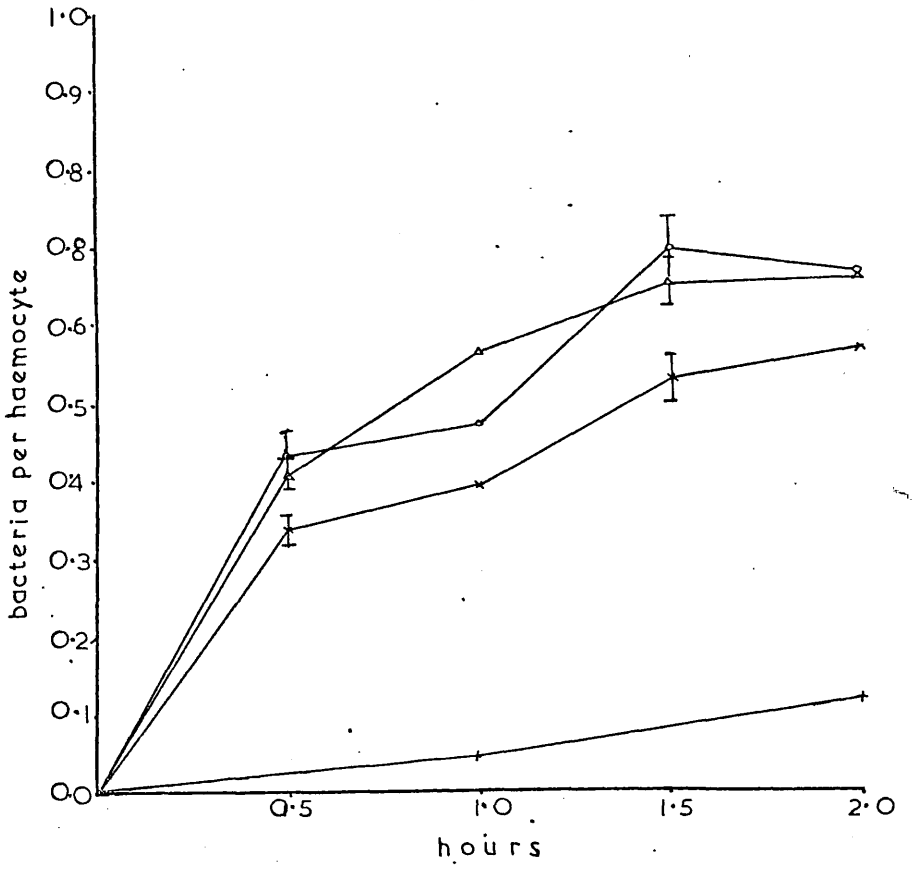


Fig. 5. iii. 1.

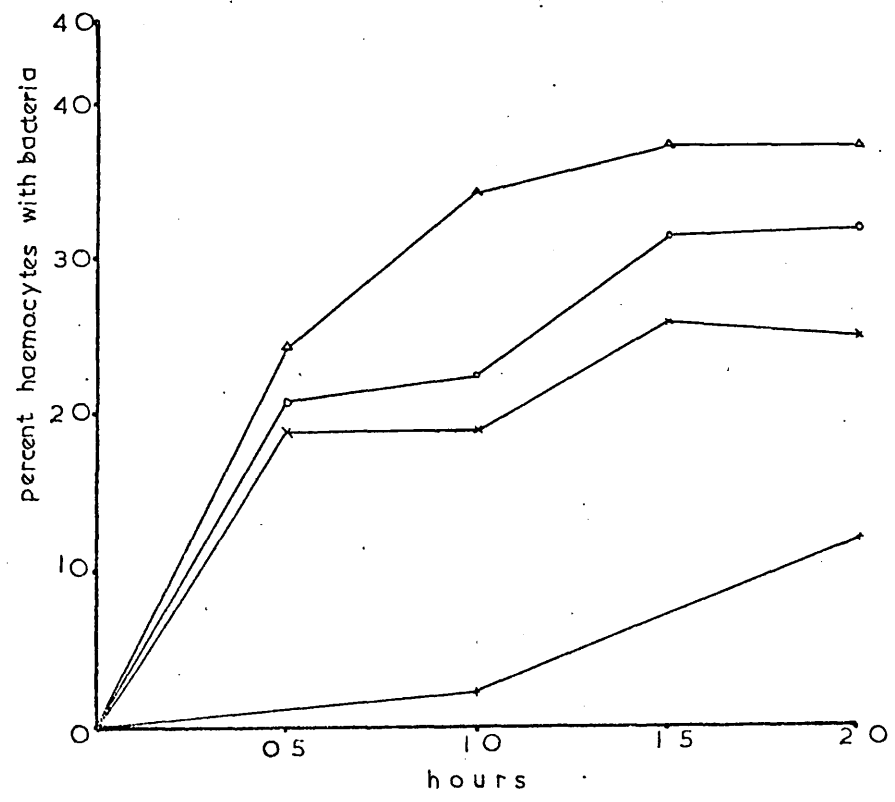


Fig. 5. iii. 2.

90 minutes the results still bore a similar relationship to one another (sea water/naive haemolymph $t = 3.081$ - $p = < 0.01$; sea water/experienced haemolymph $t = 0.917$ - $p = > 0.10$.)

The proportion of cells which took up bacteria did seem to be slightly higher when the bacteria were pre-treated with sea water (Table 5.iii.2., Fig. 5.iii.2.). After 30 minutes the number of cells which took up bacteria opsonised in naive haemolymph was significantly fewer than the number which took up bacteria soaked in sea water ($p = < 0.002$). The number of cells which took up bacteria treated with experienced haemolymph was not significantly different from the number which took up bacteria treated in either sea water ($p = > 0.10$) or in naive haemolymph ($p = > 0.10$).

As the number of both cells and bacteria in each dish and the mean number of bacteria taken up per cell was known, the concentration of bacteria at each sampling time could be calculated. From this, using the formula of Nelstrop, Taylor and Collard (1968a) a K value can be calculated (see Section 5.ii., Tables 5.iii.3. and 4.). After 2 hours of incubation the mean number of bacteria taken up by each cell indicates that when the bacteria were opsonised in experienced haemolymph or sea water, on average 2 haemocytes in 3 must have phagocytosed a bacterium, when the bacteria were opsonised in naive haemolymph only 3 cells in 5 need have phagocytosed a bacterium.

The scores of the haemocytes from the animals which were injected with bacteria enable a rough comparison to be made with the cells phagocytosing bacteria <u>in vitro</u>.

Table 5.iii.2. LEGEND

The number of haemocytes which took up bacteria when cultured *in vitro* with heat-killed 'A'. From this the mean percentage of haemocytes which took up bacteria at each sample time was calculated.

* t = total number of haemocytes which took up bacteria
  n = total number of haemocytes scored
  % = percentage of haemocytes which took up bacteria

TABLE 5.iii.2.

Bacterium treatment	expt	0.5 hr	1 hr	1.5 hr	2 hrs
SW	1	35	25	30	27
	2	19	24	54	49
	3	65	88	63	113
	4	70	84	106	84
	5	48	97	102	91
	6	57	97	98	85
	*t	294	415	453	449
	n	1199	1200	1199	1200
	%	24.5	34.6	37.7	37.4
N op	1	12	25	17	33
	2	16	21	37	30
	3	37	41	41	57
	4	48	40	67	56
	5	56	-	71	88
	6	60	64	82	67
	t	229	191	315	331
	n	1199	1000	1198	1200
	%	19.1	19.2	26.3	24.8
E op	2	25	21	48	30
	3	61	48	51	64
	4	44	61	64	-
	5	48	-	72	82
	6	40	49	82	79
	t	218	179	317	255
	n	998	800	998	800
	%	21.8	22.4	31.6	31.9

TABLE 5.iii.3.

bacterium treatment	mean h'cytes/ dish x 10^4	Bacteria remaining per dish x 10^4 0 hrs	0.5 hrs	1.0 hr	1.5 hrs	2.0 hrs
SW	402.48	$3x10^4$	29835	29769	29734	29732
N op	402.48	$3x10^4$	29863	29841	29747	29770
E op	414.28	$3x10^4$	29820	29804	29708	29722

LEGEND The mean number of heat-killed 'A' which had been previously either incubated in sea water (SW), opsonised in the haemolymph of naive animals (N op), or opsonised in the haemolymph of pre-vaccinated animals (E op) remaining after *in vitro* phagocytosis by the haemocytes, calculated from the mean number of bacteria taken up by each haemocyte (Table 5.iii.1.)

TABLE 5.iii.4.

bacterium treatment	$K_{0.5}$	$K_{1.0}$	$K_{1.5}$	$K_{2.0}$
SW	0.0047	0.0033	0.0025	0.0020
N op	0.0040	0.0023	0.0025	0.0019
E op	0.0052	0.0028	0.0028	0.0020

LEGEND The mean rate of clearance - K - of heat-killed 'A' previously either incubated in sterile sea water (SW), opsonised in the haemolymph of naive animals (N op), or opsonised in the haemolymph of pre-vaccinated animals (E op) for 1 hour, by haemocytes in vitro.

As with the *in vitro* results the mean number of bacteria taken up by each cell and the proportion of cells which had taken up bacteria was calculated (Table 5.iii.5.).

The ratio of haemocytes to bacteria and a K value for each sampling time were calculated (Table 5.iii.6.). Though it was found that no correlation existed between total uptake of bacteria at 90 minutes and cell bacteria ratio ($p = > 0.10$) these ratios are rather lower than those of the *in vitro* experiments.

To enable these results to be compared with those of Section 5.iv., the concentration of haemocytes and bacteria has been calculated as numbers mm^{-3} (Table 5.iii.7.). The mean rate of uptake 2 hours after injection requires that about one cell in nine takes up a bacterium.

Conclusions

The results demonstrate that *Patella* haemocytes are able to phagocytose bacteria *in vitro* in the absence of haemolymph.

The bacteria used for the control were stored in sterile sea water after heat-killing, so the further hour's incubation should not have much altered their surfaces. Relative to the control, opsonising the bacteria in naive haemolymph seems to have a negative effect on phagocytosis, both in the number of cells associated with bacteria and in the mean number of bacteria taken up by each cell (Figs. 5.iii.1. and 2.). The haemolymph of pre-vaccinated animals seems to have an insignificant effect on phagocytosis. This suggests that some change in the haemolymph has resulted from vaccination.

TABLE 5.iii.5.

	1 hr		2 hrs		3 hrs	
n*	bacteria/ 200 h'cytes	h'cytes with bacteria	bacteria/ 200 h'cytes	h'cytes with bacteria	bacteria/ 200 h'cytes	h'cytes with bacteria
200	12	7	24	16	16	14
200	2	1	23	17	27	21
$\bar{x}$*	0.035	2%*	0.118	11.75%	0.108	8.75%

LEGEND The mean number of bacteria taken up by each haemocyte and the percentage of haemocytes which took up bacteria 1, 2 and 3 hours post *in vivo* injection of 3×10^8 heat-killed 'A'.

*n = number of haemocytes scored
$\bar{x}$ = mean number of bacteria taken up by each haemocyte
% = mean percentage of haemocytes which took up bacteria

TABLE 5.iii.6.

hr post injection time	h'cytes/ animal x 10^4	h'cyte / bacteria	bacteria remaining	K
1	1348	22	29953	0.0007
2	1290	23	29848	0.0011
3	888	34	29904	0.0005

LEGEND The haemocyte/bacterium ratio and mean number of bacteria remaining at each time post injection. Mean rates of clearance - K - calculated from the mean uptake per haemocyte (Table 5.iii.5.).

TABLE 5.iii.7.

0 hr bacteria mm^{-3}	0 hr h'cytes mm^{-3}	3 hrs bacteria/ h'cyte	3 hrs bacteria removed mm^{-3}	% bacteria removed	K_3
150000	5877	0.108	635	0.423	0.0006

LEGEND The results from Table 5.iii.6. expressed as concentration mm^{-3} and the percentage of the vaccinated bacteria removed after 3 hours.

The bacteria were washed in sea water after opsonisation, so it is probable that some alteration of the bacterial surface has occurred. If this were due to a factor in the haemolymph which was able to adsorb to the bacteria, when animals are pre-vaccinated the factor becomes attached to the bacterial surface and its concentration in the haemolymph would be effectively reduced. This would explain the observed relative enhancement of uptake of particles opsonised in this haemolymph. That the haemolymph contains factors which are apparently able to mask the recognition sites of foreign particles is hard to account for. The anti-opsonic effect of the haemolymph could be an artefact resulting from the use of heat-killed bacteria. This treatment is known to alter the antigenic sites on their surfaces, so this altered surface might bear little resemblance to that which the internal defence mechanisms of Patella have evolved to recognise.

This phenomenon was not observed when HRBC were used (Section 5.iv.), but from the haemagglutination-inhibition experiments (Section 4.iii.) it seems the factor in the haemolymph does not attach very readily to the HRBC surface. The effect of the factor is probably not sufficiently pronounced to be evident when only little is adsorbed to the particle surface. The difference in the extents of lysis of opsonised and un-opsonised HRBC and those opsonised in experienced fluid were not significant.

That the level of phagocytosis of the haemocytes cultured in vitro is higher than that of the haemocytes from injected animals does indicate that the in vitro system did not impair the phagocytic

ability of the cells.

The uptake of bacteria by the haemocytes of injected animals may be so low because the haemocyte/bacterium ratio was rather low, so minimising the chance of any haemocyte encountering a bacterium. But overall, from the mean number of bacteria taken up by each haemocyte, only 2,540 bacteria need have been removed from the haemolymph after 3 hours (Table 5.iii.7.), leaving about 14,760 still available. So one might suppose that any haemocyte able to take up bacteria would have had a reasonable chance of so doing.

