# STUDIES ON PHAGOCYTOSIS IN THE SHORE CRAB 'CARCINUS MAENAS' (CRUSTACEA, DECAPODA)

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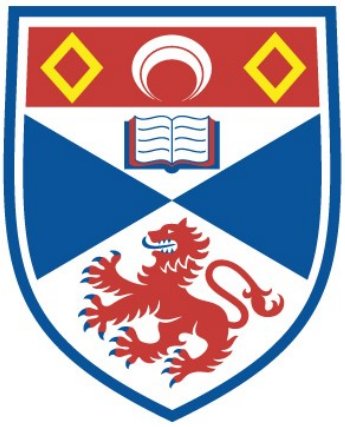

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# Studies on phagocytosis in the shore crab, *Carcinus maenas* (Crustacea, Decapoda)

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Submitted for the Degree of Doctor of Philosophy

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# Dedication

To my Mum and Dad, and brother Mitchell.

# Declaration

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b) I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on October 1990 and as a candidate for the degree of Ph.D. on October 1990.

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# ABSTRACT

Phagocytosis by hyaline cells of the shore crab, *Carcinus maenas*, was investigated *in vitro*. In particular, the project examined the role of the prophenoloxidase activating system (proPO) in opsonisation, the metabolic requirements of the cells during phagocytosis and the extent of intracellular bacterial killing. Related work investigated the mechanism and regulation of bacterial killing. Uptake was assessed using monolayers of separated hyaline cells challenged with the bacterium, *Psychrobacter immobilis*. The bacteria were pretreated with haemocyte lysate supernatant (HLS) to enhance uptake. The opsonic factor(s) were found to be freeze stable and to be generated during serine protease activation of the proPO system. Phagocytosis was also found to depend upon electron transfer and oxidative phosphorylation and to require an intact cytoskeleton for engulfment. Following ingestion, ca 84% of the bacteria were found to be killed within 3 h.

Experiments designed to investigate the mechanism of killing showed that treatment of the hyaline cells with phorbol 12-myristate 13-acetate, lipopolysaccharide, phytohaemagglutinin or concanavalin A, but not laminarin, stimulates the production of superoxide ions ($O_2^-$). The semi-granular and granular cells did not produce $O_2^-$ following stimulation. Incubation of the cells with superoxide dismutase (SOD) confirmed that $O_2^-$ was produced. Parallel experiments were conducted on a range of marine invertebrates. In all cases $O_2^-$ production was observed, showing that $O_2^-$ production is a general phenomenon for invertebrate phagocytes. However, quantification of hydrogen peroxide ($H_2O_2$) production, using a $H_2O_2$ assay, showed that crab phagocytes produced more $H_2O_2$ than tunicate phagocytes indicating that the kinetics of the response varies between species.

Using immunocytochemistry, the antioxidant enzymes, catalase, glutathione peroxidase and SOD were found to be located within the haemocytes and plasma of *C. maenas*. These enzymes may minimise the risk of damage to the host tissues by the $O_2^-$ and $H_2O_2$ produced by the hyaline cells.

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# ABBREVIATIONS

| | |
|---|---|
| AC | Anticoagulant |
| ßGBP | ß-1,3-glucan binding protein |
| BSA | Bovine serum albumin |
| CAC | Citrated cacodylate buffer |
| CAF | Cell adhesion factor |
| Con A | Concanavalin A |
| DMSO | Dimethyl sulfoxide |
| DNP | 2,4-dinitrophenol |
| EDTA | Ethylene-diaminetetra-acetic acid disodium salt |
| EGTA | Ethylene glycol-bis(ß-Aminoethyl ether) N,N,N',N'-etraacetic acid |
| FITC | Fluorescein isothiocyanate |
| GLS | Granular cell lysate supernatant |
| GPX | Glutathione peroxidase |
| GRGDS | Synthetic peptide glycine-arginine-glycine-aspartic acid-serine |
| $H_2O_2$ | Hydrogen peroxide |
| HLS | Haemocyte lysate supernatant |
| HMS | Hexose monophosphate shunt |
| HyLS | Hyaline cell lysate supernatant |
| Ig | Immunoglobulin |
| L-dopa | L-dihydroxyphenylalanine |
| LPS | Lipopolysaccharide |
| MAC | Marine anticoagulant |
| MMS | Modified marine saline |
| MS | Marine saline |
| MSII | Calcium and magnesium free marine saline |

| | |
|---|---|
| NaCl | Sodium chloride |
| $NaN_3$ | Sodium azide |
| $O_2^-$ | Superoxide ion |
| PB | Potassium phosphate monobasic buffer |
| PBS | Phosphate buffered saline |
| PBS-X | Phosphate buffered saline with 0.5% Triton X-100 |
| PHA-P | Phytohaemagglutinin |
| PI | Phagocytic index |
| PMA | Phorbol 12-myristate 13-acetate |
| PO | Phenoloxidase |
| ppA | Prophenoloxidase activating enzyme |
| proPO | Prophenoloxidase |
| PRS | Phenol red solution |
| RGD | Tripeptide arginine-glycine-aspartic acid sequence |
| S.E.M. | Standard error of the mean |
| SgLS | Semi-granular cell lysate supernatant |
| SI | Survival index |
| SOD | Superoxide dismutase |
| STI | Soybean trypsin inhibitor |
| XOD | Xanthine oxidase enzyme solution |

# Chapter 1

# General Introduction

## 1.1. Introduction

Over 90% of extant animal species are invertebrates. Invertebrates are a highly diverse group of organisms that vary greatly in size, morphology and mode of existence, and within this group are the commercially important molluscan, insect and crustacean species. Investigations of the immune responses of these organisms have occurred primarily because of their increasing economic importance in disease, aquaculture and agriculture (see review by Sindermann, 1971). The success of the aquaculture industry is dependent on the production of healthy stock. Hence an understanding of the pathogenicity and control of disease in marine invertebrates is vital. Crustaceans are important not only as a food source but also in the general productivity of the fisheries industry. They can act as vectors of disease; for instance, the crayfish, *Austropotamobius pallipes*, is host to the fungus, *Fusarium solani*, which can infect man and other invertebrates (O'Day *et al.*, 1979; Vey and Vago, 1973). Similarly other invertebrates act as vectors for some of the major parasites affecting humans. The snail *Biomphalaria glabrata* is a vector for the parasite *Schistosoma mansoni*, one of the causative agents of schistosomiasis, while mosquitoes of the genus *Anopheles* are vectors for malaria parasites.

Interest in invertebrate immunity has also developed because of the need to find ethically acceptable hosts for medical and scientific research. The immune responses and factors of invertebrates have also proved to be beneficial to medical research. Endotoxin contamination detection kits were developed from the amoebocytes of the horseshoe crab, *Limulus polyphemus*; and novel compounds with antiviral and antitumour activity have been isolated from tunicates (Rinehart *et al.*, 1983). Recently it has also been proposed that invertebrate cellular immune responses may be useful biomarkers for monitoring sublethal effects of environmental pollution (Chen *et al.*, 1991; Smith and Johnston, 1992).

## 1.2. General Strategies of Invertebrate Immunity

Invertebrate immune strategies are characterized by the lack of T lymphocytes and the absence of specific antibody-antigen interactions that mediate non-self recognition, - a feature of vertebrate immune responses. Instead invertebrate immunocompetence is maintained through inflammatory type responses such as phagocytosis, encapsulation, clotting, antibacterial, antifungal, antiviral and cytotoxic factors (see review by Ratcliffe *et al.*, 1985). Phagocytosis is carried out by both circulating and fixed cells and removes small particles that have gained entry to the body fluids. Phagocytic cells are found in nearly all animal groups (Ratcliffe and Rowley, 1981). The type of response is wide ranging from the non-specific uptake demonstrated by scavenging primitive cells such as the archaeocytes (van de Vyver, 1981) of sponges (Hildemann *et al.*, 1979) to the more specialised opsonin-mediated uptake of tunicate cells (Smith and Peddie, 1992). Larger particles or gross infections elicit encapsulation and nodule formation. Clotting minimises fluid loss and prevents infectious agents spreading through the circulation (see review by Bohn, 1986). As part of these cellular responses, cell degranulation may release antibacterial, antifungal or antiviral factors (see reviews by Smith and Chisholm, 1992; Söderhäll and Cerenius, 1992).

Host defence responses of invertebrates have been traditionally categorised as either cellular or humoral. Although this may be a convenient classification, research has revealed that in many cases the cellular and humoral responses do not act independently from each other. In fact, only a few factors occur solely in the plasma, or act independently of haemocytes and may therefore be regarded as truly humoral (see review by Smith and Chisholm, 1992). Invertebrate immune defences should therefore

be regarded as functionally multifaceted, comprising of an interaction of factors and cells in response to infection.

## 1.3. Historical Appraisal of Invertebrate Immunity

Work on invertebrates has often been hampered by the assumption that the short life-cycles and high reproductive potentials often displayed by these organisms eliminated the need to evolve immune responses to infection. However, in the last 25 years it has been recognised that these assumptions are invalid and intensive investigations into the defence mechanisms of invertebrates has now been undertaken.

Initially, researchers looked for immunoglobulin (Ig)-like factors and activities in invertebrates, or for T-cells and receptors characteristic of the antigen-specific recognition of vertebrate immune responses (Turner, 1992). Over the last 20 years research has shown that invertebrates lack T lymphocytes and do not express vertebrate-type immunoglobulins in their blood systems. In 1989, Klein published an editorial suggesting that researchers of invertebrate immune responses should regard the immune systems of invertebrates as separate from those of vertebrates, and workers should not have preconceived ideas of the types of cells and molecules present in invertebrates. However, recently, following the application of advanced molecular biology techniques, such as gene sequencing some components including molecules of the Ig superfamily and C reactive proteins, have been detected in some invertebrates (Sun *et al.*, 1990). Such work indicates that the molecular if not functional natures of these molecules have been evolutionarly conserved (see reviews by Marchalonis and Schluter, 1990; Cooper *et al.*, 1992).

The use of vertebrate terminology to describe invertebrate cells and responses, even where there is little evidence for true homology with their vertebrate namesakes, has particularly complicated the research (see review by Lackie, 1980). There has been great confusion over cell type nomenclature; a problem augmented by the huge diversity of cell types in invertebrates. As a result the literature has become very confusing with any one cell type having as many as five or six names. It is thus difficult to interpret or extrapolate results between research papers. However, Ratcliffe and Rowley (1979, 1981) suggested a general classification of 5 cell types based on functional roles rather than morphological characteristics which has clarified the situation for invertebrates as a whole. These cells are the progenitor, phagocytic, hemostatic, nutritive and pigmented (non-respiratory and respiratory) cells. Although there are these 5 functional cell types, within a phylum, biochemical and microscopical analysis may indicate more than 5 cell types. However each cell type is likely to be able to be categorised in one of the 5 functional classes.

Until recently, the lack of appropriate experimental techniques for studies of invertebrate immune responses has further hindered investigative progress. Lack of *in vitro* cell lines/culture methods and cell separation techniques hampered research into the development, maturation and interaction of the various cell types, and few, if any, bioactive molecules from invertebrate blood cells had been purified and characterized. Now, suitable culture media buffers and anticoagulants have been devised to obtain optimal conditions for culture or separation of the cells under investigation (Smith and Ratcliffe, 1978; Söderhäll and Smith, 1983; Smith and Peddie, 1992).

## 1.4. Crustacean Immune Strategies

Within the phylum Arthropoda, the class Crustacea comprises animals which are highly diverse in morphology and lifestyle. Examples of crustaceans can be found in terrestrial environments but most are aquatic with the majority marine. Crustaceans are similar to other arthropods in possessing an open circulatory system in which the haemolymph circulates by the pumping of the heart, body movements and muscular or gut contractions (McLaughlin, 1983). The open circulatory system increases the risk of excessive haemolymph loss or widespread infection following injury, but the possession of a hard tanned exoskeleton acts as an excellent first line of defence against potential pathogens. In addition, these animals display distinct defence responses that prevent or limit infection and maintain homeostasis by minimising body fluid loss (see review by Ratcliffe *et al.*, 1985).

In common with other invertebrates, humoral and cellular defences of crustaceans include phagocytosis, encapsulation, blood coagulation, antibacterial and antiviral factors, agglutinins and hydrolytic enzymes. Phagocytosis is considered to be the primary defence response of these animals, and is therefore of great importance in maintaining immunocompetence. This thesis investigates phagocytosis and so a brief overview of this response and nodule formation will be presented in sections 1.6. and 1.7., respectively.

## 1.5. Cell Types

Three distinct classes of haemocytes have been identified in the haemolymph of crustaceans. These are the hyaline, the semi-granular and the granulocyte cells (see review by Bauchau, 1981). The hyaline cells are the smallest of the three cell types (ca

7 μm in diameter), have a large nucleus, few granules, readily form pseudopodia and are actively phagocytic (Smith and Ratcliffe, 1978; Smith and Söderhäll, 1983a; Tsing *et al.*, 1989; see also review by Bauchau, 1981). The granulocytes are ca 10-15 μm in diameter with a kidney shaped nucleus and numerous large cytoplasmic granules (Smith and Ratcliffe, 1978; Smith and Söderhäll, 1983a; Tsing *et al.*, 1989; see also review by Bauchau, 1981). The semi-granular cells are intermediate in size and are also highly labile (Johansson and Söderhäll, 1985; see also review by Bauchau, 1981). Few semi-granular cells are found in the shore crab, *Carcinus maenas*, but crayfish may have a much larger population of this cell type (Johansson and Söderhäll, 1985). In crayfish the semi-granular cells may be phagocytic and are known to contain granules which are discharged following non-self stimulation (Smith and Söderhäll, 1983a; Söderhäll *et al.*, 1986). The three different classes of haemocyte may be separated by continuous density gradient centrifugation (Söderhäll and Smith, 1983; Smith and Söderhäll, 1983b). Cell morphology is not the only difference between the haemocyte types. Söderhäll *et al.* (1986) have shown that *in vitro* there are also differences in the functional role and biochemical composition of crustacean cells. These differences will be discussed in the relevant sections. In this thesis, the granulocytes will be referred to as granular cells as this is the term most commonly used in the literature.

Equivalent cells, such as the phagocytes and granule-containing cells can be found in other phyla (see reviews by Ratcliffe and Rowley, 1979, 1981). Briefly, some of the other phyla will be mentioned for future reference for the remainder of the introduction and Chapter 5. In polychaetes, there are 3 cell types of which the amoebocytes are involved in phagocytosis (see review by Dales and Dixon, 1981). With molluscs there is debate as to the number of cell types. For gastropod molluscs the controversy is whether there are two cell types from separate cell lines, or, whether there is one type with two morphological forms. Functionally the types are dissimilar; as one is highly

phagocytic while the other exhibits only low rates of uptake (see review by Sminia, 1981). In bivalve molluscs there are thought to be three cell types (see review by Cheng, 1981). In contrast, insects are thought to have 6 basic cell types (Price and Ratcliffe, 1974; Ratcliffe and Rowley, 1979) and the plasmatocyte type is considered to be the phagocyte (Wittig, 1965; Ratcliffe and Rowley, 1975). There are thought to be 6 basic cell types in echinoderms and uptake is exhibited by the phagocytic amoebocytes (see review by Smith, 1981). For the urochordates, 9 cell types have been classified into 6 categories and in *C. intestinalis* uptake is carried out by phagocytic amoebocytes (Smith and Peddie, 1992; see also review by Wright, 1981).

## 1.6. Phagocytosis

The involvement of phagocytosis in host defence has been widely recognised since the early studies by Metchnikoff (1884).

In *C. maenas*, phagocytosis is a non-specific response mediated by the hyaline cells which ingest both Gram-negative and Gram-positive bacteria *in vitro* (Smith and Ratcliffe, 1978). Phagocytosis can be divided into the following four stages in order of occurrence - chemotaxis, attachment, ingestion and killing. Chemotaxis and attachment require recognition which can either be specific or non-specific.

### 1.6.1. Chemotaxis

For crustaceans reports of chemotaxis tend to be inferred from its role in phagocytosis, nodule formation, encapsulation and cell aggregation responses (see review by Ratcliffe *et al.*, 1985). Despite the importance of chemotaxis as the first step in haemocyte/foreign body interaction, few studies have been made of the

mechanism of this response in crustaceans or of the factors and cells which mediate and regulate chemotaxis.