After 3 hours it can be calculated that only 0.423% of the initial dose of bacteria was removed from the haemolymph by the haemocytes (Table 5.iii.7.). The haemocytes cultured *in vitro* should have been able to remove 2.605% of the initial dose after 2 hours, had they been at the same concentration as the haemocytes *in vivo*.

The rates of clearance calculated from the results of both these experiments seem so low as to be incompatible with the observed sterility of *Patella*'s internal environment. This suggests that perhaps the haemocytes are not the only cells capable of taking up foreign particles. Particles injected into *Liolophura gaimardi* are principally taken up by fixed phagocytes (Crichton, Killby and Lafferty, 1973) and Bayne (1974) concluded from the very high *in vivo* clearance rates observed in *Helix pomatia*, that the haemocytes alone could not account for the number of bacteria removed over the time . If this is not the case, then effective mechanisms for keeping bacteria or potential pathogens from entering the animal must exist.

iv. The elimination of bacteria injected in vivo.

Method

Animals were vaccinated through the foot, into the haemocoel. 100 µl of washed, living bacteria suspended in sterile sea water was used in each case. Either 'A' or E. coli were used.

After 1 or 6 hours, haemolymph was aspirated from the artery leaving the heart (see Section 2.ii.). 100 µl of the aspirate was added to 900 µl of sterile sea water. The diluted haemolymph was then plated out in 25 µl drops from a fixed delivery pipette (Excalibur) onto 6 different prepared agar plates, after the method of Miles and Misra (1938), (see Sections 2.viii. and 4.ii.). For 6 animals, 6 plates were used and each plate had one drop of diluted haemolymph from each animal. Dilutions of the vaccine used were plated in a similar manner.

The plates were incubated over night at room temperature for 'A' and at 37°C for E. coli. Colony counts were made under a dissecting microscope.

Each animal was bled only once as the first bleeding often damaged the blood vessel and animals sometimes bled quite extensively after the hypodermic needle had been withdrawn.

Cell counts were made with an 'Improved Neubauer' haemocytometer (Hawksley) of all the samples of haemolymph, before they were diluted.

Results

As the haemocoelic volume of the animals was not known, the

dilution factor of the vaccine, on injection, cannot be calculated, so the fate of bacteria during the first hour of the experiment is unknown. But change from one to six hours post-vaccination can be calculated (see Table 5.iv.1.).

In some cases the initial dose of bacteria given was not known as the vaccine had been insufficiently diluted to allow colony counts to be made after plating out. As animals all received the same dose comparison of the numbers of bacteria remaining after one and six hours could be made and K values calculated. These results are tabulated and the six hour counts calculated as a proportion of the one hour counts of animals all receiving the same dose of bacteria (Table 5.iv.1.).

Cell counts of all the animals used in experiment 1 were made, so the mean number of bacteria removed by each haemocyte can be calculated (Table 5.iv.2.). The cell number was found to increase significantly from one to six hours (Section 6.ii.), so the mean of cell number at both times is used. The results calculated as concentration mm^{-3} (Tables 5.iv.3. and 4.) allow comparison to be made with the results obtained from in vitro phagocytosis (Section 5.iii.).

Conclusion

The injected bacteria generally show a decline in number over the time of the experiments.

When E. coli was incubated in cell-free haemolymph the numbers did decline slowly, from the 1 hour sample to the 4 hour sample, 21.87% of the bacteria present were lost (a mean of 7.29% per hour) (calculated from results in table 4iii3).

Table 5.iv.1. LEGEND

The number of bacteria mm^{-3} remaining after in vivo injection of living 'A' and E. coli. Mean rates of clearance - K - are also calculated.

TABLE 5.iv.1.

dose	1 hr bacteria mm^{-3}	6 hrs bacteria mm^{-3}	
EXPERIMENT 1			
10005 'A' in 100 mm^3 sterile sea water	11.73 (± 3.45)*	15.85 (± 7.57)	
	27.36 (± 8.16)	0.20 (± 0.14)	
	8.60 (± 3.03)	2.47 (± 0.46)	
	2.20 (± 0.86)	0.32 (± 0.20)	
	2.40 (± 1.11)	1.40 (± 0.32)	
	24.33 (± 8.96)	6.60 (± 1.60)	
	5.60 (± 2.87)	1.60 (± 0.44)	
	15.07 (± 6.32)	7.36 (± 2.84)	
	0.67 (± 0.20)	0.33 (± 0.19)	
	2.13 (± 0.89)		
n^o	10	9	K_{1-6} = 0.079
$\bar{x}$	10.01	4.01	1-6 hrs 59.9% of
SE	± 3.02	± 1.64	bacteria removed from haemolymph

* ± = standard error of 6 replicate counts
^{o}n = number of animals vaccinated
$\bar{x}$ = mean bacteria mm^{-3}
SE = standard error of mean

TABLE 5.iv.1. (Continued)

Vaccine	bacteria mm^{-3} 1 hr	3 hrs	6 hrs	24 hrs	
EXPT. 2					
'A'	11.73		15.85		
	22.80		0.20		
	8.60		2.47		
	2.20		0.43		
	2.40		1.40		
	24.33		6.60		
	5.60		1.60		
	0.67		7.36		
	2.13		0.33		
n*	10		9		K_{1-6} = 0.074
$\bar{x}$	9.55		4.03		1-6 hrs 57.80% of
SE	2.76		1.72		bacteria removed from haemolymph
EXPT. 3	h'cytes mm^{-3}		h'cytes mm^{-3}		
'A'	29.87	1740	5.73	6380	
	0.27	750	2.67	830	
	0.87	730	81.04	3460	
	0.13	2780	67.68	1710	
	0.00	7100	0.00	10350	
			2.27	3080	
n	5	5	6	6	360% increase in
$\bar{x}$	6.23	2620	22.43	4302	bacteria mm^{-3} over
SE	5.91	1182	12.61	1436	1-6 hrs ∴ each injected bacterium may have divided x 1.5-2 1-6 hrs

TABLE 5.iv.1. (Continued)

Vaccine	bacteria mm^{-3} 1 hr	3 hrs	6 hrs	24 hrs	
EXPT. 4					
'A'	2.67			13.12	
	1.13			35.20	
	1.20			0.60	
	0.20			xs*	
	19.33			xs	
	2.67			xs	
	16.53			xs	
	0.00			xs	
	12.47			xs	
	0.33				
	0.07				
n	11			3	316.98% increase in bacteria mm^{-3} over 1-24 hrs . ∴ each injected bacterium may have divided at least x 1.5. 1-24 hrs.
$\bar{x}$	5.14			16.31	
SE	2.19			10.12	
EXPT 5					
E. coli	1.60	0.00	0.07		
	28.73	2.87	21.33		
	6.20	99.33	1.33		
	17.93	45.73	0.00		
	60.93	7.33	42.87		
	87.00	17.60	13.47		
	17.07	22.87	9.13		
	3.47				
	74.33				
n	9	7	7	K_{1-3}= 0.036 K_{1-6}= 0.084	
$\bar{x}$	33.03	27.96	12.60	1-3 hrs 15.35% bacteria removed	
SE	10.85	13.26	5.87	3-6 hrs 54.94% " "	
				1-6 hrs 61.85% " "	
				xs = growth confluent	

TABLE 5.iv.2.

1 hr post-injection			6 hrs post-injection		
h'cytes mm^{-3}	'A' mm^{-3}	h'cytes : 'A'	h'cytes mm^{-3}	'A' mm^{-3}	h'cytes : 'A'
2040	11.73	174 : 1	3880	15.85	245 : 1
5930	27.36	217 : 1	3210	0.20	16050 : 1
3500	8.60	407 : 1	3560	2.47	1441 : 1
3610	2.20	1436 : 1	3870	0.32	12093 : 1
1060	2.40	442 : 1	4110	1.40	2936 : 1
1580	24.33	65 : 1	2090	6.60	317 : 1
910	5.60	162 : 1	3860	1.60	2412 : 1
1270	15.07	84 : 1	5650	7.36	768 : 1
1430	0.67	2134 : 1	5580	0.33	16909 : 1
1380	2.13	648 : 1			
*n 10	10	10	9	9	9
$\bar{x}$ 2271	10.00	577 : 1	3979	4.01	5908 : 1
SE 506	3.02	216	368	1.73	2336

5.986 'A' removed by 3080 haemocytes over 1-6 hours.
∴ 1 haemocyte would have removed 0.002 'A'
or 1 haemocyte in 514 takes up a bacterium.

LEGEND The results of Experiment 1 (Table 5.iv.1.) expressed as concentration of bacteria and haemocytes mm^{-3} 1 and 6 hours after vaccination with living 'A'. From this the haemocyte/bacterium ratio at each time was calculated.

*n = number of animals injected
$\bar{x}$ = mean number of 'A' mm^{-3} or haemocytes mm^{-3}
SE = standard error of mean

From the first to the third hour after *in vivo* vaccination of *E. coli*, 15.35% of the bacteria were removed from the circulation* (a mean of 7.68% per hour); these rates do not seem to be very different. From 1 to 6 hours the respective percentages of *E. coli* lost are 35.34% (7.07% per hour) *in vitro* and 61.80% (12.37% per hour) *in vivo*. A K value calculated for the *in vitro* experiment over this time (K_{1-6} = 0.038) is rather lower than that of the injected animals (K_{1-6} = 0.084). The decline of *E. coli* when injected seems to be remarkably little different from its decline in cell-free haemolymph. It would appear that the haemocytes contribute very little to decline in bacterial numbers in the first 3 hours after inoculation, a greater proportion of the bacteria were removed by haemocyte activity from the third to the sixth hour after inoculation. From the results of Section 5.ii., it seemed that the haemocytes of animals vaccinated about 18 hours previously were activated, so the results of injecting *E. coli* could indicate that the cells take some hours to become activated.

Multiplication of 'A' was inhibited somewhat, *in vivo*, since when incubated in cell-free haemolymph *in vitro*, its increase was too rapid to be quantifiable (Section 4.ii.).

From the results in Table 5.iv.2., it can be calculated that a mean of 3,080 haemocytes mm^{-3} removed 5.986 'A' mm^{-3} from the first to the sixth hour after inoculation. This gives a mean of 0.002 bacteria removed by each cell over the 5 hour period, or 1 in 514 haemocytes need have taken up a bacterium over this period. Had each haemocyte taken up as many bacteria as those used in the *in vitro* experiments (Section 5.iii.), the same number of cells should

*(see table 5 iv 1, expt. 5)

have been able to remove 2,048 'A' mm^{-3}. Since 'A' is able to multiply in Patella haemolymph (Section 4.ii.), the observed decline could represent a much higher level of phagocytosis than might be supposed from these results. The use of non-living particles could give a truer result, but would be much harder to quantify. Although the rate of division of 'A' in haemolymph is not known, E. coli was found to decline in haemolymph, but the rate at which E. coli declined, when injected, was not very different from that of 'A'. It could be that 'A' is more avidly taken up by the haemocytes, but preliminary investigations suggest that the difference in percentage uptake between E. coli and 'A' is not significant ($d = 0.205$ - $p \Rightarrow 0.1$), when the cells are incubated in vitro.

In experiment 3, a net increase in numbers of 'A' is recorded. When animals were bled 24 hours after injection (experiment 4), the concentration of 'A' was too great to quantify in 8 animals and only one showed a count lower than the animals bled after 1 hour.

From these results, it seems that Patella's ability to clear injected particles is, in the short term at least, somewhat limited.

v. Discussion.

The experiments in this section were performed in order that the possible contribution of the haemocytes to the internal defence of Patella and any haemolymph factors which might contribute, could be elucidated. Using the haemocytes in vitro (Section 5.ii. and iii.) it was observed that they are able to take up particles in the absence of any haemolymph factors. When fresh HRBC were used as the target particles, no opsonic factors were evident, whereas using heat-killed

'A', the haemolymph appeared to have an anti-opsonic effect.

Absence of haemolymph opsonins has been recorded only in two cockroaches, Periplaneta americana (Scott, 1971) and Blaberus craniifer (Anderson, Holmes and Good, 1973). The Exopterigota are considered to be the more primitive of the two main insect orders and Patella, a member of the Archaeogastropoda, is a less advanced animal than most of the molluscs which have been studied from this point of view. Scott (1971) suggests that the broad evolutionary trend seems to be towards increasing dependence on serum, or haemolymph, factors for the recognition of foreign particles by the phagocytes.

Anti-opsonic effects of haemolymph have been observed in other animals. Scott (1971) recorded a slightly reduced adherance of opsonised SRBC to Periplaneta haemocytes and using Parachaeraps bicarinatus haemocytes, the percentage of cells taking up bacteria was found to be consistently lower after opsonisation with three of the five species used (Tyson and Jenkin, 1974). Anderson and Good (1976), using Otala, found that the haemocytes tended to take up fewer fresh SRBC in the presence of haemolymph. Though neither Periplaneta nor Parachaeraps showed any opsonic activity, to try and elucidate the nature of the recognition site on the haemocytes, Scott (1971) and Tyson and Jenkin (1974) treated the cells with trypsin. In both cases this significantly reduced the association between the haemocytes and the test particles. The authors concluded that proteinaceous sites were involved. In the case of Parachaeraps, opsonising particles presented to trypsin treated cells restored the level of phagocytosis to that of untreated cells. Incubating the

trypsin treated haemocytes in haemolymph restored the phagocytic capacity of the Parachaeraps cells, but did not affect those of Periplaneta. Tyson and Jenkin (1974) conclude that the recognition factor may be free in the haemolymph or attached to the haemocytes, but apparently the haemolymph factor is effective as an opsonin only in the absence of the cell surface factor. Pre-adsorbed haemolymph did not restore the phagocytic capacity of the haemocytes, so the anti-opsonic effect might have been a result of the blocking of recognition sites on the bacteria by adsorption of this factor from the haemolymph. Treatment of Patella haemocytes with 0.002% w:v trypsin in sea water was attempted but the cells were so disrupted by the process that the experiment was abandoned.

The only case where anti-opsonic effect is discussed at all is that of Crassostrea, where Tripp and Kent (1967) noticed that dilutions of haemolymph less than 1/16 were anti-opsonic as the naturally occurring agglutinin caused clumping of the particles. To neither Patella nor Otala can this explanation apply since neither possess haemolymph agglutinins. That the anti-opsonic effect was observed only with heat-killed bacteria, not with fresh HRBC could, as suggested (Section 5.iii.) be an artefact due to alteration of the bacterial coat by heat.

The enhanced uptake of particles opsonised in experienced haemolymph was apparent only when 'A' was the test particle. Miescher, Spiegelberg and Benacerraf (1963) have shown that the amount of antibody required to facilitate the uptake of a particle is proportional to the surface area of the particle. If an opsonin were a molecule which became attached to the surface of a particle, one would certainly

expect the same to be the case. So if a factor were produced by Patella in response to injection, its concentration in the haemolymph might be insufficient to significantly affect uptake of HRBC, although bacterial uptake might be enhanced. If the anti-opsonic effect were the result of a factor in the haemolymph, as suggested (Section 5.iii.), the same could well apply. So when HRBC were the test particles, the anti-opsonic effect would be less.

The rates of phagocytosis calculated for the cells both in vitro and in vivo seem to be fairly consistent. The surprise is that the levels of uptake should be apparently lower in vivo. If the haemocytes take a few hours to become activated, as the results in Section 5.ii. indicate they may do, then the dose, or haemocyte/particle ratio could be an important factor in their activation. The animals used in Section 5.iv. were all given lower doses of bacteria than those which were injected in order that the haemocytes could be scored visually for contained bacteria (Section 5.iii.) and indeed the mean number of bacteria that need have been taken up by each haemocyte is lower in the former case, 0.002 bacteria per cell over 5 hours as opposed to 0.108 over 3 hours. The haemocytes used in vitro showed the highest levels of uptake, up to 0.704 bacteria per cell in 90 minutes. The ratio of bacteria to haemocytes was highest in the in vitro experiments though over the range of ratios used there was no significant correlation between the level of phagocytosis and the bacterium to haemocyte ratio.

The rate of clearance observed in vivo was so low as not to require that any fixed phagocytes, which would not contribute to

in vitro phagocytosis, assist the haemocytes in removing bacteria from the circulation. The highest K values were calculated for the cells used *in vitro* with HRBC, but this may be a reflection of attachment and lysis being a rather faster process than actual phagocytosis. In all cases the rate of uptake and removal is much lower than observed in some other molluscs (Bayne and Kime, 1970; Bayne, 1974; Pauley, Krassner and Chapman, 1971). The only recorded rate of clearance that seems to be slower than that of *Patella* is that of *Achartina* species used by Nelstrop, Taylor and Collard (1968b). These snails managed to clear half a dose of 4×10^9 of T_1 phage particles in 10 weeks and all of a dose of 4×10^3 particles were cleared 11 weeks after inoculation. These rates are so low that natural disintegration, or loss, of the particles could account for them and there is no necessity for the snail's defence mechanisms to be involved at all.

Neither the haemolymph nor the haemocytes of *Patella* would appear from these results, (Sections 4 and 5), to have the capacity to maintain a sterile internal environment or to resist invasion by potential pathogens, without assistance from some other mechanism(s).

SECTION 6

CHANGES IN THE ACID PHOSPHATASE LEVEL IN THE HAEMOCYTES AND HAEMOLYMPH AFTER CHALLENGE WITH BACTERIA

i. Introduction.

The haemocytes appear to be the main line of internal defence in the molluscs and phagocytosis seems to be the principle mechanism whereby foreign particles or regressing tissues are cleared. If phagocytosis is to be an effective method of clearance, the phagocytes should be able to degrade the particles taken up.

Where they have been studied, molluscan phagocytes have been found to contain a variety of lytic enzymes. The haemocytes of Crassostrea virginica contain lysozyme (Eble, 1966; Tripp and Kent, 1967) and Cheng and Rodrick (1975) found lysozyme, acid phosphatase, alkaline phosphatase, β-glucuronidase and lipase in the haemocytes of Mercenaria mercenaria and Crassostrea. The same enzymes were also found to occur in the haemocytes of Mya arenaria (Cheng 1975). Histochemical staining of Mytilus edulis haemocytes indicated the presence of acid phosphatase, β-glucuronidase, N-acetyl-β-glucosaminidase and indoxyl esterase (Moore and Lowe, 1977). Biomphalaria glabrata haemocytes contain lysozyme (Cheng, Chorney and Yoshino, 1977) and also stain positively for acid phosphatase (Jeong and Heyneman, 1976). Sminia (1972) found that Lymnaea stagnalis haemocytes contained lysosomal enzymes, including small amounts of acid phosphatase, though the fixed phagocytes, which are thought to belong

to the same cell population, contain none.

Lytic enzymes present in the haemocytes of a species are often found to be present in the haemolymph as well. In general it seems possible that the haemolymph enzymes have their origin in the haemocytes. Using Mercenaria, Cheng, Rodrick, Foley and Koehler (1975), found that in vitro, actively phagocytosing haemocytes released lysozyme into the surrounding medium. And from light microscope studies, haemocytes associated with bacteria were found to be comparatively degranulated (Foley and Cheng, 1977). From in vivo alterations in the level of lysozyme in the haemocytes and haemolymph of Biomphalaria, before and after challenge, Cheng, Chorney and Yoshino (1977) concluded that the haemolymph lysozyme had been released by the haemocytes.

Challenge with a foreign particle might be expected to have some effect on the levels and possibly the distribution of lytic enzymes if they were involved in the processes of internal defence. The levels of lysosomal enzymes in several species of molluscs seem to be elevated after the injection of a foreign particle. Aminopeptidase was significantly increased in the haemocytes of Crassostrea after in vitro challenge with heat-killed bacteria (Yoshino and Cheng, 1976), though the level in the haemolymph was not altered. Mya, after in vivo challenge, showed elevated levels of this enzyme in both the cells and the haemolymph, (Cheng and Yoshino, 1976a). In Lymnaea acid phosphatase in the haemocytes was found to increase after an injection of carbon (Sminia, 1972) and in Biomphalaria the haemocyte and haemolymph lysozyme levels rose after challenge with bacteria (Cheng, Chorney and Yoshino, 1977).

The function of haemolymph lysins in molluscs is not known, since in none of the species mentioned are they present in sufficient quantity to be bactericidal (see Section 4.i.), even after challenge. But the above results indicate that the haemocytes of these species are competent to degrade phagocytosed particles intracellularly. The haemocytes of Patella appear to be the main defence mechanism (Section 5.v.) and from the results obtained with other molluscs it would seem probable that its haemocytes would also contain lytic enzymes, as they are able to take up and digest HRBC in vitro (Section 3.iii.).

Preliminary investigations indicated that lysozyme was completely absent from the haemolymph, before and after challenge with heat-killed bacteria. A very slight lysozyme-like activity was detected in some haemocyte samples, such a small quantity could only assist in intracellular digestion, however. The activity was so low that larger samples of haemocytes would be necessary for accurate quantification, but this was not possible as the assays were done in winter when the haemocyte count of Patella seems to fall. Acid phosphatase (EC. no. 3.1.3.2.) has been detected in both haemocytes and haemolymph. The alteration in the level and distribution of this enzyme was investigated after challenge with bacteria and with sea water. The possibility of the haemocytes being the source of the haemolymph acid phosphatase was also investigated. Cultures of the haemocytes in vitro enabled increases in staining intensity to be observed semi-quantitatively and the release of acid phosphatase into the surrounding medium to be assayed.

ii. Changes in acid phosphatase levels in the haemocytes and haemolymph after the injection of bacteria.

Method

Animals were injected through the foot with a suspension of washed, living 'A' (Section 2.vii.) or with an equal volume of sterile sea water. They were left at room temperature for 1 or 6 hours before bleeding from the pallial artery.

Preliminary experiments showed that the level of acid phosphatase in the haemolymph of uninjected animals varied widely between individuals. To try to reduce the variability of the results, equal volumes of haemolymph from 3 different animals were pooled, for each sample.

The haemolymph was kept on ice until required. A cell count of each pooled sample was made using an 'Improved Neubauer' haemocytometer (Hawksley). The samples were then centrifuged for 5 minutes at 600 x g. This caused the haemocytes to form a clot in the bottom of the centrifuge tube which was removed and stored in a haematocrit tube at -20^{o}C until needed.

The level of acid phosphatase in the cell-free haemolymph was assayed using the Roche 'Diagnostica' kit for the determination of the activity of total acid phosphatase in the serum and plasma. The substrate is p-nitrophenyl-phosphate, in a citrate buffer at pH 4.9. Acid phosphatases catalyse the hydrolysis of p-nitrophenyl-phosphate to p-nitrophenyl and phosphoric acid. The p-nitrophenyl produced is proportional to the phosphate activity and can be photometrically measured in alkaline solution.

500 µl of the reagent were delivered with a fixed delivery pipette (Excalibur) into an experimental and into a blank test tube, which were then warmed at 37°C for about 5 minutes. 500 ul of haemolymph was then added to the experimental tube and both tubes were incubated at 37°C for exactly 30 minutes. After incubation, 2.5 ml of 0.1 N sodium hydroxide was added to both tubes and 500 µl of the haemolymph was added to the blank tube. Addition of the sodium hydroxide caused protein in the haemolymph to precipitate, so the tubes were centrifuged for 5 minutes at 600 x g, the supernatant was then read at 405 nm in a spectrophotometer (Beckman DB-G).

The supernatant from the experimental tube could not be read directly against the blank as the spectrophotometer would not zero with most of the blanks owing to the colour of the haemolymph. So readings of both supernatants were made against distilled water and the blank result was subtracted from the experimental.

The acid phosphatase present is measured in international units (IU). 1 IU corresponds to a substrate conversion rate of 1 µmol min^{-1} l^{-1}.

To convert the spectrophotometer readings into IU, a standard plot was made using acid phosphatase (ortho-phosphoric-monoester phosphohydrolase (EC. no. 3.1.3.2.) from potatoes (Sigma Lot 54C-5072). This had an activity of 0.5 IU mg^{-1}, standard dilutions were made up in 0.02 M McIlvaine citric acid-disodium phosphate buffer at pH 4.8 (from Hale, 1958). The activity of the haemolymph samples could then be read off from the graph as IU.

To assay the acid phosphatase level in the haemocytes, the cell clots in the haematocrit tubes were spun in a haematocrit centrifuge (Hawksley) at 12,000 x g for 4 minutes and the length of the cell column recorded. The clot was ejected into a centrifuge tube and 500 μl of sterile sea water was added for each 1 mm of cells, using a fixed delivery pipette (Excalibur).

The haemocytes were disrupted by sonication. The centrifuge tubes were held in the water bath of a sonicator (Kerry's Ultrasonics) until all trace of the clot had disappeared. After shaking, the tubes were centrifuged for 5 minutes at 600 x g and the acid phosphatase level of the supernatant determined as before.

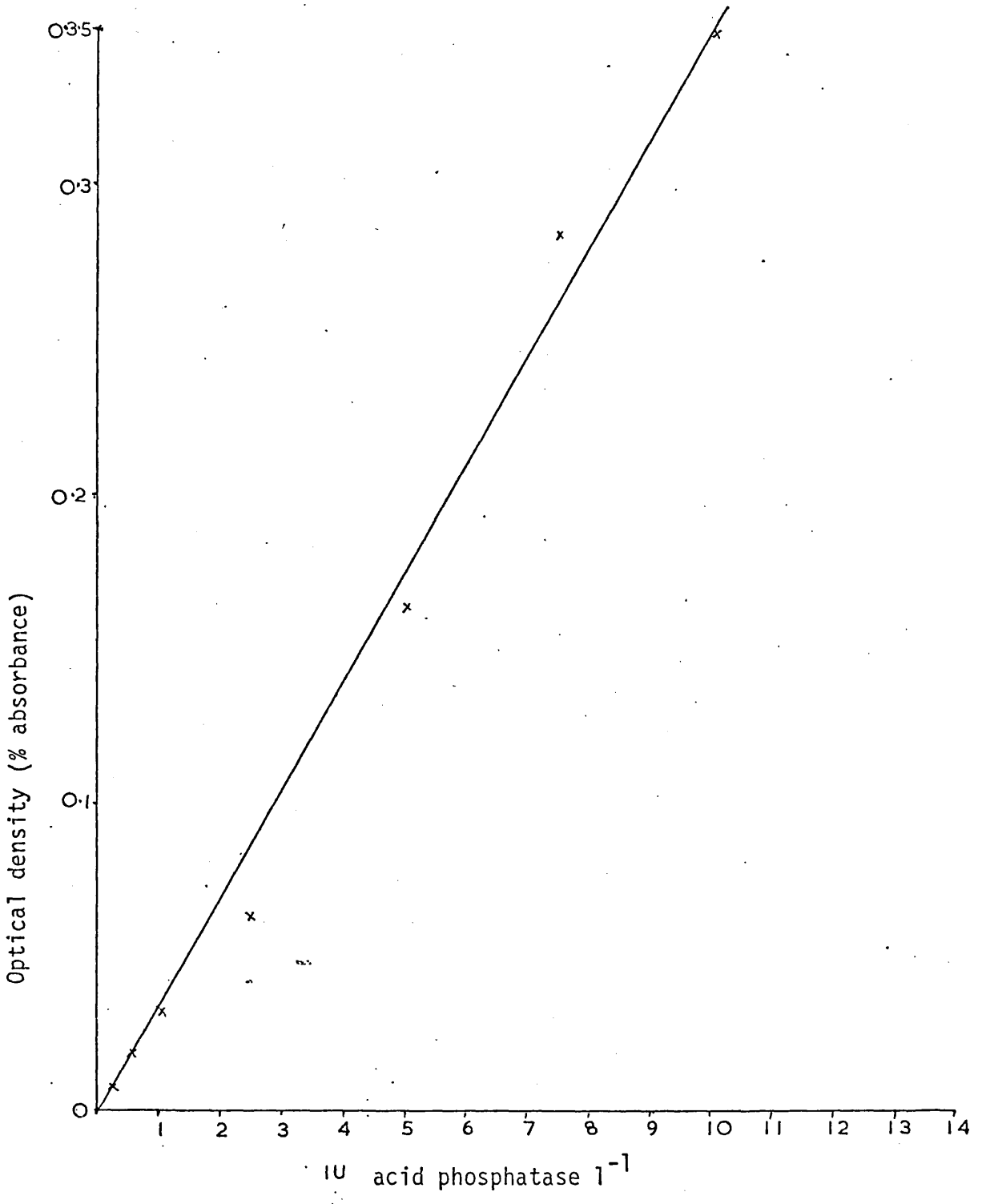
1 ml of the sample was the minimum which could be used for acid phosphatase determinations, so at least 2 mm of haemocytes were required. The acid phosphatase in all the haemocyte samples could not be determined, since some of the cell columns were less than this length.