Studies of chemotaxis of other invertebrate groups have indicated that low molecular weight factors are involved in this response. In the snail, *Viviparus malleatus*, (Schmid, 1975) and the oyster, *Crassostrea virginica*, (Howland and Cheng, 1982), for example, the haemocytes respond chemotactically to a 10-kDa protein found in bacterial cell walls. However, the chemotactic migration of snail haemocytes occurs only in the presence of snail haemolymph (Schmid, 1975), and in *C. virginica* chemotactic attraction of the haemocytes of *C. virginica* to *Bacillus megaterium* is diminished following prior exposure of the cells to this bacteria (Cheng *et al.*, 1981). Marks *et al.* (1979) showed that the bacteria, *Staphylococcus epidermidis* and *Aeromonas hydrophila*, exert concentration-dependent chemotactic attraction on the coelomocytes of the earthworm, *Lumbricus terrestris, in vitro*. The coelomocytes are also attracted to the body wall tissues of two other xenogenic species (an earthworm and an arthropod), although coelomocyte attraction to these foreign tissues declines with decreasing phylogenetic relatedness to *L. terrestris*. This study by Marks *et al.* (1979) was the first to demonstrate the presence of a migration inhibitor in the host tissue that presumably acts to regulate the response. Ottaviani *et al.* (1990) have demonstrated that the neuroactive molecules adrenocorticotropic hormone (ACTH) and ß-endorphin are chemotactic to the phagocytes of the snail, *Planorbarius corneus*. In addition, ACTH and ß-endorphin-like molecules have been detected in the cells and serum of *P. corneus* (Ottaviani *et al.*, 1991). During a study of phagocytosis in tunicates, Kelly *et al.* (1993b) demonstrated that an opsonin purified from the plasma of the solitary urochordate, *Styela clava*, acts as a powerful chemoattractant for *S. clava* haemocytes. The cells migrate towards the opsonin *in vitro* and this directional movement is lost when equal concentrations of the opsonin are present in the chemotaxis chambers.

Whether similar chemotactic responses are shown by crustacean cells is unknown but such information is essential if the fundamental basis of the interaction of cells with non-self and its regulation are to be fully understood.

### 1.6.2. Recognition

Recognition can be either specific or non-specific and is mediated either directly by membrane bound molecules, the nature of which remains equivocal for invertebrates, or indirectly through soluble factors known as opsonins. Opsonins adhere to foreign material and enhance its recognition by phagocytic cells. Opsonic factors may be freely circulating in the blood, as is the case with the lectin opsonin of the mussel *M. edulis* (Renwrantz and Stahmer, 1983). The levels of the opsonin in the blood may also be enhanced following challenge by foreign material, which results in the release of recognition factors from the cells, as occurs in crustaceans (Söderhäll *et al.*, 1986). However, the term opsonin has been widely used in the literature with reference to cases of increased uptake by invertebrate cells, but a lack of biochemical purification of the factor(s) means that few molecules can be regarded as true opsonins.

The nature of Ig-independent recognition in invertebrates has not been fully resolved but several hypothesis have been put forward. In arthropods, it has been proposed that non-self recognition is mediated through the prophenoloxidase (proPO) activating system (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979; Söderhäll, 1982; Leonard *et al.*, 1985b; Söderhäll and Smith, 1986 a,b) (see section 1.8. for an overview of this system). In the crayfish, *Pacifastacus leniusculus*, release of the proPO system by exocytosis from the semi-granular cells is mediated specifically by LPS or the ß-1,3 glucan, laminarin G, *in vitro* (Johansson and Söderhäll, 1985). Similar degranulation does not occur with the granular cells of the crayfish in response to LPS or ß-1,3-glucan,

*in vitro* (Johansson and Söderhäll, 1985), although in the crab, *C. maenas*, the granular cells exocytose in response to LPS *in vitro* (Söderhäll *et al.*, 1986) to release the proPO system. In both crayfish and crab the inactive components of the proPO system are then activated specifically by LPS or ß-1,3-glucans (Smith and Söderhäll, 1983a; Söderhäll and Häll, 1984; Söderhäll *et al.*, 1986). Activation of the proPO system generates several bioactive factors (see review by Söderhäll and Smith, 1986b) including five "sticky" proteins (Söderhäll *et al.*, 1984). These "sticky" proteins are believed to coat the foreign material and enhance the phagocytic uptake by the phagocytes (Söderhäll *et al.*, 1986). Because the "sticky" proteins are donated by a different population (the semi-granular and granular cells) to the phagocytes (at least in crab) it has been proposed that these steps constitute a cellular communication pathway (Söderhäll *et al.*, 1986). The "sticky" proteins in the haemocyte lysate supernatant (HLS) are stronger non-self signals than the bacteria themselves. Smith and Söderhäll (1983b) found that in the freshwater crayfish, *Astacus astacus*, only the semi-granular cells degranulated when challenged by bacteria incubated in crayfish saline, but bacteria that had been incubated in activated HLS, caused degranulation of both the semi-granular and granular cells as well as lysis of the semi-granular cells *in vitro*. Active phenoloxidase (PO), itself, does not appear to have opsonic properties since heat activated crab HLS does not enhance uptake (Söderhäll *et al.*, 1986). Heat treatment of the HLS induces a conformational change in prophenoloxidase producing phenoloxidase without activation of the proPO system. In addition, to the "sticky" proteins, the monomeric 76 kDa glycoprotein known as the cell adhesion factor (CAF) is also produced during the degranulation of the semi-granular and granular cells of *P. leniusculus*, *in vitro* (Johansson and Söderhäll, 1988). Cell adhesion activity is only present when PO is specifically activated by LPS or ß-1,3-glucans in the presence of calcium ions (Johansson and Söderhäll, 1988). Once the CAF is activated, it attaches to non-self surfaces and is, therefore, theoretically capable of mediating haemocyte adhesion to, and

encapsulation of, foreign particles (Kobayashi *et al.*, 1990). It is possible that this molecule could function as an opsonin when the cascade is activated. However, as yet, confirmation for the opsonic role of CAF for the phagocytes has not been published so further research is required before the exact nature of the opsonic factor(s) in this system are elucidated.

An alternative theory, popular among invertebrate immunologists, is that lectins serve as recognition molecules. Lectins are carbohydrate binding molecules which have been proposed to act as opsonins in invertebrates by attaching to membrane glycoproteins, glycolipids and polysaccharides (Renwrantz and Stahmer, 1983; Sminia *et al.*, 1979). It was initially thought that in some crustacean species haemagglutinins may be functionally analogous to the antibodies found in vertebrates as they enhance adhesion and phagocytosis of erythrocytes (McKay *et al.*, 1969). However, support for this hypothesis has not been forthcoming. Agglutinins have been identified in a whole range of crustaceans (Cornick and Stewart, 1973; Ravindranath *et al.*, 1985; Cassels *et al.*, 1986; Vasta *et al.*, 1983). Although some of these molecules have been characterized, their functional roles within the host have yet to be established. In particular for crustaceans, functional studies need to be carried out to determine whether agglutinins play a part in mediating non-self recognition by the haemocytes.

Much of the work that has been carried out to determinine the functional role of agglutinins has involved non-crustacean species. For example, Coombe *et al.* (1984) have shown that the purified HA-2 agglutinin from the colonial tunicate *Botrylloides leachii* functions as an opsonin for sheep erythrocytes when coated with the appropriate carbohydrate moities. Incubation of the erythrocytes with lactose abolishes the enhancement of uptake (Coombe *et al.*, 1984), suggesting that lactose blocks the receptors involved in opsonisation. Agglutinins have also been identified in the solitary

tunicates, *Styela plicata* and *Halocynthia hilgendorfi*. However, the agglutinin from *S. plicata* does not exhibit any opsonic activity (Fuke and Sugai, 1972). A purified agglutinin from the snail, *Helix pomatia*, was shown to be strongly opsonic for the clearance of non-self particles from the circulation (Harm and Renwrantz, 1980). However, in only two species of mollusc, the oyster, *Crassostrea gigas*, and the mussel, *Mytilus edulis*, have the agglutinins been purified and shown unequivocally to act as opsonins (Hardy *et al.*, 1977; Renwrantz and Stahmer, 1983). Recently an opsonin has been purified from the solitary urochordate *Styela clava* (Kelly *et al.*, 1993a). It is thought to share some functional similarities to C-type lectins in that it is $Ca^{2+}$ dependent and its activity is inhibited by specific carbohydrates, particularly those with a galactose configuration (Kelly *et al.*, 1993a). However, the presence of a lectin is not always an indication that it is involved in phagocytosis. In the clam, *Mercenaria mercenaria*, lectins mediate the agglutination of erythrocytes, yeast and bacteria but do not actually enhance phagocytosis (Tripp, 1992b). Another unknown recognition pathway, therefore, must be involved. Lectin activity has also been detected in the sea urchin, *Strongylocentrotus droebachiensis* (Bertheussen, 1983). However, this lectin does not have any opsonic effect (Bertheussen, 1983). Similarly, in the lobster, *Homarus americanus*, Goldenberg and Greenberg (1983) have shown that the agglutinating and opsonic activities of the haemolymph are distinct. Recently multiple lectins have been isolated and characterized from the haemolymph of the cockroach, *Blaberus discoidalis* (Chen *et al.*, 1993). As with other invertebrates the functional role of lectins in this insect still needs to be determined. In general, the role of lectins and agglutinins as recognition molecules has not been demonstrated for all invertebrates and much debate still surrounds this area of research.

There is also evidence for receptors on some invertebrate cells which bind directly to foreign material in the absence of humoral components (Bayne *et al.*, 1979; Sminia *et*

*al.*, 1979; van der Knaap *et al.*, 1983; Goldenberg *et al.*, 1984). Renwrantz and Stahmer (1983) demonstrated that the recognition factors on the haemocyte surface of the mussel *M. edulis* are divalent cation dependent and that both humoral and cell-bound agglutinin molecules are involved in phagocytosis. Possible cellular receptors for components of mammalian complement have been identified on snail and sea urchin phagocytes and evidence has been presented for the role in enhancement of phagocytic uptake (Laulan *et al.*, 1988; Bertheussen and Seljelid, 1982, respectively). Laulan *et al.* (1988) demonstrated that coating particles with IgG and C3b significantly enhances uptake by the blood cells of *Lumbricus terrestris*. Uptake of particles coated with IgM and C3d, on the other hand, was not enhanced, indicating that the appropriate receptors for IgG and C3b but not IgM and C3d may be present on the cell surface (Laulan *et al.*, 1988). Similarly, in the sea urchin, *S. droebachiensis*, the phagocytes have been shown to have surface receptors for C3bi and C3b components of mammalian complement as erythrocytes coated in these factors had greatly enhanced uptake compared to red blood cells coated in other complement fractions (Bertheussen and Seljelid, 1982). It would be interesting to identify the type of cell surface receptors present on crustacean phagocytes as this would provide more information on the interaction of non-self material with the cells.

### 1.6.3. Attachment / Adhesion

The attraction of haemocytes to non-self material is followed by attachment which may be either receptor mediated or promoted by humoral or cell derived factors. In crayfish, degranulation of the semi-granular and granular cells produces the 76 kDa CAF (Johansson and Söderhäll, 1988). Cell adhesion activity only

occurs when PO is activated by LPS or ß-1,3-glucans (Johansson and Söderhäll, 1988), although the actual mechanism of activation is unknown. Johansson and Söderhäll (1988) have proposed that the CAF is of vital significance in host defence because, once activated, it attaches to non-self surfaces and is, therefore, capable of mediating haemocyte adhesion to, and encapsulation of, foreign particles. In vertebrates, recent work has shown that many of the cell adhesion proteins such as fibronectin, collagens, and fibrinogen have a tripeptide arginine-glycine-aspartic acid (RGD) sequence at their cell recognition site and that this sequence is vital for mediating cell adhesion (Ruoslahti and Pierschbacher, 1987). Experiments using the synthetic peptide glycine-arginine-glycine-aspartic acid-serine (GRGDS) have shown that GRGDS imitates the biological activities, *in vitro*, of the CAF (Johansson and Söderhäll, 1989) i.e. attachment, degranulation and spreading of granular cells. This work is the first indication that receptors that recognise RGD may be present on the surface of invertebrate haemocytes. The subsequent sequencing of the amino acids of CAF is required to confirm this hypothesis. Further work may confirm the proposal by Johansson and Söderhäll (1989) that parasites of arthropod vectors adhere to the host cells in a similar manner to that observed in vertebrate cell/pathogen interaction with the attachment mediated through a RGD sequence.

### 1.6.4. Ingestion and Phagocytic Uptake

Phagocytosis is classically considered to be the primary cellular defence mechanism but experiments have shown variation in the rate of uptake between cells of different phyla. Crustaceans tend to have low uptake rates and values of 2-30% have been reported (see Table 1.1). These figures for phagocytosis probably represent the minimum values as *in vitro* systems are often suboptimal (Ratcliffe *et al.*, 1985). For

Table 1.1.

Table 1.1. Summary of phagocytic uptake levels *in vitro* from a range of crustacean species

Unless otherwise stated experiments used the monolayer assay and uptake levels are the percentage number of cells ingesting 1 or more particles.

Superscript a = Sheep red blood cells

b = opsonised

c = unopsonised

d = Number of red blood cells / 1000 haemocytes

| Species | Cell Type | Test particle | Opsonisation conditions | Assay conditions | Uptake level | Reference |
|---|---|---|---|---|---|---|
| *Homarus americanus* | phagocytic haemocytes | SRBC[a] | lobster serum 1 h at 20°C | 1 h at 15°C | Serum incubated SRBC ca 2 % Untreated ca 1% | Paterson and Stewart (1974) |
| | hemocytes | *Aerococcus viridans* var. *homari* | Test bacteria washed lobster hemolymph medium Animals subjected to 4 weekly injections with either artificial seawater *Pseudomonas perolens* endotoxin or *A. viridans* var. *homari* (formalin killed) | 1 h at 15°C | ASW ca 3.6% *P. perolens* endotoxin ca 11.4% *A. viridans* var *homari* ca 7.5% | Paterson *et al.*, (1976) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 h at 21°C | 10°C pH 7.6<br>75 min<br>Cells activated<br>on glass<br>coverslips for<br>6 h. | | op[b] | unop[c] | Goldenberg *et al.*, (1984) |
| | | | | | Activated<br>cells | ca 17.2% | 1.8% | |
| | | | | | Unactivated<br>cells | ca 5.3% | 0.6% | |
| *Astacus astacus* | Haemocytes | *Moraxella* sp. | Bacteria suspended in 0.1% laminarin, glucose, dextran, cellulose, chitin or crayfish saline (CFS) | moist chamber<br>2 h at 20°C<br>rocking platform | CFS<br>laminarin<br>glucose<br>dextran<br>cellulose<br>chitin | ca 5.6%<br>ca 18%<br>ca 5%<br>ca 5.6%<br>ca 8.7%<br>ca 5.2% | | Smith and Söderhäll (1983a) |
| *Parachaeraps bicarinatus* | phagocytic haemocytes | Erythrocyte | Serum 30 min at 20°C | 20°C 1.5 h | unopsonised<br>opsonised | ca 50[d]<br>ca 275[d] | | McKay and Jenkin (1970) |
| *Carcinus maenas* | Amoeboid phagocytes | *Moraxella* sp.<br>*Bacillus cereus*<br>*Gaffkya homari* | Serum 1 h at 15°C rotating drum or untreated (untreat.) | Rocking platform15°C<br>3 h constant environment cabinet | | untreat. | Serum | Smith and Ratcliffe (1978) |
| | | | | | *Moraxella* sp. | ca 15% | 7% | |
| | | | | | *B. cereus* | ca 5.3% | 5.5% | |
| | | | | | *G. homari* | ca 3.6% | 2.9% | |
| | Hyaline cells | *Moraxella* sp | Glucan or heat activated HLS or *Carcinus* saline (CS) 2 h 20°C | 20°C 1 h | Glucan HLS<br>Heat HLS<br>CS | ca 19.4%<br>ca 5.8%<br>ca 7.9% | | Söderhäll *et al.*, (1986) |

Table 1.2.

Table 1.2. Summary of phagocytic uptake levels *in vitro* from a range of non-crustacean marine species.

Unless otherwise stated experiments used the monolayer assay and uptake levels are the percentage number of cells ingesting 1 or more particles.
Superscript a = opsonised
b = unopsonised
c = Sheep red blood cells
d = Millipore filtered seawater
e = Room Temperature
f = Mean number of SRBC ingested per phagocyte

| Species | Cell Type | Test particle | Opsonisation conditions | Assay conditions | Uptake level | Reference |
|---|---|---|---|---|---|---|
| Annelida | | | | | | |
| *Arenicola marina* | coelomocyte | *Moraxella* sp.<br>*Bacillus cereus*<br>*B. megaterium*<br>*Alcaligenes aquamarinus* | serum 1 h at 15°C on a rotating drum | 15°C on platform rocker | After 2 h op.[a] unop.[b]<br>*Moraxella* ca 2.5% 3.3%<br>*B. cereus* ca 4.1% 5.1%<br>*B. megaterium* ca 2.9% 2.4%<br>*A. aquamarinus* ca 3.9% 4.2% | Fitzgerald and Ratcliffe (1982) |
| Mollusca | | | | | | |
| *Lymnaea stagnalis* | amoebocyte | SRBC[c] | 30 min incubation in Type I or II serum | 21±1°C 30 min Humidity chamber | Ringer ca 10%<br>Type I ca 80%<br>Type II ca 35% | van der Knaap *et al.*, (1983) |
| *Biomphalaria glabrata* | M-line haemocytes | yeast | Incubation in 13-16-R1 plasma | 20 min | unop ca 40%<br>op ca 86% | Fryer *et al.*, (1989) |
| *Mytilus californianus* | haemocytes | | Monolayer overlaid | 60 min 15°C | | Bayne *et al.*, (1979) |
| | | RBC | RBC adsorbed plasma | | 26.7% | |
| | | RBC | haemolymph | | 26.8% | |
| | | RBC | MFSW[d] | | 17.3% | |
| | | yeast | yeast adsorbed plasma | | 87.5% | |
| | | yeast | haemolymph | | 74.7% | |
| | | yeast | MFSW | | 84.8% | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Mercenaria mercenaria* | Large granular haemocytes | *S. aureus*<br>*E. coli*<br>yeast<br>polystyrene spheres | Particles suspended in artificial seawater (ASW) or clam serum unless type stated | Moist chamber 30 min at R.T[e]. | ASW / Serum<br>*S. aureus* 71% / 74%<br>*E. coli* 99% / 97%<br>yeast ca 90% / ca 90%<br>spheres ca 65% / ca 75%<br>human ca 70%<br>calf ca 55% | Tripp (1992a) |
| *Mytilus edulis* | haemocytes | yeast | Serum or Tris buffer | | Buffer ca 5%<br>2.5% serum ca 10%<br>10% serum ca 25%<br>50% serum ca 65% | Renwrantz and Stahmer (1983) |
| Merostomata | | | | | | |
| *Limulus polyphemus* | blood cells | carbonyl iron | | | majority of cells contained only 1 particle | Armstrong and Levin (1979) |
| Echinodermata | | | | | | |
| *Strongylocentrotus nudus* | phagocyte | fixed sheep, human RBC | Coelomic fluid 1 h at 4°C | 30 min at 12°C | unopsonised 8%<br>opsonised 10% | Ito *et al.*, (1992) |
| *S. droebachiensis* | phagocytes | SRBC | human serum 30 min at 37°C<br>mouse serum 20 min at 37°C<br>fish serum 30 min at 10°C | 15 min | unopsonised 0.3[f]<br>human serum 4.3[f]<br>mouse serum 4.0[f]<br>fish serum 4.4[f] | Bertheussen and Seljelid (1982) |
| Urochordata | | | | | | |
| *Ciona intestinalis* | vacuolar and granular amoebocytes | *Psychrobacter immobilis* | Either Buffer, cell lysate supernatant (CLS) or LPS treated | 2 h at 20°C | Buffer control ca 5%<br>CLS ca 20%<br>LPS treated CLS ca 43% | Smith and Peddie (1992) |

example in monolayer experiments cells are attached to glass surfaces, incubation temperature may affect cellular metabolism and the buffers in which the cells are prepared may be nutrient deficient. However, the potential of phagocytosis as a defence mechanism is great when the large number of circulating cells are considered (i.e. ca 25 x $10^6$ cells $ml^{-1}$ in crab) (Smith and Söderhäll 1983a). A large proportion of these are phagocytes and that individual phagocytes may ingest more than one particle (Smith and Söderhäll, 1983a).

For each phylum, numerous studies have quantified phagocytic uptake both *in vitro* and *in vivo*. It is difficult to make comparisons of the uptake values obtained because of the number of variables that apply to each set of experiments. The nomenclature of the cell types causes confusion as to the actual cells involved and, within the same phyla, different cell types may be considered to be phagocytic. A vast range of particles, both biotic and abiotic have been used as test particles and the way these particles have been treated prior to challenge with the cells undoubtedly affects results (see Tables 1.1 and 1.2). Particles may have been incubated with, for example, HLS, commercially available sugars, plasma or even different salines. The incubation time involved and the temperature at which the opsonisation is carried out also influences subsequent uptake rates. Furthermore incubation temperature, length of contact time and particle:cell ratio may also influence the phagocytic activity of the cells. Tables 1.1 and 1.2 give an indication of the variety of experimental conditions used in a range of studies. Phagocytic uptake levels are summarised in Tables 1.1 and 1.2. Several reviews have dealt with phagocytosis in invertebrates as a whole (see reviews by Ratcliffe, 1985; Ratcliffe *et al.*, 1985; Bayne, 1990).

Pretreatment of Gram-positive or Gram-negative bacteria with serum from *C. maenas* produces no enhancement of phagocytosis; indeed there may even be a slight

decrease in the uptake rates of the Gram-negative bacterium of *Moraxella* sp. (Smith and Ratcliffe, 1978). Serum is the fluid remaining when blood is allowed to clot and contains intracellular factors that have been denatured during the clotting process. Investigations have shown that in crab and crayfish, HLS containing an active prophenoloxidase (proPO) system has opsonic properties (Smith and Söderhäll 1983a; Söderhäll *et al.*, 1986). This opsonisation not only increases the phagocytic uptake by three or four times but it also significantly increases the number of particles taken up per cell (Smith and Söderhäll, 1983a).

Ingestion requires energy but little research has been carried out on the energy requirements of phagocytosis in invertebrates. Anderson *et al.* (1973) demonstrated that the glycolytic pathway provides energy for phagocytosis by the haemocytes of the insect *Blaberus craniifer*. However the hexose monophosphate shunt is not stimulated during phagocytosis (Anderson *et al.*, 1973). Cheng (1976) discovered that the glycolytic pathway is the energy source for phagocytosis by the clam, *M. mercenaria*, and that uptake does not result in an increase in oxygen consumption. The haemocytes of the oyster, *C. virginica,* are also able to phagocytose under completely anaerobic conditions (Alvarez *et al.*, 1989). The energy source for phagocytosis by crustacean cells is unknown.

### 1.6.5. Intracellular Killing

An important requirement of the non-specific inflammatory response is the destruction of foreign particles following ingestion by the phagocyte. In vertebrates, oxygen radicals generated during the respiratory burst are known to mediate intracellular killing (Krinsky, 1974; Johnston *et al.*, 1975). The respiratory burst has also been demonstrated to occur in molluscan (Dikkeboom *et al.*, 1986a,b; 1988;

Pipe, 1992) and echinoderm phagocytes (Ito *et al.*, 1992). However, this metabolic process has not yet been shown for crustacean phagocytes. It is also unknown whether there is a link between oxygen radical generation and intracellular killing following phagocytosis by invertebrate haemocytes.

Recently there has been speculation as to the role of oxygen radical production during encapsulation responses (Nappi and Vass, 1993). Phenoloxidase oxidises phenol to quinones which, via dopamine metabolism, can form melanin (see review by Nappi and Vass, 1993). Nappi and Vass (1993) have proposed that dopamine metabolism, during the formation of cellular melanotic capsules, produces $H_2O_2$ and OH radicals which may be cytotoxic against the enclosed parasite. However, research is required to determine if oxygen radicals participate in the killing of parasites within capsules.

## 1.7. Nodule and Encapsulation Type Responses

Nodule formation occurs when the invasive foreign particles are too numerous to be effectively sequestered by the circulating phagocytes. However, it has been suggested that if the foreign matter is greater than 10 μm in diameter it is too large to be ingested by a single phagocyte and immobilization by encapsulation occurs (see review by Lackie, 1980). Nodules are loosely packed clumps of cells which surround the foreign material, whereas capsules consist of multiple layers of flattened haemocytes that form a sheath-like structure around the foreign body core. In both cases the subsequent necrosis of the invasive material is accompanied by melanisation (Unestam, 1975; Smith and Ratcliffe, 1980b). There are many reports of nodule and capsule formation in crustaceans but because of the problems of dissecting nodules and capsules from these

animals many details of these responses have not been elucidated. In contrast, with insects the small body size allows easier access to the nodules and capsules and has allowed many of the details of these responses to be determined. Hence where it is relevant, or in order to clarify a point, reference will be made to insect studies.

In arthropods the formation of nodules or capsules is initiated by either random contact between the non-self material and the haemocytes (Ratcliffe *et al.*, 1985), or by the coating of the foreign matter with the exocytosed contents of the semi-granular or granular cells (Ratner and Vinson, 1983; Kobayashi *et al.*, 1990). In crustaceans degranulation of the haemocytes is triggered by the presence of the foreign material and the adhesion of the bacteria to the surface of the haemocytes may be enhanced by these degranulated factors (Smith and Ratcliffe, 1980 a,b). In *C. maenas*, nodules formed *in vitro* have been shown to consist of both phagocytic and granular cells (Smith and Ratcliffe, 1980b). In contrast Persson *et al.* (1987) showed, *in vitro*, that only the semi-granular cells of the crayfish, *Astacus leptodactylus*, encapsulate foreign material. Since this response is either inhibited or delayed if the cells are incubated with known inhibitors of cellular degranulation, the response appears to be enhanced by the degranulation of the cells (Persson *et al.*, 1987). For insects it appears to be much clearer as to which cell types are involved in each stage of the response, from studies using the larvae of the wax moth *Galleria mellonella* (Schmit and Ratcliffe, 1977). These authors demonstrated that encapsulation occurs in two stages involving two cell types (Schmit and Ratcliffe, 1977). Initial recognition of foreignness is mediated by the granular cells which exocytose on the surface of the implant (Schmit and Ratcliffe, 1977). The capsule is then formed predominantly by the phagocytic plasmatocytes (Schmit and Ratcliffe, 1977). Similarly in the insect *Clitumnus extradentatus*, a localised clot forms around the foreign material within 5 min and in the following 24-72 h the multilayered sheath of flattened cells forms (Schmit and Ratcliffe, 1978). Again

the capsule is formed in two stages (Schmit and Ratcliffe, 1978). In crustaceans, Söderhäll *et al.* (1984) have demonstrated *in vivo* that the haemocytes of the crayfish *A. astacus* exhibit a stronger encapsulation response to fungal spores coated with HLS rather than spores coated with plasma, which indicates that there are stimulatory factor(s) present in the haemocytes. They suggested that these factor(s) may be generated when the proPO system is activated (Söderhäll *et al.*, 1984). Kobayashi *et al.* (1990) demonstrated that the semi-granular cells of the crayfish *P. leniusculus* are capable of encapsulating glass beads precoated with proPO activated HLS *in vitro*. These authors showed that the *in vitro* encapsulation response was enhanced by purified 76 kDa CAF, and that PO exhibited no encapsulation promoting activity of its own (Kobayashi *et al.*, 1990). In contrast, studies on the encapsulation response of the locust *Schistocerca gregaria* (Dularay and Lackie, 1985) indicated that the proPO system was not involved. In their study Dularay and Lackie (1985) showed that negatively-charged Sepharose beads coated with at least 5 proteins from an activated proPO system were not encapsulated *in vivo* by the haemocytes. Either the stimulatory factors did not attach to the beads or, alternatively, were not present in the assay (Dularay and Lackie, 1985).

The effectiveness of the encapsulation response may not be a function simply of recognition of non-self by the cells. The nature of the foreign material such as wettability and charge may affect its adhesion to the cell (Lackie, 1983). Differences in the rate of clearance of foreign particles *in vivo* has also been related to the site of injection (Smith and Ratcliffe, 1980a). Investigations involving *C. maenas* have shown that carmine particles injected into the pericardial sinus accumulate primarily in the heart, but when the dye was injected into a cheliped it is sequestered to the gills (Smith and Ratcliffe, 1980a). Furthermore, genetic differences between individual *Drosophila melanogaster*, have been shown to influence the encapsulation response (Carton and

Boulétreau, 1985), as have variations in the number of circulating haemocytes from investigations on two insect species (Lackie, 1979).

In *C. maenas*, nodules accumulate mainly in the gills but also occur in the hepatopancreas and heart (Smith and Ratcliffe, 1980a,b; White and Ratcliffe, 1982). The accumulation of large numbers of nodules in the gills could potentially impair the respiratory capability of the animal but for the presence of nephrocytes which remove degraded bacterial or cell debris from the gills (Smith and Ratcliffe, 1981). White *et al.* (1985) demonstrated that bacterial killing of *Moraxella* sp. and *Bacillus cereus* occurs *in vivo* only following the sequestration of bacteria in nodules to the gills. *In vitro* studies show that antibacterial activity is not mediated by humoral factors, and White *et al.* (1985) suggest that bacterial death following encapsulation is cell mediated.

## 1.8. The Prophenoloxidase (proPO) Activating System

The proPO system is a complex cascade of serine proteases and other factors that are sequentially activated by proteolytic cleavage to generate several bioactive molecules (see review by Söderhäll and Smith, 1986b). The system terminates in the conversion of stable inactive prophenoloxidase to active phenoloxidase (PO) which is involved in melanization. Activation of the system is very specific. The ß-1,3-glucans of fungal cell walls, LPS or peptidoglycans all specifically activate the proPO system in arthropods (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979; Söderhäll , 1982; Leonard *et al.*, 1985b). With regard to crustaceans the system is generally contained within the semi-granular and granular cells (Söderhäll and Smith, 1983; Smith and Söderhäll, 1991). The semi-granular cells of *C. maenas* also contain proPO but these cells are low in number and highly labile so it is very difficult to isolate them for

use in *in vitro* assays (Söderhäll and Smith, 1983). The proPO system seems to be released from the haemocytes by exocytosis (Johansson and Söderhäll, 1985). In the crayfish, *P. leniusculus*, release of the proPO system by exocytosis from the semi-granular cells is mediated specifically by LPS or the ß-1,3-glucan laminarin G, *in vitro* (Johansson and Söderhäll, 1985). The granular cells of the crayfish do not degranulate in response to LPS and ß-1,3-glucan, *in vitro* (Johansson and Söderhäll, 1985). In *C. maenas*, the granular cells exocytose in response to LPS *in vitro* (Söderhäll *et al.*, 1986) thereby releasing the proPO system. The inactive components of the proPO system can then be activated specifically by LPS or ß-1,-3-glucans *in vitro* (Smith and Söderhäll, 1983a; Söderhäll and Häll, 1984; Söderhäll *et al.*, 1986). The degranulation is mediated by LPS or ß-1,3-glucans which then activates the released system (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979; Söderhäll, 1982). A ß-1,3-glucan-binding protein (ßGBP) has been purified from the plasma of crayfish and shown to mediate spreading and degranulation of crayfish granular haemocytes when bound to laminarin (Barracco *et al.*, 1991). The ßGBP is believed to be associated with the proPO system within the cells (Barracco *et al.*, 1991). Cellular degranulation results in an even greater release of the cell-bound recognition factors that then trigger the degranulation of adjacent haemocytes (Söderhäll *et al.*, 1986). This amplification of the activation of the proPO system from the cells, although beneficial in enhancing the immune defence responses could also be detrimental if it is not regulated *in vivo* as it may result in extensive internal cellular degranulation and clotting (Söderhäll *et al.*, 1986).

Calcium is very important throughout the proPO system as a regulator of biological activity (Söderhäll, 1981; Leonard *et al.*, 1985a). Activation of PO is inhibited in the absence of calcium *in vitro* (Smith and Söderhäll, 1983a; Ashida and Söderhäll, 1984; Leonard *et al.*, 1985a). High concentrations of calcium

(greater than 50 mM) reduce PO activity possibly by stabilizing the enzyme against proteolytic cleavage (Ashida and Söderhäll, 1984; Smith *et al.*, 1984). In addition to the regulation of the proPO system by calcium, there are also enzymes involved in regulating the system. Phenoloxidase seems to be activated *in vivo* by a native protease (Söderhäll, 1983). The serine protease which has been purified from crayfish haemocytes has a molecular weight of 36 kDa (Aspán *et al.*, 1990b). It activates proPO and hence the enzyme was named the prophenoloxidase activating enzyme (ppA) (Aspán and Söderhäll, 1991). The activity of ppA is known to be shortlived and peaks earlier than the PO activity (Söderhäll, 1983).

Proteolytic inhibitors have also been found in the haemocytes and cuticle of the crayfish, *A. astacus* (Häll and Söderhäll, 1982, 1983) and $\alpha_2$-macroglobulin-like activity has been found in the haemolymph of the crayfish, *P. leniusculus* (Hergenhahn and Söderhäll, 1985; Hergenhahn *et al.*, 1988). It is possible that the $\alpha_2$-macroglobulin-like activity in crustaceans is analogous to the activity of $\alpha_2$-macroglobulin in mammals and "cages" the proteases in the circulation. In this way these molecules may modulate activation of the proPO system which would otherwise be detrimental to the host (Hergenhahn *et al.*, 1988). Three other proteinase inhibitors have been purified from crayfish haemocytes of which two (the 155kDa trypsin inhibitor and the $\alpha_2$-macroglobulin-like 190kDa molecule) are believed to be involved in the regulation of proPO activation (Aspán *et al.*, 1990a) by inhibiting (to a greater or lesser extent) ppA. Apart from inhibitors, activation of the system also generates five "sticky" proteins (Söderhäll *et al.*, 1984) and the 76kDa CAF (Johansson and Söderhäll, 1988) that have been proposed to be involved in non-self recognition, opsonisation and encapsulation responses, as discussed above.