Results

The results of the acid phosphatase determinations are expressed as IU l^{-1}, for both haemocytes and haemolymph.

The time 0 determinations were done with the haemocytes and haemolymph of naive animals and so are the same in Tables 6.ii.1. and 2., for 'A' and sea water injected animals respectively.

6 hours after injection with 'A' the acid phosphatase level in haemolymph and cells was significantly higher than that of the naive animals (haemolymph, $t = 2.538$; $p < 0.02$ - haemocytes, $t = 2.908$; $p < 0.02$).



Legend.

The optical density of p-nitrophenyl released from p-nitrophenyl-phosphate by standard dilutions of acid phosphatase in citrate buffer at pH 4.8.

Since the determinations of acid phosphatase in the haemolymph and haemocytes were so widely scattered and contained a high proportion of negative results a non-parametric test was also used to test the significance of the apparent trends of increased concentration after injection with bacteria or sea water.

Kendall's S test (Kendall M., (1975) 'Rank correlation methods' pp 49-66, Charles Griffin & Co Ltd., London) was used and this indicated that the trends of increased concentration of acid phosphatase in the haemolymph and haemocytes after injection with bacteria were significant ($z = 3.245$, $p < 0.01$ and $z = 3.580$, $p < 0.001$ respectively). The increased concentration in the haemolymph after sea water injection was significant ($z = 2.140$, $p < 0.05$), but the trend in the haemocytes was not significant ($z = 1.210$, $p > 0.10$).

Table 6.ii.1. LEGEND

The number of haemocytes mm^{-3} in the haemolymph and the level of acid phosphatase in the haemolymph and haemocytes, expressed as international units (IU) l^{-1} in naive animals and in animals 1 and 6 hours after injection with living 'A'.

TABLE 6.ii.1.

	Naive			1 hr post-injection			6 hrs post-injection		
	h'cytes mm^{-3}	IU l^{-1}* h'lymph	IU l^{-1} h'cytes	h'cytes mm^{-3}	IU l^{-1} h'lymph	IU l^{-1} h'cytes	h'cytes mm^{-3}	IU l^{-1} h'lymph	IU l^{-1} h'cytes
	3770	0.0	-	2570	0.020	-	5220	1.50	1.25
	3240	0.0	0.150	2780	0.0	-	4210	3.35	-
	4240	0.0	0.0	3320	0.0	-	4620	0.05	-
	2640	0.0	0.0	2790	0.0	-	3640	2.28	0.45
	5740	0.450	-	4130	0.900	-	6310	0.45	1.35
	2460	0.0	0.200	4560	0.080	0.17	3920	5.95	-
	4100	0.050	0.0	5450	0.625	0.0	5200	0.0	-
	4570	0.450	0.400	2800	0.001	0.0	3610	0.20	1.70
	2850	0.0	0.550	2080	11.00	0.30	3540	0.01	1.10
	3210	0.0	-	1990	0.03	0.35	2610	0.05	-
	2700	0.0	-	2040	0.80	-	2340	0.40	-
	1190	0.0	-	5930	0.018	-	3880	3.55	1.55
	2630	3.25	-	3500	0.0	0.38	3210	0.30	5.45
	1700	0.0	-	3610	0.0	0.30	3560	1.00	0.0
	1580	0.0	-	1060	0.01	-	3870	0.50	0.0
				1580	0.85	-	4110	0.45	0.50
				910	0.0	-	2090	1.35	-
				1270	0.30	-	3860	0.85	0.25
				1430	1.05	-	5650	1.30	0.98
				1380	3.47	-	5580	0.50	0.65
n^{o}	15	15	7	20	20	7	20	20	13
$\bar{x}$	3108	0.280	0.186	2758	0.958	0.214	4051	1.202	1.171
SE	315.4	0.216	0.082	321.0	0.559	0.061	249.1	0.341	0.389

* The haemocyte level is that of 1 mm of packed haemocytes disrupted by sonication in 500 ul of sterile sea water.

^{o}n = number of trials. Each trial was the pooled whole haemolymph of 3 animals

$\bar{x}$ = mean

SE = standard error of mean

Table 6.ii.2. LEGEND

The number of haemocytes mm^{-3} in the haemolymph and the level of acid phosphatase in the haemolymph and haemocytes expressed as international units (IU) l^{-1} in animals 1 and 6 hours after injection with 100 μl sterile sea water.

TABLE 6.ii.2.

	1 hour			6 hours		
	h'cytes mm^{-3}	IU l^{-1}* h'lymph	IU l^{-1}* h'cytes	h'cytes mm^{-3}	IU l^{-1} h'lymph	IU l^{-1} h'cytes
	1470	0.0	-	2570	0.58	-
	3900	0.0	-	3810	0.0	0.50
	3470	0.0	-	3790	1.95	0.0
	2310	0.0	0.45	2830	0.28	0.0
	3830	2.45	-	2480	0.10	-
	2800	2.30	-	2250	0.05	-
	1890	0.90	-	4060	0.10	0.50
	1850	0.0	-	3260	0.03	-
	4470	0.0	-	3370	0.50	1.42
	3200	0.0	-	3150	10.00	-
	2780	0.0	-			
	3390	0.0	0.70			
	2990	0.0	1.20			
	3640	0.0	-			
	2100	0.0	-			
	3200	0.0	-			
	2640	0.40	-			
n^o	17	17	3	10	10	5
$\bar{x}$	2996	0.356	0.783	3157	1.359	0.484
SE	192.91	0.192	0.220	195.24	0.978	0.259

* The haemocyte level is that of 1mm packed haemocytes disrupted by sonication in 500 μl sterile sea water

n^o = number of trials. Each trial was the pooled whole haemolymphs of 3 animals

$\bar{x}$ = mean

SE = standard error of mean

Fig. 6.ii.1. LEGEND

(from Tables 6.ii.1. and 6.ii.2.) The level of acid phosphatase in IU l^{-1}, in the haemolymph (-.-.-) and haemocytes (——) of naive animals and of animals pre-injected with either heat-killed bacteria (x) or with sterile sea water (o) and the change in mean haemocyte number (---).

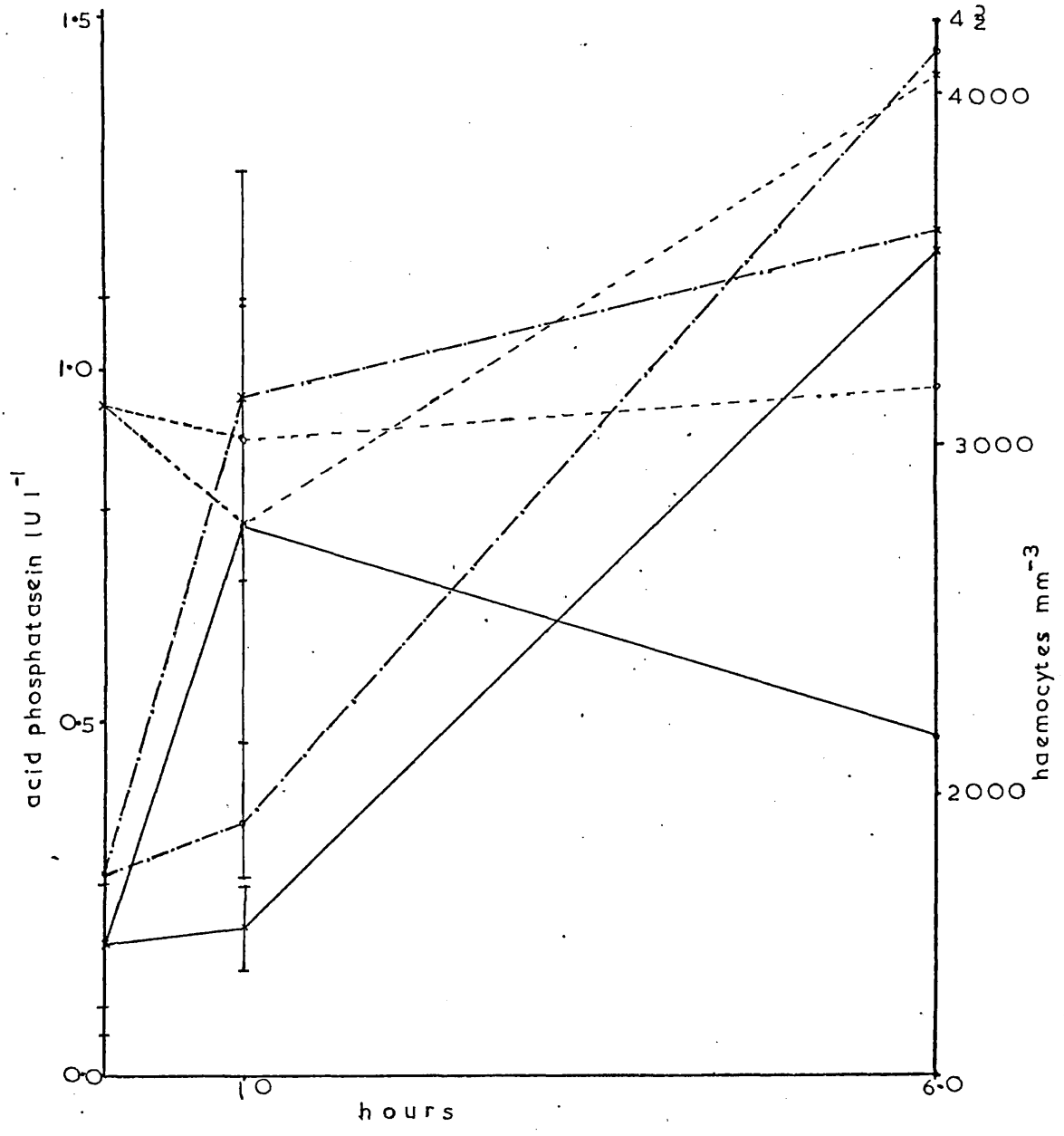

1·5
1·0
0·5
0·0
1·0
6·0
hours
acid phosphatasein IU l⁻¹
4 2
4000
3000
2000
haemocytes mm⁻³

Fig. 6.ii.1.

Though the increase in the mean level of acid phosphatase in the haemolymph was not significant 1 hour after injection, a significantly greater proportion of the samples gave a positive reading ($d = 2.536$; $p = \langle 0.02$). From Table 6.ii.2. it can be seen that though animals injected with sea water showed a small rise in haemolymph acid phosphatase, the proportion of the samples giving a positive reading was the same as that of the naive animals. The difference in the haemolymph acid phosphatase levels 6 hours after injection with either bacteria or sea water is not significant.

Although living 'A' incubated in sea water at a concentration of 1.0×10^9 ml^{-1} for 1 hour at room temperature did give a positive result, this was a higher concentration than was found in the animals after injection (Section 5.iv.). A comparison of the results using living 'A' with those of a preliminary experiment for which heat-killed bacteria were used, shows that 6 hours after injection the levels of haemolymph acid phosphatase were insignificantly different ($t = 0.953$; $df = 21$).

Over the first hour after 'A' injection, the haemocyte number fell slightly, but had risen significantly by 6 hours after the injection ($t = 2.308$; $p = \langle 0.05$). After sea water injection, no significant change in haemocyte concentration occurred (Fig. 6.ii.1.).

As the haemocyte clots were diluted according to volume, the IU l^{-1} represents a mean level of acid phosphatase within the haemocytes and was not affected by their altering numbers. So the increase in cellular acid phosphatase indicates a mean rise in the amount present in each cell. Injection of 'A' appeared to cause a significant

rise in both cell number and cellular acid phosphatase ($t = 2.908$; $p = \langle 0.02$), whereas sea water injection affected neither significantly.