Recent work has suggested that the prophenoloxidase (proPO) activating system is involved in recognition in arthropods and may participate in the cellular and humoral immune responses (Söderhäll, 1982; Ashida *et al.*, 1982; Smith and Söderhäll, 1983a,b; Leonard *et al.*, 1985b). Activation of the system is known to enhance phagocytosis (Smith and Söderhäll, 1983a; Leonard *et al.*, 1985b; Söderhäll *et al.*, 1986), initiate capsule or nodule formation (Söderhäll *et al.*, 1984; Smith *et al.*, 1984; Persson *et al.*, 1987), mediate plasma coagulation (Söderhäll, 1981) and produce fungistatic compounds (Söderhäll and Ajaxon, 1982). Moreover, the proPO activating system has been implicated in mediating the killing of a Gram-negative bacteria *Moraxella* sp., *in vitro*, in crabs (Söderhäll and Smith, 1986a). A study of *C. maenas* haemocytes by Chisholm and Smith (1992) has shown that although the antibacterial activity against Gram-negative and Gram-positive bacteria resides in the cells that contain the proPO system, PO, itself, is not involved. Possibly the antibacterial activity is mediated by some of the peptides that are generated following activation of the system (Chisholm and Smith, 1992). However, the evidence for the claims that the system is involved in cellular responses has been largely circumstantial, based on experiments using activated HLS. To substantiate these hypotheses that the proPO system is involved will require purification and characterization of proPO factors. Experiments then need to be carried out to demonstrate unequivocally the biological action of the factor *in vitro* and even more desirably *in vivo*.

## 1.9. Objectives

The preceeding sections describe the various cellular defence strategies of crustaceans and highlights that phagocytosis is important in the defence against foreign material. However, many aspects of this immune strategy are still poorly understood. In part we need to know the recognition mechanisms involved, the factors which regulate the response, the metabolic requirements and the fate of ingested material. It was decided, therefore, to investigate, *in vitro*, phagocytosis in crustaceans using the common shore crab *Carcinus maenas* as the experimental animal.

The aims of the study are:

1. To determine the role of the activation of the proPO system in opsonisation and the metabolic and cytoskeletal requirements of the cells during uptake, *in vitro* and,
2. To determine if intracellular killing occurs, identify possible mechanisms of intracellular killing and any regulatory mechanisms that are involved, *in vitro*.

# Chapter 2

## Parameters influencing phagocytosis by hyaline cells of *Carcinus maenas in vitro*

# 2.1. INTRODUCTION

Since 1884 when Metchnikoff first described phagocytosis in daphnids, there has been much research into this host defence response (See Chapter 1). However, little of the work has actually been carried out on crustacean haemocytes. Identification of the cell type involved and the types of particle which the crustacean cells can "recognise" and ingest have been established as have rates of uptake of unopsonised/opsonised particles, both *in vivo* and *in vitro* (Söderhäll *et al.*, 1986; see review by Ratcliffe *et al.*, 1985).

Studies of *C. maenas* have established that Gram-positive and Gram-negative bacteria are phagocytosed by approximately 5% and 15%, respectively of the phagocytic cells *in vitro* (Smith and Ratcliffe, 1978). From experiments using mixed cell monolayers it was shown that incubation of *Moraxella* sp. with laminarin significantly increased the proportion of phagocytosing cells and the number of bacteria ingested per cell (Smith and Söderhäll, 1983a). In addition, the level of uptake was found to increase with time and be dose-related (Smith and Söderhäll, 1983a). Smith and Söderhäll (1983a) proposed that laminarin triggers the activation of the proPO system, generating opsonic factors which enhance phagocytic uptake. In 1986 further work was carried out on the role of the proPO system in opsonisation by Söderhäll *et al.* These authors proposed that opsonisation occurs by the following process. Lipopolysaccharides or ß-1,3-glucans trigger the degranulation of the granular cells which results in the release of the proPO system. This is then activated by LPS or ß-1,3-glucans (Johansson and Söderhäll, 1985) to generate factor(s) that act as opsonins by coating the non-self material and stimulate phagocytic uptake by the hyaline cells. The hyaline cells do not contain the proPO system (Söderhäll and Smith, 1983) so Söderhäll *et al.* (1986) suggested that these results provide evidence of cellular co-operation in the defence reactions of crustaceans.

However there are many other parameters that influence phagoctyosis that have not been studied in crustaceans. In this chapter some of these parameters will be determined. It still needs to be investigated whether: -

1) the opsonic factor(s) in HLS are freeze stable, as this provides some information on the chemical nature of the factor(s);

2) the opsonic factor(s) in HLS are generated by serine protease activity. Serine proteases have been implicated in the activation of the proPO system (Söderhäll, 1981, 1983);

3) active cellular metabolism is necessary for phagocytosis. Incubating the cells with the metabolic inhibitors sodium azide ($NaN_3$) or 2,4-dinitrophenol (DNP) should determine whether electron transfer and oxidative phosphorylation (Wainio, 1970) are metabolic requirements for phagocytosis;

4) an intact cytoskeleton is required for phagocytosis by incubating the cells with cytochalasin B which disrupts actin polymerisation (Flanagan and Lin, 1980),

and 5) the divalent cations $Ca^{2+}$ and $Mg^{2+}$ are required during phagocytosis by using ethylene-diaminetetra-acetic acid disodium salt (EDTA) which chelates $Ca^{2+}$ and $Mg^{2+}$ and ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) which chelates $Ca^{2+}$.

# 2.2. MATERIALS AND METHODS

## 2.2.1. Animals

Specimens of *Carcinus maenas* were collected from St. Andrews Bay, Scotland. All the animals were maintained in tanks in an aquarium with a flow through system (32 ± 2‰; 10 ± 2°C) for two weeks prior to use and fed twice weekly on minced fish. Only healthy adult intermoult males (7 - 10 cm carapace width) were used in experiments.

## 2.2.2. Buffers

To minimise endotoxin contamination which may activate haemocyte degranulation (Söderhäll *et al.*, 1986), buffers were prepared using glassware which had been rendered pyrogen free by dry heating in an oven for 1 h at 160°C and all solutions were 0.2 μm filtered prior to use.

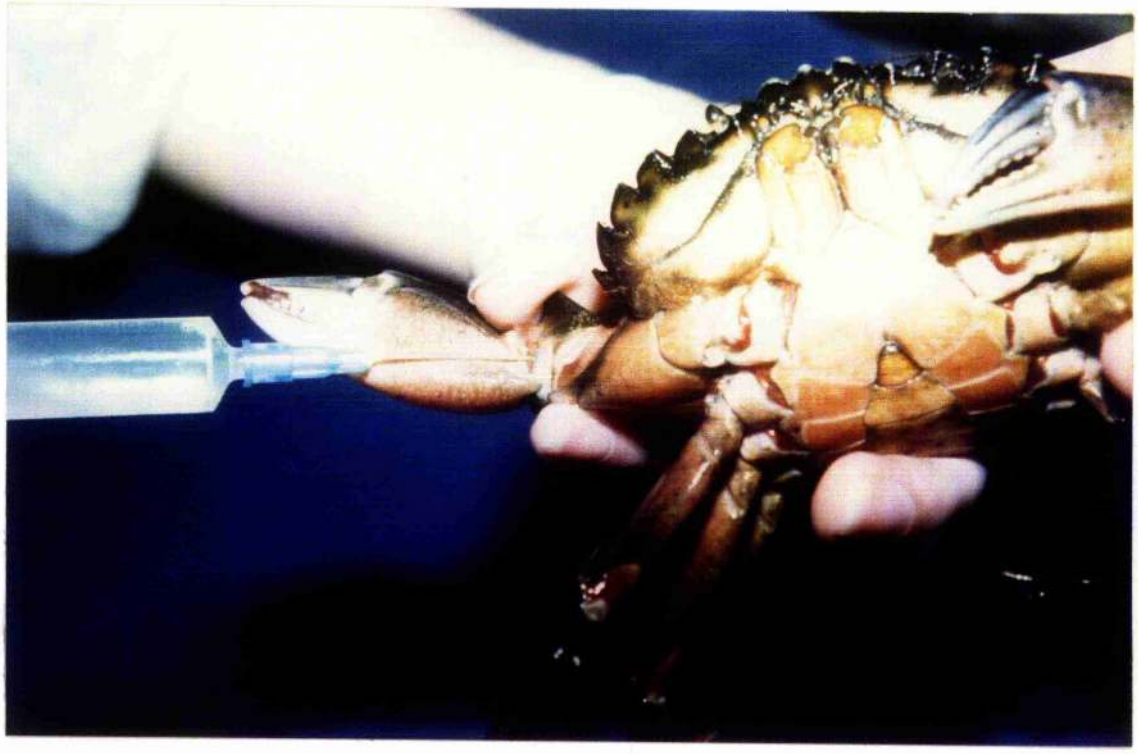
## 2.2.3. Harvesting of the Cells

Haemolymph removal was carried out as described by Smith and Ratcliffe (1978). In every case the area to be sampled was surface sterilized with 96% ethanol and care was taken to avoid pyrogen contamination of the haemolymph. Haemolymph samples were withdrawn from the unsclerotized membrane between the chela and carpus of the chelipeds (Smith and Ratcliffe, 1978) into a sterile syringe and diluted 1:1 with anticoagulant (AC) (0.45 M sodium chloride, 0.1 M glucose, 0.03 M trisodium citrate, 0.026 M citric acid, 0.01 M EDTA, pH 4.6; Söderhäll and Smith, 1983) (Figure 2.1). Each animal was subjected to a single bleed.

Figure 2.1.

Figure 2.1. Harvesting of haemocytes from the shore crab *Carcinus maenas* via the unsclerotized part of a cheliped.

Haemolymph (ca 2 ml) was withdrawn into a sterile syringe containing 2 ml of AC as described in section 2.2.3.



## 2.2.4. Preparation of Haemocyte Lysate Supernatant (HLS)

Diluted haemolymph samples (ca 20 ml) pooled from 5 crabs for each HLS preparation were centrifuged at 1,900 g for 10 min at 4°C. The haemocyte pellet was then washed once in citrated cacodylate buffer (CAC) (0.45 M sodium chloride, 0.1 M trisodium citrate, 0.01 sodium cacodylate. pH 7.0) (Smith and Söderhäll, 1991) before being centrifuged again at 1,900 g for 10 min at 4°C. The resulting cell pellet was homogenized in 2 ml of 0.2 μm filtered marine saline (MS) (0.5 M sodium chloride, 0.045 M tris(hydroxymethyl) methylamine, 0.026 M magnesium chloride hexahydrate, 0.012 M calcium chloride hexahydrate, 0.011 M potassium chloride, 0.1 M hydrochloric acid. pH 7.4) for 10 min using an ice-cold, pyrogen free, glass piston homogenizer. The homogenate was then finally spun in polycarbonate tubes at 44,100 g for 22 min (2 min run-up time) at 4°C. The supernatant, designated HLS, was made up to 5 ml with MS.

## 2.2.5. Protein Determination

The protein concentration of the HLS was determined according to the method of Bradford (1976) using bovine serum albumin as the standard. For all HLS preparations the protein concentrations were adjusted to give approximately 1 mg $ml^{-1}$.

## 2.2.6. Phenoloxidase (PO) Assay

Samples of HLS were checked for phenoloxidase activity spectrophotometrically at 490 nm using a modification of the method of Söderhäll and Smith (1983). Briefly, 200 µl of HLS were incubated with 200 µl of the elicitor, trypsin (1 mg $ml^{-1}$) (Sigma, Dorset, UK), for 30 min at 20°C before the addition of 200 µl of L-dihydroxyphenylalanine (L-dopa; Sigma) (3 mg $ml^{-1}$) as the substrate. For the controls, MS was substituted for trypsin. Following a 10 min incubation period, a further 600 µl of MS were added to the reaction mixture and the absorbance read at 490 nm on a 4053 kinetics spectrophotometer (LKB, Uppsala, Sweden). Enzyme activities are expressed as the change in absorbance at 490 nm $min^{-1}$ $mg^{-1}$ protein.

## 2.2.7. Separation of the Phagocytes

To separate the cells, diluted haemolymph (ca 4 ml) was loaded immediately onto chilled preformed 60% Percoll (Pharmacia, Uppsala, Sweden) continuous density gradients in 3.2% sodium chloride (NaCl) (pH 7.0) and spun at 2,000 g for 10 min at 4°C (Söderhäll and Smith, 1983). The cells were harvested and diluted with 3.2% NaCl to approximately 1 - 9 x $10^7$ cells $ml^{-1}$, as counted on an Improved Neubauer haemocytometer. The different cell types in each band were identified according to the criteria given in Bauchau (1981). For the monolayer experiments, only phagocytic hyaline cells were used.

## 2.2.8. Preparation of Hyaline Cell Monolayers

Glass coverslips were cleaned by boiling in Decon 90 then rinsed in distilled water. The coverslips were then rendered pyrogen-free by dry heating in an oven for 1 h at 160°C. When cool, the coverslips were placed in 6-well covered multiwell plates (Sterilin, Feltham, UK), and 200 µl of cells pipetted onto each one. The cells were then left to attach for 30 min at room temperature. To remove non-adherent cells, the coverslips were gently washed twice with MS.

## 2.2.9. Bacteria

The Gram-negative marine bacterium *Psychrobacter immobilis* formerly *Moraxella sp.* (NCIMB 308) was used as the challenge particle for the *in vitro* phagocytosis assays. *P. immobilis* was cultured in Bacto Marine Broth 2216 (Difco Labs, Michigan, USA) on an orbital mixer for 24 h at 20°C. A sample (ca 12 ml) of the bacteria was heat killed by boiling for 10 min then centrifuged at 1,900 g for 10 min, washed twice in 3.2% NaCl and finally resuspended in MS. The bacterial turbidity was adjusted to give an absorbance of 0.5 at 570 nm. This gives a bacterial concentration of ca 1 - 2 x $10^7$ $ml^{-1}$.

## 2.2.10. Opsonisation of Bacteria

Prior to the addition of the bacterial suspension, 1 ml of HLS was incubated in a sterile bijou with an equal volume of laminarin (a ß-1,3-glucan from *Laminaria digitata,* Calbiochem, La Jolla CA, USA) (1 mg $ml^{-1}$) for 15 min to activate the proPO system. The unopsonised controls contained an equivalent volume of MS (2 ml) and no HLS. Two millilitres of the bacterial suspension (ca 1 - 2 x $10^7$ $ml^{-1}$)

were then added to each bijou. The bijoux were incubated for 2 h at room temperature and shaken every 30 min. The bacteria were then washed 3 times with 3.2% NaCl at 1,900 g and finally resuspended in 1 ml of MS to give a bacterial concentration of 2 - 4 x $10^7$ bacteria $ml^{-1}$.

### 2.2.11. Phagocytosis Assay

Each monolayer was overlaid with 100 µl of unopsonised or opsonised bacteria and the monolayers were incubated in a humid chamber on a rocking platform at room temperature. After 1 h the monolayers were washed 3 times with MS to remove the non-phagocytosed bacteria. The cells were then fixed in 10% seawater/formalin before examination under phase contrast optics of a Leitz Diaplan microscope (Wetzlar, Germany).

#### 2.2.11.1. Freeze Stability of Opsonic Factors in HLS

To identify whether the opsonic properties of the HLS are conserved following freezing, samples of HLS were stored at -18°C and -70°C for a period of time. Ten millilitres of HLS were prepared as above using 10 crabs. One millilitre of the fresh HLS was used immediately for opsonisation as above. The remainder was divided out and stored at either -18°C or -70°C. After a period of a week, a month or three months, samples from both temperatures were thawed at ambient temperature, used to opsonise the bacteria and phagocytosis assays carried out. Protein levels and PO activity of each sample which was either fresh or had been frozen were also determined.

## 2.2.11.2. Inhibition Experiments

To determine the effect of potential inhibitors of opsonisation, cellular metabolism or cytoskeleton formation on phagocytic uptake the following experiments were carried out.

a) ProPO system inhibitors

The serine protease inhibitors, soybean trypsin inhibitor (STI, Sigma) and benzamidine (Sigma), were made up in MS and used at the concentrations 5 mM and 100 mM, respectively. These inhibitors were used to determine whether the opsonic factors in the HLS are activated through serine protease activity. For the experimental bijoux, 1.4 ml of HLS were incubated with 1 ml of inhibitor for 15 min prior to the addition of 1 ml of laminarin for a further 15 min incubation. The positive control was the same as the experimental except 1 ml of MS replaced the 1 ml of inhibitor. For negative controls, the HLS, inhibitor and laminarin were replaced with 3.4 ml of MS. Before the addition of 3 ml of bacterial suspension to each bijou, 0.4 ml of the reaction mixtures were removed and assayed for PO activity. The bijoux were incubated for 2 h at room temperature and shaken every 30 min. The bacteria were then centrifuged at 1,900 g for 10 min at 4°C washed 3 times in 3.2% NaCl and finally resuspended in 1 ml of MS to give a final concentration of 3 - 6 x $10^7$ bacteria $ml^{-1}$, prior to use in a phagocytosis assay.

b) Metabolic inhibitors

Three monolayers of hyaline cells, per crab, were covered with 100 µl of sodium azide ($NaN_3$), an inhibitor of electron transfer, (0.001, 0.01 or 0.1 M dissolved in MS) for 15 min. Two other monolayers were covered with 100 µl of MS to act as

controls. After 15 min, the monolayers were washed twice with MS. One of the control monolayers was then overlaid with unopsonised and the other with opsonised bacteria. The $NaN_3$ incubated cells were challenged with opsonised bacteria. The remainder of the phagocytosis assay was carried out as above. The same method was utilised for 2,4-dinitrophenol (DNP; Sigma) which affects oxidative phosphorylation. The DNP was dissolved in ethanol to $10^{-1}$ M then made up in MS to a range of concentrations $10^{-4}$ - $10^{-2}$ M.

c) Cytoskeletal inhibitor

Cytochalasin B (Sigma) an inhibitor of actin polymerisation was resuspended in dimethyl sulfoxide (DMSO) (1 mg $ml^{-1}$) then made up in MS, prior to use, to give a range of concentrations from 0.1 µg - 10 µg $ml^{-1}$. It was then incubated with cell monolayers using the same method as for $NaN_3$.

Cell viability was checked after incubation with each inhibitor using a fluorescein vital stain by overlaying the cells with 100 µl of carboxyfluorescein diacetate (3 µg $ml^{-1}$ in MS). In all cases over 98% viability was maintained. Final concentrations of ethanol and DMSO used to resuspend DNP and cytochalasin B, respectively, did not on their own affect phagocytosis.

## 2.2.11.3. Divalent Cation Requirements for Phagocytic Uptake

Stock solutions (0.1 M) of either EDTA (chelates $Ca^{2+}$ and $Mg^{2+}$) or EGTA (chelates $Ca^{2+}$) were made up in calcium and magnesium free MS (MSII). Before each experiment, the EDTA and EGTA were diluted to $10^{-3}$, $10^{-4}$ or $10^{-5}$ M with MSII. To each cell monolayer was added 100 µl of each concentration of either EDTA or EGTA and incubated for 10 min. The monolayers were then washed twice

in MSII before incubation, as above, with opsonised bacteria which had been resuspended in 1 ml of MSII. Control monolayers were incubated with MS or MSII in place of EDTA or EGTA then incubated with opsonised or unopsonised bacteria.

### 2.2.12. Quantification of Phagocytosis

Phagocytosis was quantified by scoring the number of cells containing one or more intracellular bacteria. Intracellular bacteria were distinguished from extracellular according to the criteria given in Smith and Ratcliffe (1978) and a minimum of 200 cells were counted per treatment per animal. For the experiments to determine if the opsonic factor(s) are freeze stable, a minimum of 300 cells were counted per treatment per animal. The phagocytic index (PI) was calculated as below.

$$PI = \frac{\text{Number of cells containing intracellular bacteria}}{\text{Number of cells counted}} \times 100$$

### 2.2.13. Statistical Analysis

Differences in the levels of bacterial uptake by the cells subjected to different treatments were analysed statistically by 1 or 2 way analysis of variance. Differences were considered to be significant when $p \leq 0.05$ (Sokal and Rohlf, 1981).

## 2.3. RESULTS

### 2.3.1. Freeze Stability of Opsonic Factors in HLS

Table 2.1 is a summary of the effect of freezing the HLS on its opsonic properties for various time intervals at -18°C (A) and -70°C (B) on phagocytosis. Opsonisation of the bacteria in freshly prepared HLS resulted in an increase in the level of uptake from 3.11 to 10.52% compared to the unopsonised (MS-treated bacteria). This 2-3 fold increase in the uptake of opsonised bacteria was also observed when the HLS had been frozen at either -18°C or -70°C for 1 week, 1 month or 3 months. For both temperatures at each time interval there was a significant increase in the level of phagocytosis of HLS incubated compared to MS-treated bacteria ($p \leq 0.001$, $n \geq 7$). Freezing the HLS at either -18°C or -70°C had no significant effect on its opsonic properties after 1 week ($p = 0.696$), 1 month ($p = 0.969$) or 3 months ($p = 0.684$). There was no significant effect of freezing the samples on the level of PO activity ($p \geq 0.233$ in all cases, $n \geq 7$) when compared to freshly prepared HLS (Table 2.1 C). The phagocytic uptake was greatest for both sets of samples at 3 months. This may be a reflection in the timing of these experiments as the phagocytosis assays for the 3 month readings were carried out during the summer months. The PO activity reading was higher for the -18°C sample after 1 month but the reason for this is not known.

### 2.3.2. Effect of Serine Protease Inhibitors on the Activation of Opsonic Factors in HLS

Treatment of bacteria with HLS significantly increased phagocytic uptake from 3.56 to 8.71% ($p \leq 0.001$, $n = 5$) (Fig. 2.2, STI). Pretreatment of the HLS with the serine protease inhibitor, STI, significantly reduced uptake to a level comparable with bacteria treated with MS ($p = 0.174$, $n = 5$) (Fig. 2.2). A similar effect was observed

Table 2.1. A & B

Table 2.1. A & B. Summary of mean PI of bacteria incubated with HLS stored at -18°C (A) and -70°C (B) and PO levels of activity in HLS (C).

A & B phagocytic uptake levels.

a - Phagocytic Index as explained in Materials and Methods (Section 2.2.12.).

b - cells challenged with bacteria incubated with HLS.

c - cells challenged with bacteria treated with MS.

d - number of animals.

e - values are the mean PI ± standard error of the mean (S.E.M.).

*** Difference in the uptake levels of HLS-incubated bacteria and MS-treated bacteria is highly significant at $p \leq 0.001$.

A

| Storage Time of HLS | Mean PI[a] (exp)[b] | Mean PI (con)[c] | n[d] |
|---|---|---|---|
| Fresh | 10.52 ± 0.57[e***] | 3.11 ± 0.44 | 7 |
| 1 Week | 10.99 ± 1.69*** | 4.40 ± 0.96 | 9 |
| 1 Month | 11.15 ± 1.03*** | 3.43 ± 0.58 | 7 |
| 3 Months | 13.13 ± 1.24*** | 8.05 + 1.55 | 10 |

B

| Storage Time of HLS | Mean PI[a] (exp)[b] | Mean PI (con)[c] | n[d] |
|---|---|---|---|
| Fresh | 10.52 ± 0.57[e***] | 3.11 ± 0.44 | 7 |
| 1 Week | 9.89 ± 1.71*** | 4.41 ± 0.94 | 9 |
| 1 Month | 10.48 ± 2.06*** | 4.00 ± 1.14 | 7 |
| 3 Months | 15.33 ± 1.19*** | 6.83 ± 1.15 | 10 |

Table 2.1. C

Table 2.1. C. Phenoloxidase levels of activity in HLS.

a - Phenoloxidase activity expressed as the change in absorbance at 490 nm per min per mg protein.

b - number of different HLS samples.

c - values are the means ± S.E.M.

C

| Storage conditions of HLS | | PO Activity[a] | n[b] |
|---|---|---|---|
| Fresh | | 0.51 ± 0.17[c] | 3 |
| 1 Week | -18°C | 0.49 ± 0.23 | 5 |
| | -70°C | 0.31 ± 0.13 | 5 |
| 1 Month | -18°C | 0.65 ± 0.26 | 4 |
| | -70°C | 0.29 ± 0.11 | 5 |
| 3 Months | -18°C | 0.41 ± 0.05 | 4 |
| | -70°C | 0.30 ± 0.09 | 4 |

Figure 2.2. and Table 2.2.

Figure 2.2. Effect of incubating bacteria with HLS treated with the inhibitors STI (5 mM) or benzamidine (100 mM) on phagocytic uptake by the hyaline cells of *C. maenas*.

Monolayers of hyaline cells were challenged for 1 h with bacteria that had been incubated in MS (negative control) or HLS treated with either inhibitor (experimental) or laminarin (positive control) before the cells were fixed in 10% seawater/formalin. Two hundred cells were counted per monolayer per treatment per animal and the PI calculated. Values are the mean PI ± S.E.M. n = 5 animals.

a - Experimental. HLS incubated for 15 min with inhibitor then for 15 min with laminarin before bacteria were added.

b - Negative control. MS for 30 min before bacteria were added.

c - Positive control. HLS incubated for 15 min with MS, then for 15 min with laminarin before bacteria were added.

** Difference between the negative and positive control is significant at $p \leq 0.01$.

*** Difference between the negative and positive control is significant at $p \leq 0.001$.

There is no significant difference in the uptake of bacteria incubated in HLS treated with inhibitor (experimental) or with bacteria treated with MS (negative control).

Table 2.2. Phenoloxidase levels of activity in HLS.

a - Experimental. HLS incubated for 15 min with inhibitor then for 15 min with laminarin before bacteria were added.

c - Positive control. HLS incubated for 15 min with MS, then for 15 min with laminarin before bacteria were added.

d - Phenoloxidase activity expressed as the change in absorbance at 490 nm per min per mg protein.

e = Values are the mean PO activity ± S.E.M. for 5 different HLS samples.

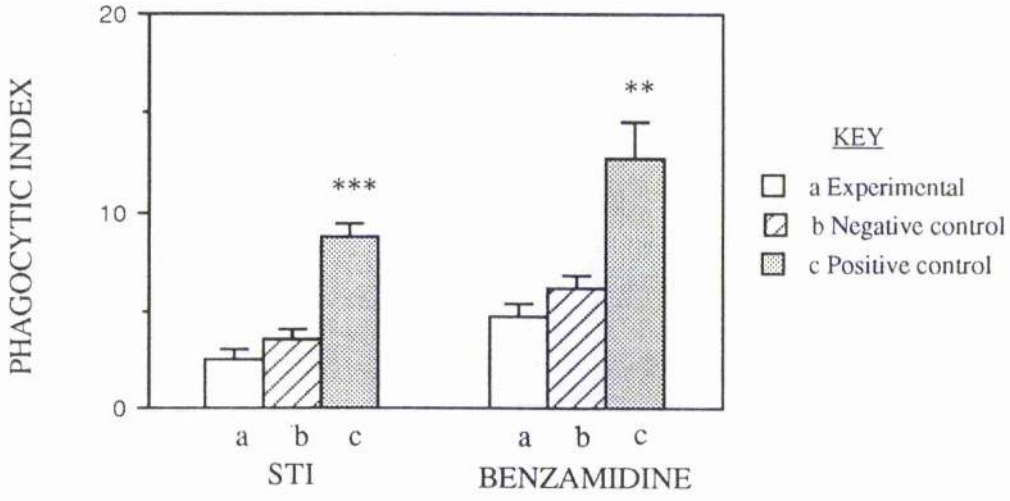



Table 2.2.

| Inhibitor | PO Activity[d] | |
|---|---|---|
| | Experimental[a] | Positive control[c] |
| STI (5 mM) | 0.29 ± 0.1 | 0.45 ± 0.13 |
| Benzamidine (100 mM) | 0.29 ± 0.05 | 0.40 ± 0.06 |

with benzamidine where opsonisation of the bacteria doubled the uptake from 6.09 to 12.71% ($p = 0.009$, $n = 5$) but pretreatment of the HLS with benzamidine decreased the uptake from 12.71 to 4.73% (Fig. 2.2). There was no significant difference in the uptake levels of bacteria incubated with HLS that had been pretreated with benzamidine and the MS-treated bacteria ($p = 0.146$, $n = 5$) (Fig. 2.2). Incubation of HLS with STI or benzamidine reduced PO activity by about a third but the difference compared to the untreated HLS was not significant ($p = 0.394$, $n = 5$, STI; $p = 0.226$, $n = 6$, benzamidine) (Table 2.2).

### 2.3.3. Effect of Inhibitors of Cellular Metabolism or Cytoskeleton Formation on Phagocytic Uptake

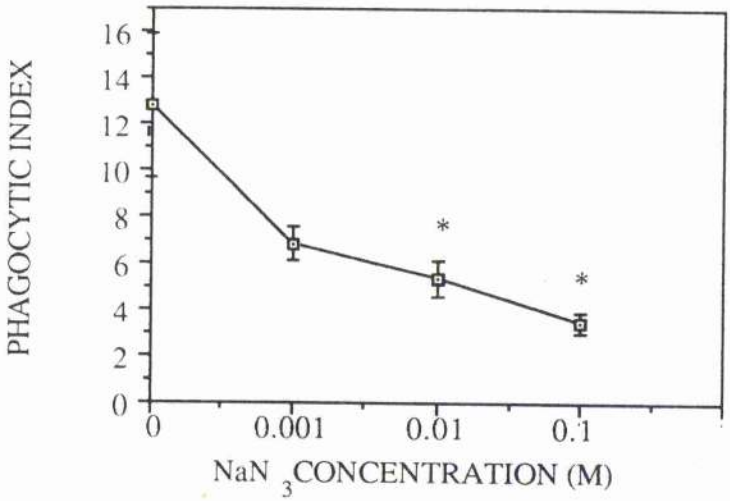
Monolayers of cells were incubated with inhibitors of cellular energy metabolism to investigate the energy pathways that are required for phagocytosis in crustaceans. Incubation of the hyaline cells with $NaN_3$, an inhibitor of electron transfer, produced a reduction in the phagocytic uptake of opsonised bacteria (Fig. 2.3). Approximately 13% of the cells ingested HLS-incubated bacteria but this declined to 6.8% when the cells had been preincubated for 15 min with 0.001 M $NaN_3$ (Fig. 2.3). Increasing the concentration of $NaN_3$ to 0.01 or 0.1 M further reduced phagocytic uptake to 5.35 and 3.47%, respectively (Fig. 2.3). Uptake of bacteria treated with MS was 4.9%. Using 1-way analysis of variance it was found that the difference between the uptake of MS-treated and HLS-incubated bacteria by cells that were not incubated with $NaN_3$ was highly significant ($p = 0.036$, $n = 6$) (Fig. 2.3). Treatment of the cells with $NaN_3$ significantly reduced the uptake of HLS-incubated bacteria for 0.01 and 0.1 M $NaN_3$ ($p = 0.045$ and $p = 0.015$, respectively, $n = 6$) compared to the untreated cells.

Figure 2.3.

Figure 2.3. Effect of various concentrations of $NaN_3$ on phagocytic uptake by the hyaline cells of *C. maenas*.

Monolayers were incubated with 100 µl of $NaN_3$ (0.001, 0.01 or 0.1 M in MS) for 15 min. The cells were then challenged with opsonised bacteria for 1 h before fixation in 10% seawater/formalin. Two hundred cells were counted per monolayer treatment per animal and the PI calculated. Values are the mean PI values ± S.E.M. n = 6 animals.

* Difference between the phagocytic uptake levels of untreated and treated cells is significant at $p \leq 0.05$.


PHAGOCYTIC INDEX
16
14
12
10
8
6
4
2
0
0
0.001
0.01
0.1
*
*
NaN 3 CONCENTRATION (M)

Dinitrophenol, an uncoupler of oxidative phosphorylation, also inhibited phagocytosis (Fig. 2.4). Uptake of HLS-incubated bacteria compared to MS treated bacteria was about double (PI = 8.23 and 4.16%, respectively) ($p = 0.012$, $n = 6$). Following treatment of the cells with DNP, the uptake of HLS-incubated bacteria declined (Fig. 2.4) and uptake was significantly lower than uptake by untreated cells challenged with HLS-incubated bacteria ($p \leq 0.026$ in all cases, $n = 6$). At $10^{-4}$ M DNP, the PI was 4.83% whereas at $10^{-3}$ M, the PI was 4.44% and at the highest concentration of DNP ($10^{-2}$ M), the PI was 4.65%. There was no significant difference in the level of uptake at the three concentrations of DNP ($p \geq 0.844$, $n = 6$).

Figure 2.5 shows the effect of incubating cytochalasin B, an known inhibitor of actin polymerisation, on phagocytic uptake by hyaline cells. Incubation of the bacteria with HLS increased uptake by untreated cells from 2.8 to 6.7% ($p \leq 0.001$, $n = 4$). When the cells were incubated with cytochalasin B, the uptake was reduced from 6.7 to 3.36% at 0.1 $\mu g\ ml^{-1}$, to 2.73% at 1 $\mu g\ ml^{-1}$ and to 2.32% at 10 $\mu g\ ml^{-1}$. This reduction in uptake compared to the untreated cells was highly significant ($p = 0.018$, 0.1 $\mu g\ ml^{-1}$; $p = 0.03$, 1 $\mu g\ ml^{-1}$; $p \leq 0.001$, 10 $\mu g\ ml^{-1}$, $n = 4$).

### 2.3.4. Effect of Divalent Ions on Phagocytosis

Incubation of the cells with either EDTA or EGTA at all of the concentrations tested resulted in the detachment of the cells from the coverslips. The effect of EDTA or EGTA on phagocytosis could, therefore, not be determined as so few cells remained on the coverslips.

Figure 2.4.

Figure 2.4. Effect of various concentrations of DNP on phagocytic uptake by the hyaline cells of *C. maenas*.

Monolayers were incubated with 100 µl of DNP (0.0001, 0.001 or 0.01 M) for 15 min. The cells were then challenged with opsonised bacteria for 1 h before fixation in 10% seawater/formalin. Two hundred cells were counted per monolayer per treatment per animal and the PI calculated. Values are the mean PI values ± S.E.M. n = 6 animals.

* Difference between the phagocytic uptake levels of untreated and treated cells is significant at $p \leq 0.05$.