The change in the proportion of the total acid phosphatase present in the haemocytes and haemolymph after injection indicates that the presence of bacteria and of sea water does have an effect on the distribution of the enzyme (Table 6.ii.3.). From this it may be seen that 1 hour after injection with 'A' the proportion in the haemocytes had dropped, but where the sea water was used the reverse was the case. The distribution 6 hours after sea water injection is similar to that 1 hour after 'A' injection and the proportions are about equal 6 hours after 'A' injection (Fig. 6.ii.2.).

Conclusions

The results suggest that injection of bacteria results in an elevated level of acid phosphatase in the haemocytes and haemolymph. That neither cell number nor cellular acid phosphatase respond significantly to sea water injection suggests that this is a response to the foreign particles, not only to the trauma of injection, though 6 hours after sea water injection the level of haemolymph acid phosphatase was not significantly different from that 6 hours after 'A' injection.

The changes in the distribution of acid phosphatase between the haemocytes and the haemolymph (Fig. 6.ii.2.) suggest that the enzyme may be released from the cells into the haemolymph and then the level built up again in the cells, from the first to the sixth hour after injection. That 1 hour after 'A' injection and 6 hours after sea water injection the haemolymph acid phosphatase level had risen signif-

TABLE 6.ii.3.

	Naive		1 hr		6 hrs	
Injection	h'lymph	h'cytes	h'lymph	h'cytes	h'lymph	h'cytes
Bacteria	60.09%	39.91%	81.74%	18.26%	50.64%	49.36%
Sea water	60.09%	39.91%	31.49%	68.51%	73.74%	26.26%

LEGEND The distribution of acid phosphatase between haemocytes and haemolymph expressed as a percentage of the total, (from Tables 6.ii.1 and 2.) for naive animals and animals which had been injected either with living bacteria or with sterile sea water 1 and 6 hours previously

Fig. 6.ii.2. LEGEND

(from Table 6.ii.3.) The distribution of acid phosphatase between the haemocytes () and the haemolymph () in naive animals and in animals previously injected with either heat-killed bacteria or sterile sea water.

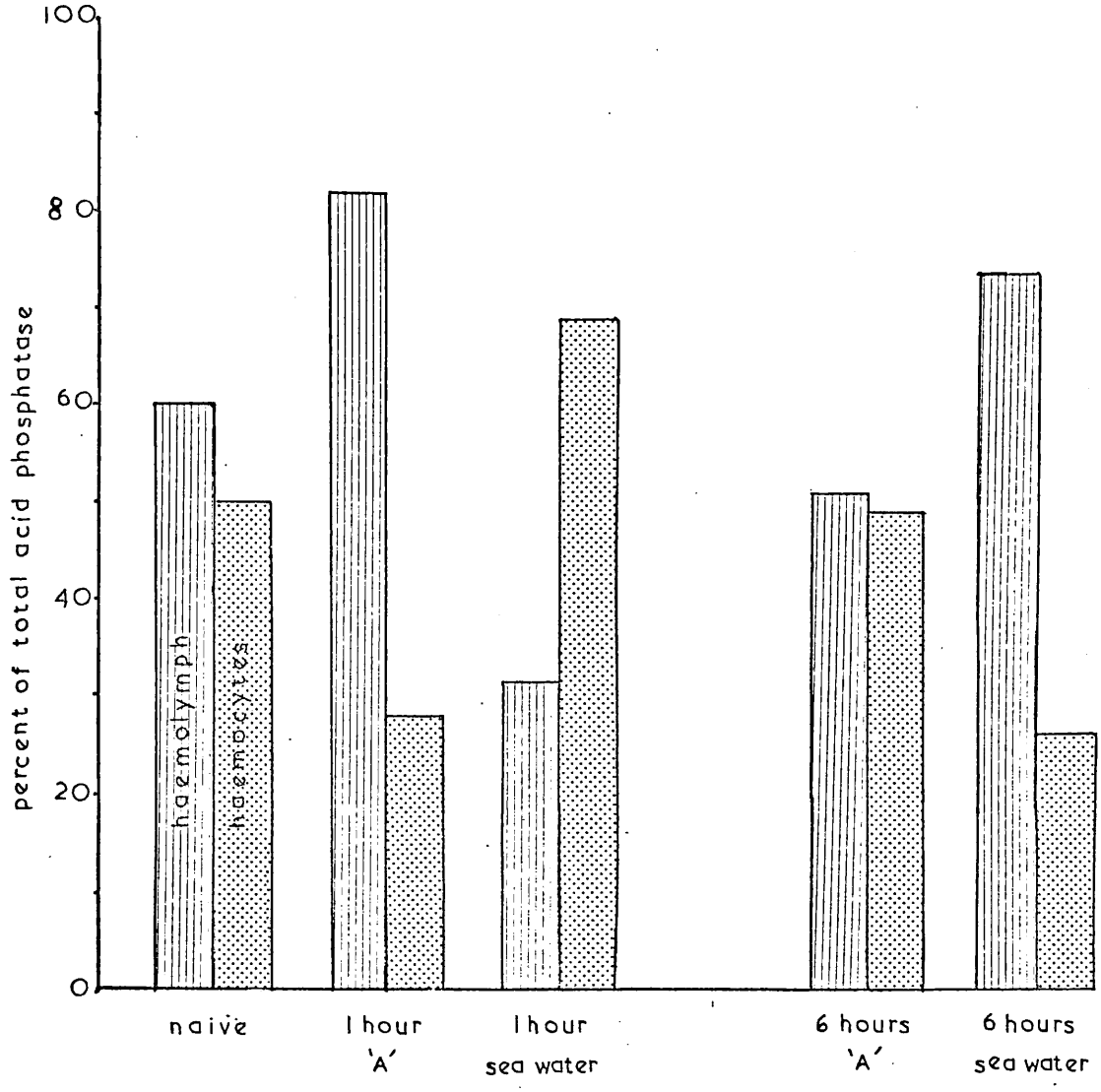

100
80
60
40
20
0
percent of total acid phosphatase
haemolymph
salivary gland
naive
1 hour 'A'
1 hour sea water
6 hours 'A'
6 hours sea water

Fig. 6.ii.2.

icantly, though the cellular levels were not altered, could indicate that the haemocytes are not the only source of the enzyme in the haemolymph. It seems probable that the build-up of acid phosphatase in the cells is not a very fast process, the increase of staining intensity over 1 hour *in vitro* was only 17% (Section 6.iii.) for naive haemocytes. If the rise in haemolymph acid phosphatase were due to the haemocytes alone then a fall in the cellular level over the first hour might be expected, unless the fall in cell number during this time signifies a rupture of some cells and a consequent release of acid phosphatase into the haemolymph.

Pennell (1964) gives the acid phosphatase level of human red cells as 543 IU $cell^{-1} \times 10^{11}$, for human platelets the level is 6.5 IU $cell^{-1} \times 10^{11}$ (Gross, Lohr and Waller, 1966) and from the results of Baggiolini, Hirsch and DeDuve (1970) it was calculated that rabbit leucocytes contain about 34,115 IU $cell^{-1} \times 10^{11}$. For the same number of haemocytes, the mean for *Patella* is 8.85 IU for naive cells and 29.15 IU 6 hours after injection with 'A'. The haemocytes are evidently much lower in acid phosphatase than rabbit leucocytes, as might be expected, but the level is comparable with that of human platelets.

The rise in haemocyte number observed 6 hours after 'A' injection is probably a specific response to foreign particles, since no such rise occurred after injection with sea water. The animals were bled from a different site to that in which they were injected, so the elevated cell number would not be the result of haemocytes collecting at a wound site, as Bayne and Kime (1970) suggested might be the case with their observations of *Helix pomatia*.

As is the case with some other molluscs studied (Cheng and Rodrick, 1975; Yoshino and Cheng, 1977; Cheng and Yoshino, 1976b) the standard deviations of these results are large, indicating their wide variability. In the cases of Crassostrea and Mercenaria it was suggested that this might be due to their open circulation, which is in direct communication with the exterior (Cheng and Rodrick, 1975), but this cannot be the case with Patella since no such communication exists, but it could depend on the prior history of the individual animal which, of course, is unknown.

iii. Localisation of acid phosphatase in haemocytes cultured in vitro.

Method

A haemocyte monolayer was prepared as before (Section 2.v.). 0.8 ml of pooled whole haemolymph was left at room temperature to allow the cells to settle onto a coverslip in the bottom of a 5 cm Petri dish and a cell count was made of the pooled haemolymph. The haemolymph was removed after 20 minutes and replaced with 100 µl of sterile sea water containing 3×10^8 heat-killed 'A' and 3 ml of sterile sea water. The dish was agitated for 1 hour on an oscillating table (Gallenkamp), the coverslip was then removed, rinsed in sea water and fixed in Baker's formol-calcium. A sample of the pooled haemolymph was also left on a slide to allow the cells to settle under the same conditions. These cells were fixed after 20 minutes.

The fixed monolayers on the slides and coverslips were stained by the simultaneous azo-dye coupling method, using substituted napthols (after Burstone, 1958, modified by Barka, 1960: see Bancroft, 1975)

for 1 hour at 15°C to localize any acid phosphatase in the heamocytes, methyl green was used as a counter-stain.

This procedure was repeated using whole haemolymph from animals which had been injected with heat-killed 'A' about 18 hours before.

100 haemocytes on each coverslip and slide were scored visually for the intensity of positive staining for acid phosphatase under standardized bright field illumination on a Zeiss Photomicroscope II.

In order to see if any acid phosphatase was secreted by the cells into the surrounding medium, as soon as the bacteria and sea water had been added to the dish, a 1 ml sample of the culture medium was removed. Another sample was removed after 1 hour. A control dish containing haemocytes and sterile sea water but no bacteria, was also sampled after 1 hour. These samples were stored at -20°C until the levels of acid phosphatase in them could be determined by the method described above (Section 6.ii.).

Results

The staining method used gives a red to magenta colour where acid phosphatase is present (Plates 6.iii.1. and 2.). In some cases it is possible to localise the acid phosphatase exactly within the haemocyte, but more usually the colour is diffused throughout the cytoplasm. The methyl green counter-stain colours the nuclei and enables cells with no positive acid phosphatase stain to be seen.

The haemocytes were scored visually, under the microscope, for the intensity of their stain. Cells with no apparent colour were scored as '0', where the cells were stained positively they

Plate 6.iii.1. LEGEND

Haemocytes from a naive animal showing positive staining for acid phosphatase (red colour) after 1 hour of in vitro culture with bacteria.

Plate 6.iii.2. LEGEND

Haemocytes from a naive animal showing positive staining for acid phosphatase (red colour) after 1 hour of in vitro culture with bacteria.

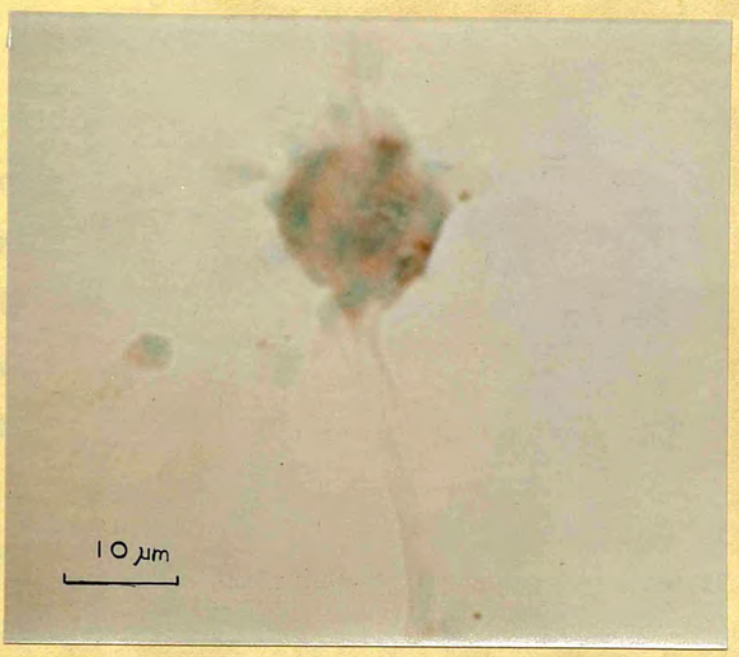


PLATE 6.iii.1.

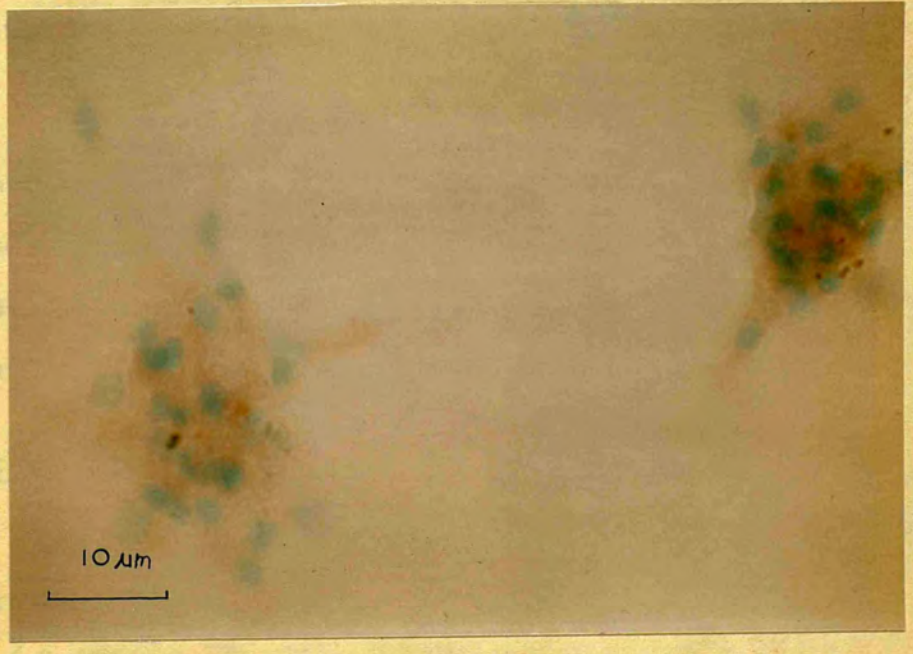


PLATE 6.iii.2.

were scored as 'stain positive - '+', or 'stain strongly positive' - '++'.