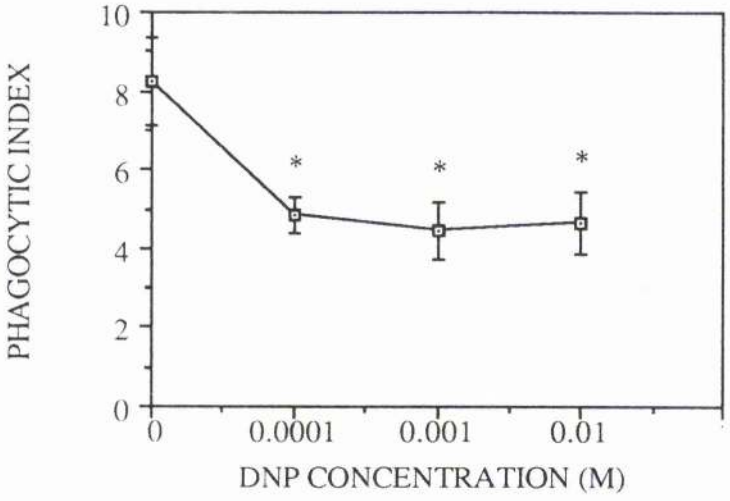

PHAGOCYTIC INDEX
10
8
6
4
2
0
*
*
*
0
0.0001
0.001
0.01
DNP CONCENTRATION (M)

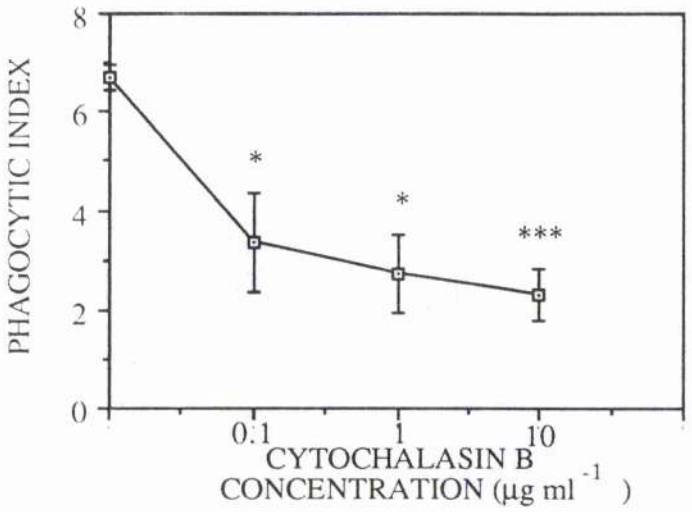
Figure 2.5.

Figure 2.5. Effect of various concentrations of cytochalasin B on phagocytic uptake by the hyaline cells of *C. maenas*.

Monolayers were incubated with 100 µl of cytochalasin B (0.1, 1, 10 µg $ml^{-1}$) for 15 min. The cells were then challenged with opsonised bacteria for 1 h before fixation 10% seawater/formalin. Two hundred cells were counted per monolayer per treatment per animal and the PI calculated. Values are the mean PI values ± S.E.M. n = 4 animals.

* Difference between the phagocytic uptake levels of untreated and treated cells is significant at $p \leq 0.05$.

*** Difference between the phagocytic uptake levels of untreated and treated cells is significant at $p \leq 0.001$.


PHAGOCYTIC INDEX
8
6
4
2
0
*
*
***
0.1
1
10
CYTOCHALASIN B
CONCENTRATION (µg ml -1 )

## 2.4. DISCUSSION

Using separated hyaline cell monolayers, these experiments have investigated some of the features of phagocytic uptake by crustacean haemocytes *in vitro*. Phagocytic uptake levels of Gram-negative bacteria by the hyaline cells of *C. maenas* have already been determined (Smith and Ratcliffe, 1978; Smith and Söderhäll, 1983a). The results of the present set of experiments confirmed that uptake rates in *C. maenas* are low in agreement with uptake by the phagocytes of other crustacean species (Chapter 1, Table 1.1). In this study approximately 1.5% of the hyaline cells phagocytosed opsonised *P. immobilis* in comparison to 4% of the cells ingesting unopsonised bacteria.

Initially work was carried out to determine if the opsonic factors of the HLS were freeze stable. From the data that was collected over the 3 month period it was apparent that the opsonic factor(s) are freeze stable at either -18°C or -70°C. Söderhäll *et al.* (1986) have demonstrated PO, itself, does not enhance phagocytic uptake by the hyaline cells of *C. maenas*. As part of the freezing experiments, described here, quantification of PO levels in the samples were made. For one of the assays, PO activity was not detected yet levels of uptake of 2.09% and 8.62% were recorded for MS treated and HLS incubated bacteria, respectively. This provides further evidence that it was unlikely that phenoloxidase itself acts as an opsonin in *C. maenas* as the opsonic effect was observed in the absence of PO.

Söderhäll (1981,1983) has demonstrated that serine protease activity is associated with activation of the proPO system. In the present study some experiments were carried out to determine if serine protease activity was involved in the generation of opsonic factor(s) from the HLS. Pretreatment of the HLS with either STI or

benzamidine reduced the opsonic effect of the HLS. As STI and benzamidine are serine protease inhibitors these results suggest that the opsonic factor(s) of the HLS are generated as a result of serine protease activity. Similar results were obtained for the sea squirt *Ciona intestinalis* (Smith and Peddie, 1992).

Both $NaN_3$ and DNP are known to inhibit cellular metabolism (Wainio, 1970). Dintitrophenol acts by uncoupling oxidative phosphorylation causing an increase in the rate of electron flow from NADH down the electron transport chain. This leads to greater mitochondrial oxygen consumption and the cessation of ATP synthesis. With *C. maenas*, the inhibition of phagocytosis following treatment of the cells with either $NaN_3$ or DNP indicates that cellular metabolic energy is required for phagocytic activity.

Also in this study, incubation of the hyaline cells with cytochalasin B, a known inhibitor of actin polymerisation and phagocytosis in vertebrate cells (Stossel and Cohn, 1976), was found to inhibit phagocytosis by *C. maenas* hyaline cells. Cytochalasin B acts on the fast assembly end of the microfilament to prevent further polymerization (Flanagan and Lin, 1980). In vertebrate cells, phagocytic engulfment is frequently accompained by numerous microfilament associations at the point of phagocytosis (Fulton, 1984). Failure of the microfilaments to polymerise would hence inhibit engulfment. The haemocytes of the oyster *Crassostrea virginica* also fail to engulf beads following treatment with cytochalasin B (Alvarez *et al.*, 1989).

In mammalian cells, the direct binding of ligands to receptors such as the tripeptide RGD sequence of adhesive proteins and the successive engulfment is dependent on divalent cations (see review by Ruoslahti and Pierschbacher, 1987). For this reason the cells were incubated with the divalent cation chelators, EDTA and

EGTA, to determine whether $Ca^{2+}$ and $Mg^{2+}$ are required for phagocytosis in the shore crab. EDTA selectively chelates to $Ca^{2+}$ and $Mg^{2+}$ whereas EGTA only chelates $Ca^{2+}$. Chelation of $Ca^{2+}$ was found to cause the cells to detach from the glass coverslips, suggesting that $Ca^{2+}$ ions are required for attachment. Johansson and Söderhäll (1988) have previously demonstrated that $Ca^{2+}$ ions are required for crayfish haemocytes to adhere to the 76 kDa CAF. In contrast, Armstrong (1980) showed that the adhesion of *Limulus* haemocytes *in vitro* did not require the addition of extraneous $Ca^{2+}$. However, once adhesion to glass coverslips had occurred, incubation of the cells in 5 mM EDTA, prevented spreading (Armstrong, 1980). With the mussel, *M. edulis*, Renwrantz and Stahmer (1983) found that addition of $Ca^{2+}$ ions to the saline used to prepare the yeast suspension increased the phagocytic activity to a level comparable to yeast incubated with haemolymph. Decreasing the $Ca^{2+}$ and $Mg^{2+}$ concentrations resulted in an exponential decrease in the number of phagocytosing haemocytes (Renwrantz and Stahmer, 1983). Renwrantz and Stahmer (1983) proposed that their results indicated the presence of divalent cation-dependent recognition molecules on the surface of *M. edulis* haemocytes. Using haemocytes harvested from the wax moth *Galleria mellonella*, Brookman *et al.* (1988) found that cellular attachment was not impaired when monolayers were prepared using a $Ca^{2+}$ free monolayer buffer. They found that the subsequent rate of phagocytosis was increased when the $Ca^{2+}$ concentration of the overlay buffer was increased (Brookman *et al.*, 1988). Brookman *et al.* (1988) suggested that calcium dependent membrane bound recognition molecules similar to that in *M. edulis* (Renwrantz and Stahmer, 1983) may be present on *Galleria mellonella* phagocytes. Similarly Zelk and Becker (1992) have demonstrated that calcium is required for phagocytosis by the gastropod mollusc *Biomphalaria glabrata* haemocytes. These authors proposed that $Ca^{2+}$ ions are not functioning as opsonins but may be required for activation of receptors involved in non-self recognition on the haemocyte membranes (Zelk and

Becker, 1992). The role of $Ca^{2+}$ and $Mg^{2+}$ ions in crustacean haemocyte non-self recognition still needs to be elucidated. Possibly using a cell suspension assay to quantify phagocytosis in the absence of $Ca^{2+}$ and $Mg^{2+}$ ions would be more appropriate as cell adhesion to an artificial substratum would not be required.

As was discussed in Chapter 1 the process of phagocytosis can be broken down into various stages. In the set of experiments described in the present chapter, it has been shown that recognition of bacteria can be enhanced by opsonic factor(s) that are generated by serine protease activation of the HLS. The enhanced recognition mediated by the opsonins in turn leads to higher uptake rates. In addition, ingestion requires cellular metabolic energy and intact microfilaments. However the final killing stage of phagocytosis has not been investigated. Although in vertebrates it is accepted that ingested particles are enclosed in a phagocytic vacuole which contains killing factors such as lysozyme and various degradative enzymes very little information is known as to the fate of bacteria that are engulfed by crustacean hyaline cells. In the following chapter it will be determined whether ingested bacteria are killed within the hyaline cells and what parameters influence the bactericidal activity.

# Chapter 3

## Bactericidal activity of hyaline cells of *Carcinus maenas* during phagocytosis *in vitro*

# 3.1. INTRODUCTION

Humoral antibacterial factors are well documented for a range of invertebrate phyla (see reviews by Smith and Chisholm, 1992; Ratcliffe *et al.*, 1985). In the shore crab, *C. maenas*, Chisholm and Smith (1992) demonstrated that antibacterial factor(s) reside exclusively in the granular cells. The activity is 90% effective within 60 min against a range of Gram-positive and Gram-negative bacteria *in vitro* (Chisholm and Smith, 1992). Phenoloxidase and an activating serine protease were not responsible for the observed antibacterial activity (Chisholm, 1993). However, the mechanisms of intracellular killing following phagocytosis have not been so extensively investigated in invertebrates. Much work has been done on molluscs so some mention will be made of the appropriate studies.

Studies of both crustaceans and molluscs have found that intra- and extracellular levels of degradative enzymes increase following challenge with foreign material (Tsing *et al.*, 1989; Cheng and Yoshino, 1976; Yoshino and Cheng, 1976). Acid phosphatase, which acts by dephosphorylation, activity has been detected in the haemocytes of the shrimp, *Penaeus japonicus*, (Tsing *et al.*, 1989). The haemocytes which were identified as containing acid phosphatase activity were morphologically similar to the hyaline cells of *C. maenas* (Smith and Ratcliffe, 1978) although Tsing *et al.* (1989) referred to them as haemocytes with small granules (SGH). This is another indication of the difficulties that alternative nomenclature can create in the interpretation of the results from a variety of investigations. Similarly, increases in acid phosphatase levels in the haemocytes and haemolymph of the mollusc, *Patella vulgata*, has been observed to be associated with phagocytosis (Cooper-Willis, 1979). However, the elevated levels of acid phosphatase in the haemolymph were not sufficient to inhibit bacterial growth (Cooper-Willis, 1979).

Lysozyme has also been shown to be released in greater quantities from actively phagocytosing cells of the clam, *Mercenaria mercenaria* (Cheng *et al.*, 1975).

From investigations of bacterial clearance in *C. maenas in vivo*, White and Ratcliffe (1980) demonstrated that, contained within the lysosomes of *C. maenas* haemocytes are acid phosphatase and ß-glucuronidase which act by degrading proteoglycans and glycoproteins. Increased acid phosphatase activity *in vivo* was associated with haemocyte clumps surrounding bacterial aggregates within 15 min of injection of the bacteria, peaked 1-3 h later but had declined by one day later (White and Ratcliffe, 1980). In contrast, increased ß-glucuronidase activity of the nephrocytes was not observed until 6 h after the injection and peaked a day later (White and Ratcliffe, 1980). Nephrocytes are known to be involved in bacterial clearing (Smith and Ratcliffe, 1981) so possibly these enzymatic activities are linked to bacterial and cell degradation.

From these studies, it is known that crustacean cells do contain degradative enzymes, but bactericidal activity has not been shown to be linked to phagocytosis. The aims of this set of experiments are;

1) to establish that bactericidal activity occurs following the ingestion of the bacteria by the hyaline cells of *Carcinus maenas*,

and 2) to determine whether bacterial concentration, inhibition of phagocytosis and incubation time of the cells with the bacteria influence bactericidal activity.

# 3.2. MATERIALS AND METHODS

## 3.2.1. Harvesting and Separation of the Hyaline cells

The withdrawal of haemolymph and subsequent separation of the hyaline cells were previously described in Chapter 2 (Section 2.2.3. and 2.2.7.). The hyaline cells were resuspended in 3.2.% NaCl to give a concentration of ca 1 x $10^6$ $cells^{-1}$ as counted on an Improved Neubauer haemocytometer.

## 3.2.2. Bacteria

*Psychrobacter immobilis* was cultured as described previously in Chapter 2 (Section 2.2.9) except that in the present set of experiments the bacteria were not heat killed prior to washing. From the stock culture, a sample (ca 12 ml) of bacteria were centrifuged at 1,900 g for 10 min, washed twice in 3.2% NaCl and finally resuspended in modified MS (MMS) (0.5 M sodium chloride, 11 mM potassium chloride, 20 mM calcium chloride hexahydrate, 0.6 mM di-sodium hydrogen orthophosphate dodecahydrate, 0.05 M tris(hydroxymethyl)methylamine, 1 M hydrochloric acid. pH 7.4) (Chisholm, 1993). Magnesium ions were omitted because these ions have a deleterious effect on bacterial growth (Chisholm, 1993). The bacterial suspension was adjusted with MMS to give an absorbance value of 0.5 at 570 nm. This is a bacterial concentration of ca 1 - 2 x $10^7$ $ml^{-1}$ which gives a bacteria:phagocyte ratio of 20:1 in the bactericidal assay. For lower bacteria:phagocyte ratios (see below), the bacterial suspension (1 - 2 x $10^7$ $ml^{-1}$)was diluted with MMS to give ratios of 2:1 and 1:5. Where a higher bacteria:phagocyte ratio (see below) was required the bacterial suspension was adjusted to an absorbance

of 0.7 at 570 nm, (ca 1 - 2 x $10^9$ $ml^{-1}$). By dilution, the bacterial suspension was adjusted to give a bacteria:phagocyte ratio of 200:1.

### 3.2.3. Bactericidal Assay

Quantification of bactericidal activity was carried out using modifications of the method of Sharp and Secombes (1993). Briefly, the assay was carried out on coverslips placed in a 6-well covered multiwell plate (Sterilin). Two hundred microlitres of cell suspension (1 x $10^6$ $ml^{-1}$) were pipetted onto each coverslip and the cells allowed to attach for 30 min, prior to the removal of the supernatant. For the experimental group, the cells were incubated with 200 µl of bacterial suspension for 3 h at 18°C on a rocking platform (3 h), while the control group received 200 µl of bacterial suspension after 3 h (0 h). All the cells were then centrifuged at 150 g for 5 min at 15°C. The supernatant was then removed and 100 µl of 0.2% Tween 20 (Sigma) added to each coverslip to lyse the cells. This concentration of Tween 20 which was found to lyse the cells of the monolayer and allow the release of the internalised bacteria, did not have a detrimental effect on subsequent bacterial growth. Two hundred microlitres of sterile marine broth (Difco) were then added to each well to support the growth of the surviving bacteria. After 24 h at 18°C the absorbance of the supernatant from each well was measured at 570 nm on a microplate reader (Dynatech, MR5000, Billingshurst, W. Sussex. U.K.). The wells were read against blank wells containing 0.2% Tween and marine broth in the appropriate proportions. The percentage of surviving bacteria was calculated as below.

$$\text{SURVIVAL INDEX (SI)} = \frac{\text{Absorbance of supernatant at 570 nm (3 h)}}{\text{Absorbance of supernatant at 570 nm (0 h)}} \times 100$$

A SI of 0 indicates that all the bacteria were killed on the experimental coverslips, whereas a value of 100 indicates bacterial survival on the experimental coverslips was equal to that of the controls. A SI of over 100 indicates greater bacterial growth in the experimental than the control group.