The haemocytes which had been allowed to settle onto a slide, then fixed immediately gave a time '0' result, so comparison of this score with that of haemocytes from the same sample after incubation with bacteria in sea water would enable an approximate measure of the change in staining intensity to be made. From this the changes in acid phosphatase levels within the cells may be inferred (Table 6.iii.1.). These results, calculated as a proportion of the haemocytes scored (Table 6.iii.2.) at that time, are shown in Fig. 6.iii.1.

As 'A' did not seem to stain by this method, no correlation of staining intensity with bacterial uptake could be made.

Acid phosphatase determinations were made on 4 time '0' samples of the sea water used as a culture medium for the haemocytes, 2 with naive cells and 2 with experienced cells, 4 1 hour samples and 1 control 1 hour sample. All were negative.

The number of haemocytes in each dish could be calculated from the cell counts (Table 6.iii.3.). The lowest detectable quantity of acid phosphatase, using this method is about 1×10^{-5} IU, or 0.0014 μg in 500 μl. If the haemocytes were secreting acid phosphatase, each cell must have produced less than 5×10^{-10} μg.

Conclusions

From the results in Table 6.iii.1. it seems that the change in staining intensity over 1 hour is significant ($\chi^2 = 95.32$; $p = < 0.001$) for the naive haemocytes. Though the proportion of cells

TABLE 6.iii.1.

h'cytes incubation	n	Stain Intensity* 0	+	++
Naive				
0	200	92	108	0
1 hr	200	84	46	70
Experienced				
0	200	33	72	95
1 hr	200	69	59	72

LEGEND The intensity of positive staining for acid phosphatase of haemocytes from naive animals and animals which had been previously injected with bacteria before and after 1 hour *in vitro* incubation with heat-killed 'A'.

* stain intensity expressed as absent - '0'
present - '+'
strong - '++'

TABLE 6.iii.2.

h'cytes incubation	Stain Intensity* 0	+	++
Naive			
0	46%	54%	0%
1 hr	42%	23%	35%
Experienced			
0	16.5%	36%	47.5%
1 hr	34.5%	29.5%	36%

LEGEND The results from Table 6.iii.1. expressed as the percentage of haemocytes scored which showed absence or presence of acid phosphatase.

* stain intensity expressed as absent - '0'
present - '+'
strong - '++'

Fig. 6.iii.1. LEGEND

(from Table 6.iii.2.) The percentage of haemocytes from naive animals and from animals previously injected with heat-killed bacteria which did not stain (□), stained positively (▨) and stained strongly (▩) for the presence of acid phosphatase, before and after incubation in vitro with heat-killed bacteria.

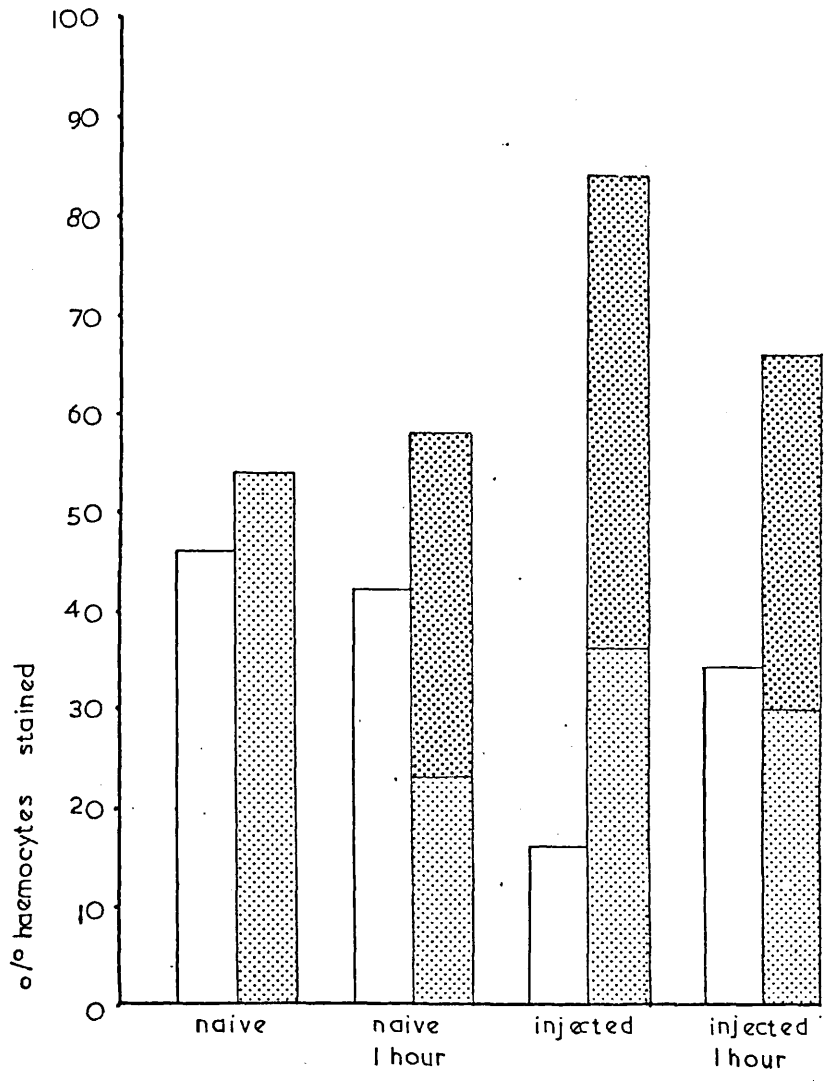

100
90
80
70
60
50
40
30
20
10
0
% haemocytes stained
naive
naive
1 hour
injected
injected
1 hour

Fig. 6.iii.1.

TABLE 6.iii.3.

h'cytes Expt.	h'cytes mm^{-3}	h'cytes/ dish x 10^3	h'cyte: 'A' ratio	IU l^{-1}
Naive				
1	2680	2144	1 : 93	0.0
2	3570	2856	1 : 70	0.0
Experienced				
1	3480	2784	1 : 72	0.0
2	3450	2760	1 : 72	0.0

LEGEND The acid phosphatase levels (as International Units (IU) l^{-1}) in sea water after use for incubation of haemocytes from naive animals or animals which had been previously injected with bacteria were incubated *in vitro* in the presence of heat-killed 'A' for 1 hour.

not staining did not change significantly over the hour ($p = \rangle 0.10$), the intensity of staining did increase. This could suggest either, that the haemocytes which contained no detectable acid phosphatase were unaffected by culture with bacteria and haemocytes already containing the enzyme were stimulated to produce more, or that haemocytes containing acid phosphatase lost it and cells containing none were stimulated to synthesise it.

If the increase in acid phosphatase in the haemolymph is a result of secretion by the haemocytes, then assuming a mean haemocoelic volume of 3 ml per animal, (from the results of Section 6.ii.), 1 hour after injection the mean number of haemocytes would have been $8,274 \times 10^3$, in each animal. Over this period the increase in acid phosphatase in the haemolymph represents the addition of 4.068 ug in a volume of 3 ml. This could be accounted for if each heamocyte were to have secreted 49×10^{-8} μg into the haemolymph. If the haemocytes used <u>in vitro</u> had secreted an equal quantity into the surrounding medium, the level in the culture medium would have been detectable after 1 hour, 23×10^{-9} μg is the minimum each cell could have secreted which would have allowed detection.

When the haemocytes of pre-injected animals were used the results indicated that the total number of cells showing a positive staining reaction was significantly fewer after 1 hour of incubation in vitro ($\chi^2 = 16.28$; $p = \langle 0.001$). This suggests that the haemocytes probably do loose acid phosphatase into the sea water, though from this experiment the amount of acid phosphatase lost cannot be calculated. These results could indicate that the haemocytes are

not the only cells which might be a source of haemolymph acid phosphatase.

That acid phosphatase produced by the haemocytes is all used in degredation of phagocytosed bacteria is not impossible, as the proportion of the naive haemocytes which stained strongly after 1 hour was 35% and the number of cells taking up bacteria after 1 hour in similar circumstances was 34.6% (Section 5.iii.).

The difference between the number of naive and the number of experienced haemocytes showing an absence of stain is significant (χ^2 = 19.570; p = $<$ 0.001). If haemocytes which do not stain are exhausted of their acid phosphatase, then the decline in positive staining observed when experienced cells were used might be expected. This would also explain the similarity in the results obtained from the naive and experienced haemocytes after 1 hour of incubation (χ^2 = 3.108; p $\geq$ 0.05).

iv. Discussion.

From these results it seems that Patella does respond to foreign particles in its internal environment. The reactions observed are probably a response to the bacteria and not only to the presence of sea water in the system.

Though injection of bacteria was found to cause a significant rise in the level of acid phosphatase in the haemolymph, this elevated level was not found to be sufficient to inhibit the growth of bacteria (Section 4.iii.). Cheng (1975) suggests that the alteration of the bacterial surface by enzyme action may affect their phagocytosis by

the haemocytes. It could be that though not sufficiently concentrated to be cidal, the haemolymph acid phosphatase might have an opsonin-like effect, if enzymic action increased the apparent foreigness of the bacterium to the haemocyte. And, indeed, haemolymph of pre-injected animals was found to have a greater opsonic effect than that of naive animals (Section 5.iii.). If this were the main function of the lytic enzymes found in the haemolymph, only fairly low levels would be necessary and the general lack of bactericidins observed in molluscan haemolymphs would be more understandable. This could also account for the enhanced rates of clearance of secondary challenge with an organic particle observed in some molluscs (Acton and Evans, 1967; Bayne, 1974; Pauley, Krassner and Chapman, 1971). Elevated haemolymph lysin levels would lead to more extensive alteration of the particle surface, increasing their dissimilarity to 'self', so to faster recognition by the haemocytes and to enhanced rates of phagocytosis and clearance.

The initial fall in the proportion of acid phosphatase in the haemocytes and the concomitant rise in the proportion in the haemolymph (Section 6.ii.) seems to indicate that the cells may be a source of this enzyme in the haemolymph. When haemocytes were cultured *in vitro* in the presence of bacteria, no acid phosphatase was detectable in the surrounding medium. Some of the enzyme was probably lost from the haemocytes, since the number of cells from pre-injected animals giving a positive staining reaction fell significantly after 1 hour of *in vitro* incubation.

Degranulation has been observed to occur in *Mercenaria* haemocytes *in vitro*, cells associated with bacteria were extensively degranulated

and some degranulation was observed in most of the cells (Foley and Cheng, 1977). When fixed and stained no granules were evident in the haemocytes of Patella (Section 3.iii.), but the decreased staining reaction of the experienced haemocytes in vitro might indicate that some degranulation had occurred. That the haemocytes showed increased acid phosphatase staining after in vitro incubation with bacteria and that the experienced cells showed greater intensity of staining than the naive, both suggest that the haemocytes are able to respond to the presence of foreign particles.

Foley and Cheng (1977) suggested that degranulation indicated the release of enzymes from the haemocytes into the haemolymph. But Mercenaria is the only mollusc in which the haemocytes have definitely been observed to cause the elevation of a lytic enzyme in the surrounding medium (Cheng, Rodric, Foley and Koehler, 1975). Aminopeptidase levels were found to be as high in the visceral mass and head/foot portion of Biomphalaria as in the haemolymph (Yoshino and Cheng, 1977), the haemocytes showed the lowest level, so there is no particular reason to suppose that the haemocytes are the principal or only source of the haemolymph aminopeptidase. As with Patella haemocytes, which were stimulated to increase synthesis of acid phosphatase by the presence of bacteria, Yoshino and Cheng (1976) found that incubation of the whole haemolymph of Crassostrea with bacteria or sea water, in vitro, caused a significant rise in the haemocyte aminopeptidase level, but did not affect that of the haemolymph. It would seem from these results that the haemocytes are unlikely to be the only source of haemolymph lysins. The results from Patella suggest that the haemocytes are not the primary source of acid phosphatase

in the haemolymph, in fact, the results could indicate that acid phosphatase in the haemolymph may be from some quite other origin. The digestive gland could be a possible source as in most gastropods it seems to be very rich in lytic enzymes. Elevated levels of acid phosphatase and alkaline phosphatase have been observed in the digestive glands of both Biomphalaria (Michelson and DuBois, 1973; Von Brand and Files, 1947) and of Helisoma trivolvis (Cheng, 1964) after trematode infection.

A rise in haemocyte number after injection has been observed in other molluscs. Sminia (1974) suggests that the fixed phagocytes and the haemocytes of Lymnaea are members of the same cell population and that cells can alternate between the two states, injection with foreign particles caused an increase in the haemocyte count and a fall in tissue phagocytes. Bayne and Kime (1970) found that colchicine did not prevent a rise in cell number after Helix pomatia was injected with bacteria, which suggests that the cells were already in existence and were not the result of enhanced mitotic activity. It seems probable that the fixed and free phagocytes of Patella are also a single population. Davies and Partridge (1972) observed haemocyte-like cells lining the haemocoel, so the rise in cell number after vaccination could be due to the release of fixed phagocytes from the haemocoel lining. Animals maintained for 20^{o}C for a few hours were found to have elevated cell counts (Davies and Partridge, 1972), so this may be a fairly generalised response to stress in Patella.

SECTION 7

GENERAL DISCUSSION

From the results of this work it is hard to see how Patella is able to maintain a sterile internal environment, though it can apparently do so, since the haemolymph plated out never showed any growth, nor when the haemolymph of vaccinated animals was plated out were any but the injected bacteria observed to grow. It is evident that phagocytosis is the main defence mechanism in Patella. The results in Sections 4 and 5 indicate that in the haemolymph of naive animals there are neither agglutinins, opsonins nor bacteristatic factors. It seems probable that the presence of foreign particles does activate the haemocytes, the results in Section 5.ii. show that experienced haemocytes lysed more HRBC in vitro than did the naive cells. Acid phosphatase synthesis appeared to be stimulated after in vitro incubation of the haemocytes in the presence of bacteria

Activation of the haemocytes is probably the usual reason for the enhanced rates of clearance observed in some molluscs after prevaccination. Aplysia californica (Pauley, Krassner and Chapman, 1971) and Crassostrea virginica (Acton and Evans, 1967) both showed faster clearance of the second injection of a foreign particle, although agglutinin and opsonin levels remained unaltered. A similar result was obtained by McKay and Jenkin (1970a, b, c) using Parachaeraps bicarinatus.

The results in Section 5.iii. suggest that prior experience

results in an elevation of the opsonic activity in the haemolymph, but as agglutinins are absent before and after challenge, the opsonic factor cannot be an agglutinin. Challenge seemed to result in elevated levels of acid phosphatase in the haemolymph, though lysozyme was consistently absent (Section 6.ii.).

Agglutinins have been found in members of all the molluscan classes, but they do not seem to occur with any consistency among the families. Boyd, Brown and Boyd (1966) and Brown, Almodovar, Bhatia and Boyd (1968) tested a total of 36 molluscan species for the presence of agglutinins in their body fluids. Of the pulmonate species, 10 agglutinated A, B and O HRBC, and 11 gave negative results, though they found that a population of the fresh water snail Bulimulus guadalupensis from Florida gave a negative result, whereas the same species collected in Puerto Rica gave a positive result. Nine prosobranch species were tested and six gave negative results. Two species of chiton both gave positive results, as did one of the four lamellibranch species tested. Aplysia protea and Octopus vulgaris both gave negative results. Subsequent work on Otala lactea (Anderson and Good, 1976) and similar work on Helix aspersa (Prowse and Tait, 1969) has shown that the haemolymph of these two species is opsonic, although the agglutinins are contained in the albumen gland and not found in the haemolymph. The albumen gland extract of Otala was found to be opsonic (Anderson and Good, 1976) but it is unlikely that it would normally play a part in internal defence. The haemolymph of Eledone cirrosa is also lacking in agglutinins but haemolymph factors are an absolute requirement for in vitro phagocytosis by the haemocytes (Stuart, 1968).

Bacterial agglutinins have been found in Aplysia (Pauley, Krassner and Chapman, 1971) and in Mercenaria mercenaria (Arimoto and Tripp, 1977). The agglutinins in all the species mentioned above were haemagglutinins against HRBC and in other molluscs only haemagglutinins against vertebrate red cells have been found. But the results suggest that agglutinins probably do not play a vital part in molluscan internal defence. If they did play an important role, a more general distribution might be expected. The results also suggest that agglutinins and opsonins are separate entities since both effects are not invariably present in a species.

If, as suggested in Section 6.iv., opsonic effects were due to the action of lytic enzymes in the haemolymph on the particle surface, then the concomitant decrease of opsonin and agglutinin, as observed by Pauley, Krassner and Chapman (1971) after vaccination of Aplysia, could be the result of depletion of the enzymes in the haemolymph and agglutinins, by adsorption to the particle surface, would also be reduced. If opsonisation were also the result of the adsorption of a haemolymph factor onto the particle, then a decrease in both opsonising and agglutinating capacity of the haemolymph would be expected. The rise in acid phosphatase in the haemolymph observed after the challenge of Patella with bacteria was in no case sufficient to inhibit the growth of bacteria (Section 4.iii. and 6.ii.). The acid phosphatase might function as an inhibitor to bacterial multiplication and so give the haemocytes a slightly greater chance of clearing the haemocoel. Bayne and Kime (1970) observed that the haemolymph of Helix pomatia contained many non-viable bacteria, after the animal had been injected. This could

have been the result of increased levels of lytic enzymes in the haemolymph. But in the case of Patella the concentration of lysins seemed to be too low to be cidal, so unless the enzymes were acting in an opsonic capacity it is hard to understand what the function of the acid phosphatase increase could be.

The defence mechanisms of Patella seem to be less elaborate and certainly less effective than those of other gastropods which have been studied. A comparison of the apparently low level of haemolymph involvement in the internal defence of Patella with the greater dependence on haemolymph factors found in some pulmonates may indicate that among the gastropods, at least, an increase in the dependence of internal defence on haemolymph factors for recognition has occurred. But since little is known of other prosobranchs or of the opisthobranchs, there is at the moment no possibility of substantiating this suggestion.

As Bayne (1974) suggests, it is probable that internal defence in the different molluscan classes is not entirely similar and extrapolation from results obtained with one class, to another class is probably unjustified. Fixed phagocytes have been found to play an important part in pulmonate clearance, they occur in the connective tissue of Lymnaea stagnalis (Sminia, 1972) and in the digestive gland of Helix pomatia (Bayne, 1974), whereas in lamellibranchs phagocytosis is by the haemocytes alone and fixed cells take no part (Reade and Reade, 1976). Octapoid cephalopods also seem to depend quite heavily on fixed phagocytes in the white body (Stuart, 1968; Bayne, 1973a). The white body is also a leukopoetic organ (Cowden and Curtis, 1974), no equivalent organ has been recognised among the lamellibranchs or

gastropods. Though Lie,Heyneman and Yan (1975) suggest that an organ in the mantle cavity of Biomphalaria glabrata may be the site of haemocyte production.

The Gastropoda include at least 1,640 genera and 230 families and the Lamellibranchia about 60 families (Morton, 1967). Something of the mechanisms of internal defence is known in species which are members of only about five gastropod and three lamellibranch families. Except in the case of the Helicidae, no more than one species in each family has been studied. Further work on molluscan internal defence should probably be directed at gaining a much wider knowledge of the capabilities of the species in different classes and sub-classes. Experiments which have elucidated the mechanisms that exist in the species which have been studied could now well be applied to a wide range of species, selected with the intention of clarifying the evolution of internal defence within the Mollusca.

LITERATURE CITED

ACTON, R.T. & EVANS, E.E. (1967) - "Comparative immunological studies with the oyster, Crassostrea virginica." In Vitro, 3, 139-145.

ACTON, R.T. & WEINHEIMER, P.F. (1974) - "Haemagglutinins: primitive receptor molecules operative in invertebrate defence mechanisms." Contemp. Topics. Immunobiol., 4, 271-282.

ANDERSON, R.S., HOLMES, B. & GOOD, R.A. (1973) - "In vitro bactericidal capacity of Blaberus craniifer haemocytes." J. Invert. Pathol., 22, 127-135.

ANDERSON, R.S. & GOOD, R.A. (1976) - "Opsonic involvement in phagocytosis by mollusc haemocytes." J. Invert. Pathol., 27, 57-64.

ARIMOTO, R. & TRIPP, M.R. (1977) - "Characterisation of a bacterial agglutinin in the haemolymph of the hard clam Mercenaria mercenaria." J. Invert. Pathol., 30, 406-413.

BAGGIOLINI, M., HIRSCH, J.G. & DE DUVE, C. (1970) - "Further biochemical and morphological studies of granule fractions from rabbit heterophil leucocytes." J. Cell Biol., 45, 586-597.

BALDO, B.A. & UHLENBRUCK, G. (1975) - "Anti-galactan activity in Tridacna maxima (Roding) haemolymph." Immunology, 29, 1161-1170.

BANCROFT, J.D. (1975) - "Histochemical Techniques." Butterworths, London, pp 246-248.

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

BANG, F.B. (1971) - "A factor in crab amoebocytes which stimulates in vitro clotting of crab blood." J. Invert. Pathol., 18, 280-283.

BANG, F.B. & KRASSNER, S.M. (1958) - "Antibacterial activity of Phascolosoma gouldii blood." Biol. Bull., 115, 343.

BASLOW, M.H. (1969) - "Marine Pharmacology." Williams & Wilkins, Baltimore. pp 8-16.

BAYNE, C.J. (1973a) - "Internal defence mechanisms of Octopus dofleini." Malacol. Rev., 6, 13-17.

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

BAYNE, C.J. (1974) - "On the immediate fate of bacteria in the land snail Helix." Contemp. Topics Immunobiol., 4, 37-45.

BAYNE, C.J. & KIME, J.B. (1970) - "In vivo removal of bacteria from the haemolymph of the land snail Helix pomatia (pulmonata stylommatophora)". Malacol. Rev., 3, 103-113.

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

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

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

BARKER J.R. (1958) "Principles of biological microtechnique" p. 273 Methuen and Co. London

CAMPBELL, D.H., GARVEY, J.S., CREMER, N.E. & SUSSDORF, D.H. (1963) - "Methods in Immunology." W.A. Benjamin, Inc., New York.

CANTACUZENE, J. (1919) - "Etude d'une infection experimentale chez Ascidia mentula." C.R. Soc. Biol., 82, 1019-1022.

CARR, I. (1973) - "The Macrophage. A revue of ultrastructure and function." Academic Press, London, N.Y.

CHADWICK, J.S. (1975) - "In vitro growth of bacteria in haemolymph of Galleria mellonella." J. Invert. Pathol., 25, 331-335.

CHENG, T.C. (1964) - "Studies on phosphatase systems in hepato-pancreatic cells of the molluscan host Echinoparyphium sp. and in the rediae and cercariæ of this trematode." Parasitology, 54, 73-79.

CHENG, T.C. (1975) - "Functional morphology and biochemistry of molluscan phagocytes." Ann. N.Y. Acad. Sci., 266, 343-379.

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

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

CHENG, T.C. & FOLEY, D.A. (1975) - "Haemolymph cells of the bivalve mollusc Mercenaria mercenaria: An electron microscope study." J. Invert. Pathol., 26, 341-351.

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

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

CHENG, T.C. & SAUNDERS, B.G. (1962) - "Internal defence mechanisms in molluscs and an electrophoretic analysis of a naturally occurring serum haemagglutinin in Viviparus malleatus Reeve." Proc. Pa. Acad. Sci., 36, 72-83.

CHENG, T.C. & YOSHINO, T.P. (1976a) - "Lipase activity in the serum and haemolymph cells of the soft-shelled clam Mya arenaria during phagocytosis." J. Invert. Pathol., 27, 243-245.

CHENG, T.C. & YOSHINO, T.P. (1976b) - "Lipase activity in the haemolymph of Biomphalaria glabrata (Mollusca) challenged with bacterial lipids." J. Invert. Pathol., 28, 143-146.

CORNICK, J.W. & STEWART, J.E. (1968) - "Interaction of the pathogen Gaffkya homari with natural defence mechanisms of Homarus americanus." J. Fish Res. Bd. Canada, 25, 695-709.

CORNICK, J.W. & STEWART, J.E. (1973) - "Partial characterisation of a natural agglutinin in the haemolymph of the lobster Homarus americanus." J. Invert. Pathol., 21, 255-262.

COWDEN, R.R. & CURTIS, S.K. (1974) - "The octopus white body : an ultrastructural survey." Contemp. Topics Immunobiol., 4, 77-90.

CRICHTON, R., KILLBY, V.A.A. & LAFFERTY, K.J. (1973) - "The distribution and morphology of phagocytic cells in the chiton Liolophura gaimardi." Aus. J. exp. Biol. med. Sci., 51, 357-372.

CUSHING, J.E., EVANS, E.E. & EVANS, L.Z. (1971) - "Induced bactericidal responses of Albalones." J. Invert. Pathol., 17, 446-448.

DAVIES, P.S. & PARTRIDGE, T. (1972) - "Limpet haemocytes. I. Studies on aggregation and spike formation." J. Cell Sci., 11, 757-769.

EBLE, A.F. (1966) - "Seasonal distribution of alkaline and acid phosphatase and non specific esterase in the normal and diseased American oyster as revealed by enzyme histochemistry." Am. Zoologist, 6, 339.

EVANS, E.E., CUSHING, J.E. & EVANS, M.L. (1973) - "Comparative Immunology: Sipunculid bactericidal responses." Infection & Immunity 8, 355-359.

FOLEY, D.A. & CHENG, T.C. (1972) - "Interaction of molluscs and foreign substances: the morphology and behaviour of haemolymph cells of the american oyster, Crassostrea virginica, in vitro." J. Invert. Pathol., 19, 383-394.

FOLEY, D.A. & CHENG, T.C. (1974) - "Morphology, haematologic parameters and behaviour of haemolymph cells of the quahaug clam Mercenaria mercenaria." Biol. Bull., 146, 343-356.

FOLEY, D.A. & CHENG, T.C. (1977) - "Degranulation and other changes of molluscan granulocytes associated with phagocytosis." J. Invert. Pathol., 29, 321-325.

FURMAN, R.M. & PISTOLE, T.G. (1976) - "Bactericidal activity of haemolymph from the horseshoe crab Limulus polyphemus." J. Invert. Pathol., 28, 239-244.

GINGRICH, R.E. (1964) - "Acquired humoral immune response of the large milkweed bug Onchopeltus fasciatus (Dallas) to injected materials." J. Ins. Physiol., 10, 179-194.

GROSS, R, LÖHR, G.W. & WALLER, H.D. (1966) - "Biochemistry of Blood Platelets." Academic Press, (eds. Kowalskie & Niewiarowskis) pp 161-172.