This assay was then modified to allow various parameters of the bactericidal activity to be investigated.

a) Effect of bacterial concentration

In order to examine the effect of bacterial concentrations on the bactericidal efficiency of the hyaline cells, *P. immobilis* was washed in 3.2% NaCl as above. The bacteria were then resuspended in MMS to give bacteria:phagocyte ratios of 1:5, 2:1, 20:1 and 200:1. The bactericidal assay was carried out as above.

b) Effect of a phagocytosis inhibitor on bactericidal activity

The phagocytic inhibitor, cytochalasin B, was used at a concentration of 10 μg $ml^{-1}$. One hundred microlitres of cytochalasin B were incubated with the cell monolayers for 15 min as described in Chapter 2 (Section 2.2.11.2.c). The cells were washed twice with MMS prior to the addition of the bacteria. For controls, monolayers incubated with MMS instead of cytochalasin B were also prepared. The bactericidal assay was then carried out as before for 3 h. Control monolayers were also prepared, as above, for quantification of phagocytosis, to ensure that cytochalasin B did inhibit uptake (Chapter 2, Section 2.2.11.). The level of uptake was expressed as the phagocytic index (PI) calculated as before (Chapter 2, Section 2.2.12.). Cytochalasin B, a cytoskeletal inhibitor, does not have a detrimental effect on *P. immobilis*, as a distinguishing feature of prokaryotes is the absence of a

cytoskeleton (Alberts *et al.*, 1983). Preliminary assays confirmed that cytochalasin B did not affect bacterial growth. In these experiments, the bacteria grew up equally well whether incubated with cytochalasin B at the appropriate concentration or with MMS.

c) Time study of bactericidal activity and phagocytic uptake

The bactericidal assay was carried out as above except that incubation of the experimental group coverslips was for 1, 3 or 5 h. In conjunction with the bactericidal assay, monolayers were also prepared for phagocytosis assays (Chapter 2, Section 2.2.11.). Hyaline cell monolayers were incubated with 200 µl of bacteria for 1, 3 or 5 h, then washed 3 times with MMS prior to the fixation of the cells in 10% seawater/formalin. Phagocytosis was then quantified as previously described (Chapter 2, Section 2.2.12.).

### 3.2.4. Statistical Analysis

Differences between treatments were analysed statistically by paired Student's t-test on combined data from a minimum of 4 animals. Differences were considered to be significant when $p \leq 0.005$ (Sokal and Rohlf, 1981).

## 3.3. RESULTS

After a 3 h incubation period with the hyaline cells of *C. maenas*, the SI of *P. immobilis* was approximately 16% in comparison to the 100% SI of the controls (Fig. 3.1). This difference was highly significant ($p \leq 0.001$, $n = 5$).

### 3.3.1. Effect of Bacterial Concentration

The experiments designed to investigate the effect of bacterial concentration on cellular killing showed that the haemocytes were only effective in killing *P. immobilis* at a bacteria: phagocyte ratio of 20:1 and 200:1 (Fig. 3.2). At 20:1, the SI was 18% and at 200:1 the SI was 45% after 3 h incubation (Fig. 3.2). At the lower bacteria:phagocyte ratios of 1:5 and 2:1, *P. immobilis* survived just as well as the controls with SIs of 113% and 102%, respectively (Fig. 3.2). However, the effect of bacterial concentration on the SI was not significant ($p \geq 0.067$, for all cases), possibly because the standard errors of the data were large.

### 3.3.2. Effect of a Phagocytosis Inhibitor on Bactericidal Activity.

Figure 3.3 shows the effect of cytochalasin B on the SI of the bacteria. This reagent inhibits actin polymerization and hence impairs the engulfment of the bacteria. Pretreatment of the haemocytes with cytochalasin B resulted in about 80% survival of the bacteria after 3 h, whereas only 6% of the bacteria survived when the cells were incubated with MMS (Fig. 3.3). This difference in the SI was highly significant ($p = 0.006$, $n = 4$). Over the same time period, cytochalasin B significantly reduced the PI of the hyaline cells from approximately 6.3% to 2.8% ($p \leq 0.001$, $n = 5$).

Figure 3.1.

Figure 3.1. Survival index of *P. immobilis* after incubation with the phagocytic hyaline cells of *C. maenas*.

For the experimental group, monolayers of phagocytic hyaline cells were incubated with 200 µl of bacterial suspension (1 - 2 x $10^7$ $ml^{-1}$, bacteria:phagocyte ratio of 20:1) for 3 h (3 h). The controls received 200 µl of bacteria after 3 h (i.e. 0 h). The cells on each coverslip were then lysed with 100 µl of 0.2% Tween 20, and 200 µl of sterile marine broth were added to support bacterial growth. After 24 h at 18°C, the absorbance of the supernatant was measured at 570 nm and the SI calculated. Values are the mean SI values ± S.E.M. n = 5 animals. For the SI at 0 h no error bars are shown as the SI is calculated as a percentage of the 0 h data.

*** Differences between the experimental (3 h) and control group (0 h) are significant at $p \leq 0.001$.

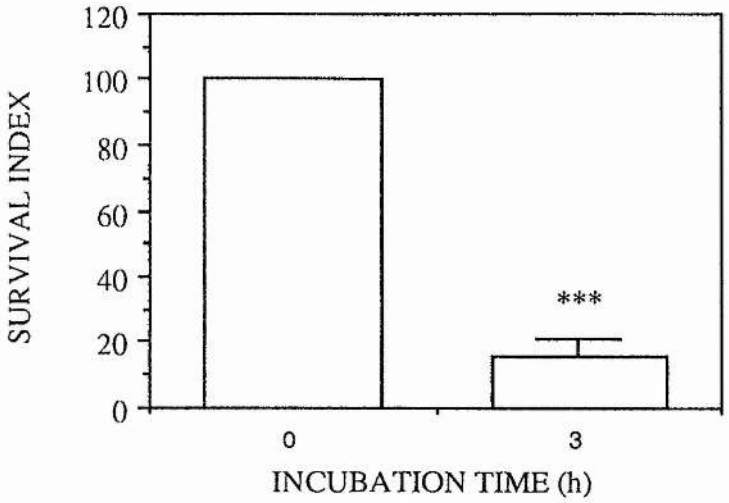

120
100
80
60
40
20
0
SURVIVAL INDEX
***
0
3
INCUBATION TIME (h)

Figure 3.2.

Figure 3.2. Survival index of different concentrations of *P. immobilis* after incubation with the phagocytic hyaline cells of *C. maenas*.

For the experimental groups, monolayers of phagocytic hyaline cells were incubated with 200 µl of bacterial suspension at various bacteria:phagocyte ratios (1:5, 2:1, 20:1 and 200:1) for 3 h. The controls received 200 µl of bacteria at each concentration after 3 h (i.e. 0 h). The cells on each coverslip were then lysed with 100 µl of 0.2% Tween 20, and 200 µl of sterile marine broth were added to support bacterial growth. After 24 h at 18°C, the absorbance of the supernatant was measured at 570 nm and the SI calculated. Values are the mean SI values ± S.E.M. n = 5 animals. There was no significant difference in the SI at the different bacterial concentrations after 3 h incubation time.

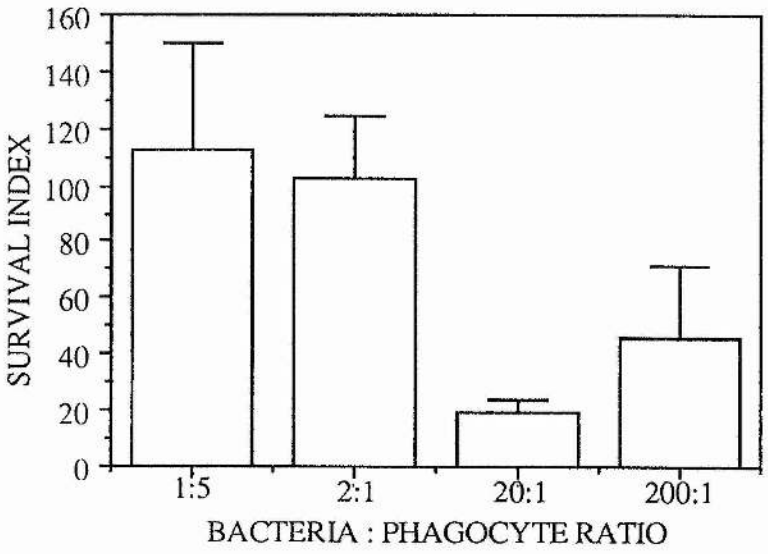
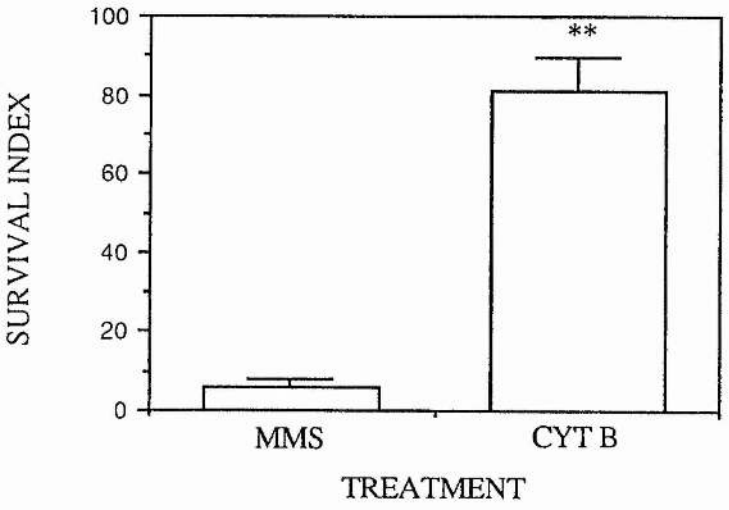

160
140
120
100
80
60
40
20
0
SURVIVAL INDEX
1:5
2:1
20:1
200:1
BACTERIA : PHAGOCYTE RATIO

Figure 3.3.

Figure 3.3. Survival index of *P. immobilis* after incubation with the phagocytic hyaline cells of *C. maenas* which had been pretreated with cytochalasin B.

For the experimental groups, monolayers of phagocytic hyaline cells were incubated for 15 min with cytochalasin B (10 $\mu g\ ml^{-1}$), washed twice with 3.2% NaCl and then incubated with 200 µl of bacterial suspension (1 - 2 x $10^7\ ml^{-1}$, bacteria:phagocyte ratio of 20:1) for 3 h. The controls received 200 µl of bacteria after 3 h (i.e. 0 h). In conjunction, a set of monolayers were incubated with MMS instead of cytochalasin B and then the bactericidal assay carried out as before. The cells on each coverslip were then lysed with 100 µl of 0.2% Tween 20, and 200 µl of sterile marine broth were added to support bacterial growth. After 24 h at 18°C, the absorbance of the supernatant was measured at 570 nm and the SI calculated. Values are the mean SI values ± S.E.M. n = 4 animals.

** Differences between the experimental (cytochalasin B incubated) and control (MMS treated) group after 3 h incubation are significant at $p \leq 0.01$.


100
80
60
40
20
0
SURVIVAL INDEX
**
MMS
CYT B
TREATMENT

### 3.3.3. Time Study of Bactericidal Activity and Phagocytic Uptake

Figure 3.4 A and B shows the effect of incubation time on SI and PI, respectively. Following a 1 h incubation, the SI was 45% but this had risen to 60% and 71% after 3 and 5 h, respectively. Using paired t-tests it was found that there was no significant difference in the SI at 1 h compared with 3 or 5 h, or 3 h compared with 5 h ( $p > 0.420$, in all cases) (Fig. 3.4A). The PI increased from 2% after 1 h to 5.1% and 8.3% for 3 and 5 h incubation times, respectively (Fig. 3.4B). The difference in PI was only significant between 1 and 3 h ($p = 0.015$, $n = 6$) with no statistical difference in the phagocytic uptake levels at 1 and 5 h ($p = 0.081$, $n= 6$). There was also no statistical difference in the uptake when the cells and bacteria were incubated together for either 3 or 5 h ($p = 0.321$, $n = 6$).

Figure 3.4. A & B.

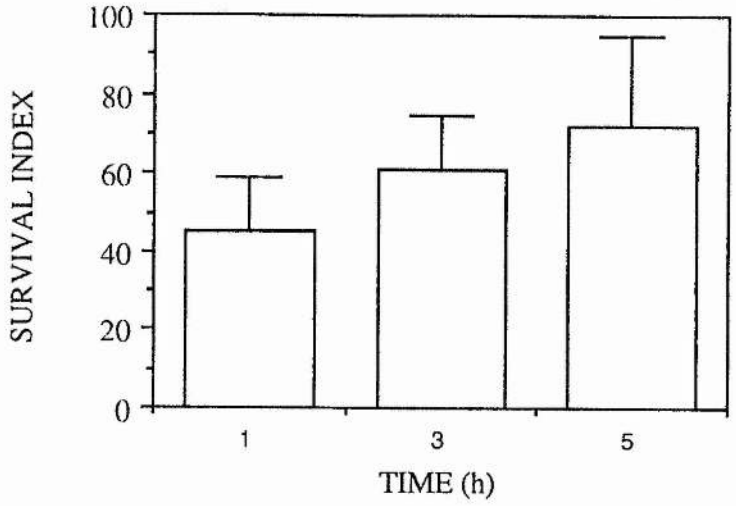
Figure 3.4 A,B. Survival index and phagocytic index of *P. immobilis* after incubation for various time intervals with the phagocytic hyaline cells of *C. maenas*.

For the experimental groups, monolayers of phagocytic hyaline cells were incubated with 200 µl of bacterial suspension (1 - 2 x $10^7$ $ml^{-1}$, bacteria:phagocyte ratio of 20:1) for 1, 3 or 5 h. The controls received 200 µl of bacteria after 1, 3 or 5 h (i.e. 0 h). The cells on each coverslip were then lysed with 100 µl of 0.2% Tween 20, and 200 µl of sterile marine broth were added to support bacterial growth. After 24 h at 18°C, the absorbance of the supernatant was measured at 570 nm and the SI calculated. Phagocytic uptake was quantified by counting 200 cells per monolayer per treatment per animal. Values are the mean SI values ± S.E.M. n = 6 animals.

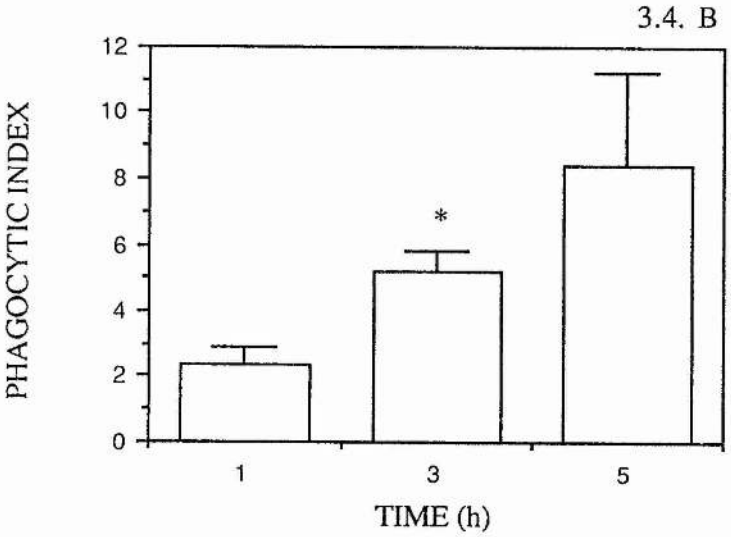
There was no significant difference in the SI at the various time intervals.

* Differences between levels of phagocytic uptake at 1 and 3 h are significant at $p \leq 0.05$.

3.4. A


SURVIVAL INDEX
100
80
60
40
20
0
1
3
5
TIME (h)

3.4. B


PHAGOCYTIC INDEX
12
10
8
6
4
2
0
*
1
3
5
TIME (h)

## 3.4. DISCUSSION

The aim of these experiments was to investigate the fate of bacteria engulfed by the phagocytic hyaline cells of the shore crab, *C. maenas*. The results show that following internalisation, the bacteria are killed by the hyaline cells.

The efficiency of the bactericidal action of the cells was not significantly affected by the bacteria:cell ratio. However, at the lower bacteria concentrations, the majority of the bacteria survived incubation with the cells, possibly because there was insufficient contact between the cells and *P. immobilis* to elicit a response. The phagocytic uptake level after 3 h of unopsonised bacteria is only approximately 4 - 5% (Fig. 3.4. B ) at the bacteria:phagocyte ratio of 20:1. At the bacteria:phagocyte ratio of 2:1 and 1:5 phagocytic uptake is likely to be less than 4%. Increasing the bacteria:phagocyte ratio to 200:1 reduced the bactericidal efficiency of the cells, leaving the ratio of 20:1 optimum for intracellular killing. *In vivo*, when a bacterial infection is too great the cells respond by encapsulating clumps of the bacteria rather than by phagocytosis (White and Ratcliffe, 1980). Possibly a ratio of 200:1 represents a level of infection that the hyaline cells are unable to phagocytose effectively. However, this experiment used separated cells which were attached to coverslips so the cells were unable to initiate nodule formation responses. White *et al.* (1985) have demonstrated that within haemocyte clumps in *C. maenas* bacteria are killed.