GURR, E. (1960) - "Encyclopaedia of microscopic stains." Leonard Hill (Books) Ltd, London.

HALE, L.J. (1958) - "Biological Laboratory Data." Methuen & Co., London.

HALL, D.O. & HAWKINS, S.E. (1975) - "Laboratory manual of Cell Biology." English Universities Press Ltd. pp 256-258.

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

HARDY, S.W., FLETCHER, T.C. & GERRIE, L.M. (1976) - "Factors in haemolymph of the mussel Mytilus edulis L., of possible significance as defence mechanisms." Biochem. Soc. Trans., 4, 473-475.

JENKIN, C.R. & ROWLEY, D. (1970) - "Immunity in invertebrates. The purification of a haemagglutinin to rat and rabbit erythrocytes from the haemolymph of the murray mussel (Velesunio ambiguus)." Aus. J. exp. Biol. med. Sci., 48, 129-137.

JEONG, K.H. & HEYNEMAN, D. (1976) - "Leucocytes of Biomphalaria glabrata : morphology and behaviour of granulocytic cells in vitro." J. Invert. Pathol., 28, 357-362.

JOHANNSEN, R., ANDERSON, R.S., GORD, R.A. & DAY, N.K. (1973) - "A comparative study of the bactericidal activity of horseshoe crab (Limulus polyphemus) haemolymph and vertebrate serum." J. Invert. Pathol., 22, 372-376.

JOHNSON, H.M. (1964) - "Human blood group A_1 specific agglutinin of the butter clam Saxidomus giganteus." Science, 146 548-549.

JOHNSON, P.T. & CHAPMAN, F.A. (1970) - "Comparative studies on the in vitro response of bacteria to invertebrate body fluids. II Aplysia californica (Sea Hare) and Ciona intestinalis (tunicate)." J. Invert. Pathol., 16, 259-267.

JONES, G.E. & PARTRIDGE, T. (1974) - "Limpet haemocytes. III Effects of cytochalasin B and colchicine on cell spreading and aggregation." J. Cell Sci., 16, 385-399.

KESSEL, R.G. & SHIH, C.Y. (1974) - "Scanning Electron Microscopy in Biology." Springer-Verlag, Berlin, Heidelberg, New York.

KRASSNER, S.M. & FLORY, B. (1970) - "Antibacterial factors in the sipunculid worms Golfingia gouldii and Dendrostomum pyroides." J. Invert. Pathol., 16, 331-338.

LAFFERTY, K.J. & CRICHTON, R. (1973) - "Immune responses of invertebrates." Frontiers of Biol., 31, 300-320.

LEVIN, J. (1967) - "Blood coagulation and endotoxin in invertebrates." Fed. Proc., 26(6), 1707-1712.

LEVIN, J. & BANG, F.B. (1964) - "The role of endotoxin in the extracellular coagulation of Limulus blood." Bull Johns Hopkins Hosp., 115, 265-274.

LEVVY, G.A., HAY, A.J. & MARSH, C.A. (1957) - "Properties of Limpet β glucuronidase." Biochem. Journal, 65, 203-208.

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

LO BUGLIO, A.F., COTRAN, R.S. & JANDLE, J.H. (1967) - "Red cells coated with Immunoglobulin G: Binding and sphering by mononuclear cells in man." Science, 158, 1582-1585.

MCDADE, J.E. & TRIPP, M.R. (1967a) - "Mechanism of agglutination of red blood cells by oyster haemolymph." J. Invert. Pathol., 9, 523-530.

MCDADE, J.E. & TRIPP, M.R. (1967b) - "Lysozyme in the haemolymph of the oyster Crassostrea virginica." J. Invert. Pathol., 9, 531-535.

MCKAY, D., JENKIN, C.R. & ROWLEY, D. (1969) - "Immunity in the invertebrates. I Studies on the naturally occurring haemagglutinins in the fluid from invertebrates." Aus. J. exp. Biol. med. Sci., 47, 125-134.

MCKAY, D. & JENKIN, C.R. (1970a) - "Immunity in the invertebrates. The role of serum factors in the phagocytosis of erythrocytes by haemocytes of the freshwater crayfish (Parachaeraps bicarinatus)." Aus. J. exp. Biol. med. Sci., 48, 139-150.

MCKAY, D. & JENKIN, C.R. (1970b) - "Immunity in the invertebrates. The fate and distribution of bacteria in normal and immunised crayfish (Parachaeraps bicarinatus)." Aus. J. exp. Biol. med. Sci., 48, 599-607.

MCKAY, D. & JENKIN, C.R. (1970c) - "Immunity in invertebrates. Correlation of the phagocytic activity of haemocytes with resistance to infection in the crayfish (Parachaeraps bicarinatus)." Aus. J. exp. Biol. med. Sci., 48, 609-617.

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

MICHELSON, E.H. & DU BOIS, L. (1973) - "Increased alkaline phosphatase in the tissues and haemolymph of the snail Biomphalaria glabrata infected with Schistosoma mansoni." Comp. Biochem. Physiol., 44B, 763-767.

MIESCHER, P.A., SPIEGELBERG, H. & BENACERRAF, B. (1963) - "Studies on the mechanism of immune phagocytosis of sensitized bacteria and red cells by the reticulo-endothelial system in mice." Editions du centre National de la Recherche Scientifique, 115, 463-475.

MILES, A.A. & MISRA, S.S. (1938) - "The estimation of the bactericidal power of the blood." J. Hyg., 38, 732-749.

MILLER, V.H., BALLBACK, R.S., PAULEY, G.B. & KRASSNER, S.M. (1972) - "A preliminary physico-chemical characterisation of an agglutinin found in the haemolymph of the crayfish Procambarus clarkii." J. Invert. Pathol., 19, 83-93.

MOEBUS, K. (1972) - "Seasonal changes in antibacterial activity of North Sea water." Mar. Biol., 13, 1-13.

MOORE, M.N. & LOWE, D.M. (1977) - "The cytology and cytochemistry of the haemocytes of Mytilus edulis and their response to experimentally injected carbon particles." J. Invert. Pathol., 29, 18-30.

MORTON, J.E. (1967) - "Molluscs." Hutchinson University Library. pp 171-225.

NELSTROP, A.E., TAYLOR, G. & COLLARD, P. (1968a) - "Studies on phagocytosis. I Antigen clearance studies in rabbits." Immunol., 14, 325-337.

NELSTROP, A.E., TAYLOR, G. & COLLARD, P. (1968b) - "Studies on phagocytosis. III Antigen clearance studies in invertebrates and poikilothermic vertebrates." Immunol., 14, 347-356.

PARTRIDGE, T. & DAVIES, P.S. (1974) - "Limpet haemocytes. II The role of spikes in locomotion and spreading." J. Cell Sci., 14, 319-330.

PATTERSON, W.D. & STEWART, J.E. (1974) - "In vitro phagocytosis by haemocytes of the American lobster (Homarus americanus)." J. Fish Res. Bd. Canada, 31(6), 1051-1056.

PATTERSON, W.D., STEWART, J.E. & ZWICKER, B.M. (1976) - "Phagocytosis as a cellular immunoresponse mechanism in the American lobster Homarus americanus." J. Invert. Pathol., 27, 95-104.

PAULEY, G.B., GRANGER, G.A. & KRASSNER, S.M. (1971) - "Characterisation of a natural agglutinin present in the haemolymph of the California sea hare, Aplysia californica." J. Invert. Pathol., 18, 207-218.

PAULEY, G.B. & KRASSNER, S.M. (1972) - "Cellular defence reactions to particulate materials in the California sea hare, Aplysia californica." J. Invert. Pathol., 19, 18-27.

PAULEY, G.B. KRASSNER, S.M. & CHAPMAN, F.A. (1971) - "Bacterial clearances in the California sea hare, Aplysia californica." J. Invert. Pathol., 18, 227-239.

PEMBERTON, R.T. (1970) - "Blood group A reactive substance in the common limpet (Patella vulgata)." Vox Sang., 18, 71-73.

PENNELL, R.B. (1964) - "Composition of normal human red cells." In ' The Red Blood Cell'. eds. Bishop C. & Surgenor, D.M. Academic Press. pp 29-69.

PISTOLE, T.G. (1976) - "Naturally occurring bacterial agglutinin in the serum of the horseshoe crab Limulus polyphemus." J. Invert. Pathol., 28, 153-154.

PROWSE, R.H. & TAIT, N.N. (1969) - "In vitro phagocytosis by amoebocytes from the haemolymph of Helix aspersa (Müller). 1. Evidence for opsonic factor(s) in the serum." Immunol., 17, 437-443.

RABINOVITCH, M. & DE STEFANO, M. (1970) - "Interactions of red cells with phagocytes of the wax-moth (Galleria mellonella) and mouse." Exp. Cell Res., 59, 272-282.

READE, P. & READE, E. (1976) - "Phagocytosis in invertebrates: Studies on the haemocytes of the clam Tridacna maxima." J. Invert. Pathol., 28, 281-290.

RENWARANTZ, L.R. & CHENG, T.C. (1977a) - "Identification of agglutinin receptors on haemocytes of Helix pomatia." J. Invert. Pathol., 29, 88-96.

RENW RANTZ, L.R. & CHENG, T.C. (1977b) - "Agglutinin-mediated attachment of erythrocytes to haemocytes of Helix pomatia." J. Invert. Pathol., 29, 97-100.

RENW RANTZ, L. & UHLENBRUCK, G. (1974) - "Blood group like substances in some marine invertebrates. III Glycoproteins with blood-group A specificity in the cephalopods Sepia officinalis L. and Loligo vulgaris Lam." J. exp. Zool., 188, 65-70.

SCARPINO, P.V. & PRAMER, D. (1962) - "Evaluation of factors affecting the survival of Escherichia coli in sea water. VI Cysteine." Appl. Microbiol., 10, 436-440.

SCHWAB, G.E. & REEVES, P.R. (1966) - "Comparison of the bactericidal activity of different vertebrate sera." J. Bacteriol., 91, 106-112.

SCOTT, M.T. (1971) - "Recognition of foreigness in invertebrates. II In vitro studies of cockroach phagocytic haemocytes." Immunol., 21, 817-828.

SIMON, G. & OPPENHEIMER, C.H. (1968) - "Bacterial changes in sea water samples, due to storage and volume." Z. Allg. Mikrobiol., 8, 209-214.

SMINIA, T. (1972) - "Structure and function of blood and connective tissue cells of the freshwater pulmonate Lymnaea stagnalis studied by electron microscopy and enzyme histochemistry." Z. Zellforsch., 130, 497-526.

SMINIA, T. (1974) - "Haematopoiesis in the freshwater snail Lymnaea stagnalis studied by electron microscopy and autoradiography." Cell Tiss. Res., 150, 443-454.

STANISLAWSKI, E., RENWRANTZ, L. & BECKER, W. (1976) "Soluble blood group reactive substances in the haemolymph of Biomphalaria glabrata (Mollusca)." J. Invert. Pathol., 28, 301-308.

STUART, A.E. (1968) - "The reticulo-endothelial apparatus of the lesser octopus, Eledone cirrosa." J. Path. Bact., 96, 401-412.

TRIPP, M.R. (1960) - "Mechanisms of removal of injected microorganisms from the American oyster Crassostrea virginica (Gmelin)." Biol. Bull. 119, 273-282.

TRIPP, M.R. (1966) - "Haemagglutinin in the blood of the oyster Crassostrea virginica." J. Invert. Pathol., 8, 478-484.

TRIPP, M.R., BISIGNANI, L.A. & KENNY, M.T. (1966) - "Oyster amoebocytes *in vitro*." J. Invert. Pathol., 8, 137-140.

TRIPP, M.R. & KENT, V.E. (1967) - "Studies on oyster cellular immunity." In Vitro, 3, 129-135.

TYSON, C.J. & JENKIN, C.R. (1974) - "Phagocytosis of bacteria *in vitro* by haemocytes from the crayfish (*Parachaeraps bicarinatus*)." Aus. J. exp. Biol. med. Sci., 52, 341-348.

UNKLES, S. (1976) - "Antibacterial defence mechanisms in *Echinus*." M.Sc. thesis. Glasgow University.

VON BRAND, T. & FILES, V.S. (1947) - "Chemical and histological observations on the influence of *Schistosoma mansoni* infection on *Australorbis glabratus*." J. Parasitol., 33, 476-482.

UHLENBRUCK, G., REIFENBERG, U. & PROKOP, O. (1969) - "Isolation of a blood group A substance from *Tubifex rivulorum*." Experientia, 25/11, 1180-1181.

WARFEL, A.H. & ELBERG, S.S. (1970) - "Macrophage membranes viewed through a scanning electron microscope." Science, 170, 446-447.

WEINHEIMER, P.F., ACTON, R.T. & EVANS, E.E. (1969) - "Attempt to induce a bactericidal response in the oyster." J. Bacteriol., 97, 462-463.

WEIR, D.M. (1973) -"Handbook of experimental Immunology." Blackwell Scientific Publications.

WELLS, R.M.G., (1973) - "Carbonic anhydrase activity in *Arenicola marina* (L.)." Comp. Biochem. Physiol., 46A, 325-331.

YOSHINO, T.P. & CHENG, T.C. (1976) - "Experimentally induced elevation of aminopeptidase activity in haemolymph cells of the american oyster Crassostrea virginica." J. Invert. Pathol., 27, 367-370.

YOSHINO, T.P. & CHENG, T.C. (1977) - "Aminopeptidase activity in the haemolymph and body tissues of the pulmonate gastropod Biomphalaria glabrata." J. Invert. Pathol., 30, 76-79.