In the present study, inhibition of phagocytosis with cytochalasin B significantly reduced the bactericidal activity of the cells indicating that the killing response is associated with the engulfment of the bacteria. It was expected that by increasing the incubation time, uptake would be significantly enhanced and that this, in turn, would

result in greater bactericidal activity. However, increasing the incubation time did not significantly increase the uptake and the bactericidal activity was not affected. Previously, Smith (1978) showed that uptake of *Bacillus cereus* increases from ca 0.5% at 30 min to a maximum of ca 4% at 3 h. However, that study was conducted using monolayers of mixed cell populations, whereas in the present investigation, uptake was determined on monolayers of separated hyaline cells. The failure to observe increased phagocytosis with time may be explained by the lack of opsonins in the monolayer culture.

These experiments confirm that phagocytosis by *C. maenas* hyaline cells is followed by the subsequent killing of the engulfed bacteria. However, the mechanism of bactericidal activity was not investigated during these experiments. In molluscs, it is known that, if stimulated, the phagocytic cells are capable of a respiratory burst (Nakamura *et al.*, 1985; Dikkeboom *et al.*, 1988) similar to the mammalian phagocytes (Babior, *et al.*, 1973). In mammals, the microbicidal nature of the reactive oxygen intermediates as part of the respiratory burst have been well documented (Allen *et al.*, 1972; Babior *et al.*, 1973). In invertebrates, Nottage and Birkbeck (1990) examined the response of the mussel *M. edulis* haemocytes to *Vibrio alginolyticus* and suggested that production of toxic oxygen intermediates by the haemocytes was largely responsible for microbial killing. Anderson *et al.* (1992a) demonstrated that the haemocytes of the oyster, *Crassostrea virginica*, produce $O_2^-$ ions, and that production increases when the cells are infected with the protozoan parasite *Perkinsus marinus* (Anderson *et al.*, 1992b). They suggested that greater oxidative stress due to excess $O_2^-$ production may contribute to the pathogenesis of the disease (Anderson *et al.*, 1992b). Other investigators of molluscan immune responses have speculated as to the role of oxidative killing in molluscan haemocytes (Adema *et al.*, 1991b) but this requires further study. It

seems logical for the next set of experiments to be carried out with the aim of demonstrating whether crustacean haemocytes are capable of producing a respiratory burst following stimulation as this may be a possible bactericidal mechanism for the cells.

# Chapter 4

## Superoxide production by hyaline cells of *Carcinus maenas in vitro*

# 4.1. INTRODUCTION

Many investigations have examined phagocytosis in invertebrates (see review by Bayne, 1990), but most studies have been concerned with recording phagocytic indices and rates *in vitro* in different species, or monitoring the effects of variables such as temperature and opsonisation on levels of uptake (Goldenberg *et al.*, 1984; Fryer *et al.*, 1989) (see Chapter 1). Few studies have examined the metabolic events associated with phagocytosis in invertebrates, or of the activation of their phagocytes by foreign materials. The previous chapter demonstrates that ingested bacteria are killed within the hyaline cells of *C. maenas*, but the bactericidal mechanism has not yet been determined (Chapter 3).

The metabolic process, termed the respiratory burst, is well documented for mammalian phagocytes (Allen *et al.*, 1972; Babior *et al.*, 1973). During phagocytosis, the membrane bound enzyme, NADPH oxidase is stimulated and catalyses the transfer of single electrons from NADPH to extracellular oxygen (Babior *et al.*, 1973). The $O_2^-$ ions generated dismutate spontaneously or are catalysed by the enzyme superoxide dismutase (SOD) to give hydrogen peroxide ($H_2O_2$) (Fridovich, 1978). Although $O_2^-$ formation is required for the killing of bacteria during phagocytosis (Babior, 1978), $O_2^-$ itself is not deleterious to bacteria (Klebanoff, 1974). However, $O_2^-$ and $H_2O_2$ react with each other to give the highly toxic hydroxyl radical (OH) and singlet oxygen ($^1O_2$) (Krinsky, 1974; Tauber and Babior, 1977).

Recently, the phagocytic cells of some invertebrates have also been observed to produce reactive oxygen intermediates following the appropriate stimulation. Using the techniques of chemiluminescence and nitroblue tetrazolium reduction, Anderson *et*

*al.* (1973) and Cheng (1976) have reported that, in insects and molluscs respectively, the haemocytes appear to be incapable of producing superoxide and other reactive oxygen moieties. However, Nakamura *et al.* (1985) have shown that haemocytes of the bivalve mollusc, *Patinopecten yessoensis*, produce $H_2O_2$, *in vitro*, while Shozawa (1986) and Connors and Yoshino (1990) have reported $O_2^-$ production by the haemocytes of the gastropod mollusc *Biomphalaria glabrata*. Dikkeboom *et al.* (1985; 1986a,b; 1987; 1988) have shown that the haemocytes of a variety of molluscan species produce $O_2^-$ and $H_2O_2$. Pipe (1992) examined the generation of $O_2^-$ and $H_2O_2$ by the haemocytes of the mussel *Mytilus edulis*, while Ito *et al.* (1992) have reported the production of $H_2O_2$ by the phagocytes of the sea urchin, *Strongylocentrotus nudus*. Takahashi *et al.* (1993) demonstrated $O_2^-$ production by *Crassostrea gigas* haemocytes using chemiluminescence and electron spin resonance spin trapping, and subsequently separated the cells into two distinct subpopulations according to the ability to produce $O_2^-$. However, it has not yet been determined whether crustacean phagocytic cells also have the metabolic capability to produce a respiratory burst.

In mammalian studies, one of the techniques used to investigate the respiratory burst response is the reduction of ferricytochrome c (Fe III) by $O_2^-$ to ferrocytochrome c (Fe II) (Johnston *et al.*, 1975). Using a modification of this method proposed by Pick and Mizel (1981) the aims of this set of experiments are to determine:

1) whether the phagocytic hyaline cells of *C. maenas* produce superoxide radicals following non-self stimulation;

and 2) some of the basic features of this metabolic process, *in vitro*.

# 4.2. MATERIALS AND METHODS

## 4.2.1. Harvesting and Separation of the Haemocytes

The withdrawal of haemolymph and subsequent separation of the different cell types were previously described in Chapter 2 (Sections 2.2.3. and 2.2.7.).

## 4.2.2. Elicitors

The following were tested as elicitors of the respiratory burst:- a) laminarin (a ß-1,3-glucan from *Laminaria digitata,* Calbiochem); b) lipopolysaccharide (LPS) (from *Escherichia coli* 0111:B4 phenolic extraction, Sigma); c) phytohaemagglutinin (PHA-P) (sterile filtered L9132, Sigma); d) phorbol 12-myristate 13-acetate (PMA) (Sigma), and e) concanavalin A (con A) (Type VI specific affinity chromatographically purified, Sigma).

Soluble elicitors were used in this investigation instead of bacteria and yeast as preliminary work had shown that particulate matter affected the optical density of the reaction mixtures: soluble elicitors therefore give better reproducibility with the superoxide anion assay. Phorbol 12-myristate 13-actetate is a known activator of the respiratory burst response in fish (Secombes *et al.*, 1988) and con A has been shown to stimulate $H_2O_2$ production in scallop amoebocytes (Nakamura *et al.*, 1985). In addition, LPS and laminarin were used as possible natural elicitors since these are derived from microbial cell walls and are known to induce cellular defence responses in crustaceans (Smith and Söderhäll, 1983a; Smith *et al.*, 1984). Phytohaemagglutinin was chosen as an elicitor because it is known to have a variety of bioactive effects (Goldstein and Hayes, 1978).

### 4.2.3. Superoxide Anion Assay

The reaction was carried out in 96 well, flat bottomed microtitre plates; one per animal. For each treatment quadruplicate wells were prepared. All of the chemicals were made up in 0.2 μm filtered MS. The experimental mixture in each well consisted of 25 μl of cells, 25 μl of elicitor (5 μg $ml^{-1}$, final concentration), 20 μl of catalase (4 μg $ml^{-1}$, final concentration) and 50 μl of ferricytochrome c (160 μM, stock solution). For the controls, 0.2 μm filtered MS was substituted for the elicitor. The PMA was resuspended in DMSO at 2 mg $ml^{-1}$ then made up in MS prior to use to the required concentration of 5 μg $ml^{-1}$. Checks for cell viability at the end of the incubation period ensured that DMSO and the other reagents in the reaction mixtures did not have a detrimental effect on the haemocytes. All reagents were independently incubated with ferricytochrome c to ensure that no interaction occurred between these compounds. The plates were incubated at 20°C and read at 550 nm at 5 min intervals on a microplate reader (Dynatech, MR5000). All wells were read against quadruplicate blanks containing ferricytochrome c and 0.2 μm filtered MS. Two readings were made for each plate at 5 minute intervals prior to the addition of the elicitor to ensure that the absorbance values of the wells were stable and did not vary between the experimental and control wells. Careful addition of the elicitor or MS for the controls minimised turbulence in the wells and the plates were not shaken before each reading. Individual well values within treatments did not deviate by more than 0.005 absorbance units. Each treatment was repeated for a minimum of five animals and the mean absorbance values for the quadruplicate wells were calculated for each treatment.

The assay was then modified to allow various parameters of the $O_2^-$ production to be investigated.

a) Effect of various catalase concentrations

The assay was carried out with the inclusion of 20 µl of 0, 4 or 8 µg $ml^{-1}$, final concentration, of catalase (from bovine liver EC 1.11.1.6, Sigma) with the elicitor LPS to ascertain the effect of $H_2O_2$ oxidation of ferrocytochrome c (Fe II).

b) Determination of the dose response of PMA or LPS

To determine the optimum concentration for stimulation of the respiratory burst, PMA or LPS was used at final concentrations in the range of 0 - 5 µg $ml^{-1}$ in the superoxide anion assay. Twenty-five microlitres of either PMA or LPS were added to the experimental mixture in each well.

c) Different cellular response to PMA

A comparison was made of the ability of PMA to stimulate the hyaline, semi-granular or granular cells by using the cell separation technique as described in Chapter 2 (section 2.2.7). Twenty-five microlitres of each cell type were then substituted in the assay.

d) Effect of incubation temperature

The effect of incubation temperature on the response was also determined by incubating the reaction mixtures at a range of temperatures (4, 10, 18, 25 and 37°C) for 30 min before the absorbance values were measured.

e) Determination of energy requirements

To investigate whether $O_2^-$ production is dependent on the hexose monophosphate shunt (HMS), as in fish (Secombes *et al.*, 1988), the effect of glucose on ferricytochrome c reduction by crab hyaline cells was examined. Each animal was bled twice, once into AC and then into AC without glucose. Assays were also carried

out for each haemocyte preparation with 0, 10 or 100 mM levels of glucose added to the MS in which the ferricytochrome c was dissolved.

f) Confirmation of $O_2^-$ production

To confirm that the reduction of ferricytochrome c was at least in part due to $O_2^-$, SOD (EC1.15.1.1 from bovine erythrocytes, Sigma) was included in the reaction mixture. The SOD was rehydrated in distilled water and then made up in MS. Twenty microlitres were added to each well to give a final concentration of 100 units $ml^{-1}$. In the control wells, 20 µl of MS were substituted for the SOD. As an additional control, to confirm the specific effect of SOD on ferricytochrome c reduction, parallel wells were set up containing haemocytes, PMA, ferricytochrome c and SOD which had been heat-inactivated by boiling for 30 min. All samples were then incubated and read as above.

g) Effect of different elicitors

Laminarin, con A, PHA-P and LPS were made up in MS to give a final working concentration of 5 µg $ml^{-1}$. Twenty-five microlitres of each elicitor were then added to the experimental mixture in each well to examine the effect of different elicitors on the hyaline cells in the superoxide anion assay.

### 4.2.4. Statistical Analysis

Differences between treatments were analysed statistically by paired Student's t-test on combined data from 5 - 7 animals depending on the experiment. Differences were considered significant when $p \leq 0.05$ (Sokal and Rohlf, 1981).

## 4.3 RESULTS

Stimulation of the hyaline cells with PMA resulted in a reduction of ferricytochrome c to give an absorbance value of ca 0.053. Cells stimulated with MS gave an absorbance of ca 0.031 after 30 min. The difference between absorbance values for the experimental and control wells was significant with $p = 0.044$ after 5 min and $p = 0.005$ after 30 min. The time course of this response is shown in Figure 4.1.

### 4.3.1. Effect of Various Catalase Concentrations

Figure 4.2 shows the effect of 0, 4 and 8 $\mu g\ ml^{-1}$ of catalase on the $H_2O_2$ mediated oxidation of ferrocytochrome c. Catalase significantly increased the absorbance values in the LPS treated wells from ca 0.029 to ca 0.040 for 4 $\mu g\ ml^{-1}$ and to ca 0.044 for 8 $\mu g\ ml^{-1}$ ($p < 0.001$ for both concentrations compared to the catalase-free controls) (Fig. 4.2).

### 4.3.2. Determination of the Dose Response of PMA or LPS

To determine the optimum concentration of elicitor, the response to increasing doses of PMA or LPS was examined. Optimum stimulation by PMA occurred between 0.2 - 5 $\mu g\ ml^{-1}$, and by LPS between 0.1 - 5 $\mu g\ ml^{-1}$ (Fig. 4.3). Accordingly, 5 $\mu g\ ml^{-1}$ of elicitor was chosen for all further assays.

### 4.3.3. Different Cellular Response to PMA

Comparison of the abilities of the three haemocyte types to produce a respiratory burst showed that for the hyaline cells, stimulation with PMA for 30 min resulted in

Figure 4.1.

Figure 4.1. Reduction of ferricytochrome c at 550 nm by separated hyaline cells of *C. maenas in vitro*.

Twenty-five microlitres of cells (1 - 9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM ferricytochrome c were challenged with 25 µl of PMA (5 µg $ml^{-1}$, final concentration), for controls with 25 µl of 0.2 µm filtered MS. The change in absorbance was measured at 5 min intervals at 550 nm at 20°C for the first 30 min after stimulation. Values are means in absorbance at 550 nm ± S.E.M. n = 5 animals.

* Differences between the experimental (PMA-incubated) and control (MS-treated) group are significant at $p \leq 0.05$.

** Differences between the experimental (PMA-incubated) and control (MS-treated) group are significant at $p \leq 0.01$.

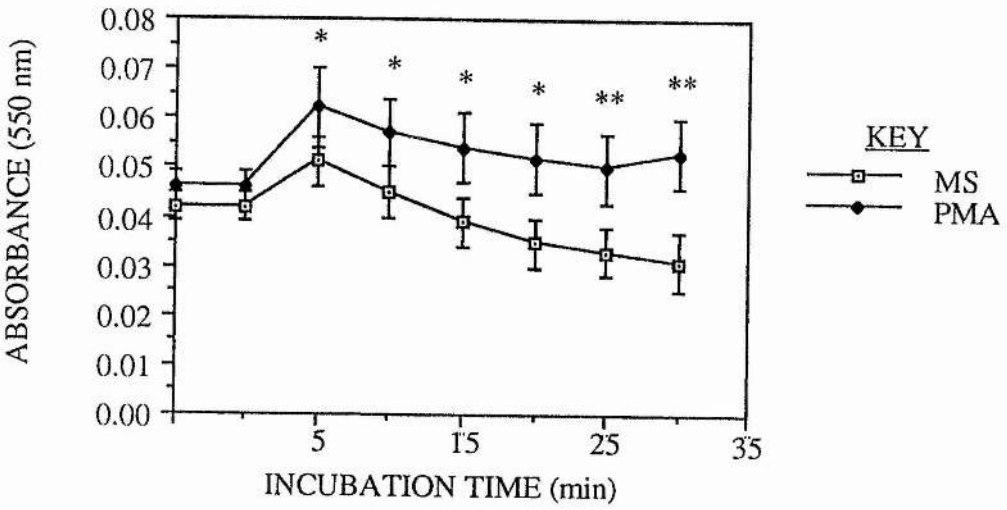

ABSORBANCE (550 nm)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
*
*
**
**
5
15
25
35
INCUBATION TIME (min)
KEY
MS
PMA

Figure 4.2.

Figure 4.2. Reduction of ferricytochrome c at 550 nm by separated hyaline cells of *C. maenas* incubated with varying concentrations of catalase *in vitro*.

Twenty-five microlitres of cells (1 - 9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of MS or catalase ( 4 or 8 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM of ferricytochrome c were challenged with 25 µl of LPS (5 µg $ml^{-1}$, final concentration) or, for controls with 25 µl of 0.2 µm filtered MS. Values are means in absorbance at 550 nm ± S.E.M. after 30 min incubation at 20°C. n = 6 animals.

In every case, there is a significant difference ($p \leq 0.05$) between the LPS-incubated and MS-treated controls.

*** Differences between the experimental (catalase-incubated) and buffer-treated controls are significant at $p \leq 0.001$.

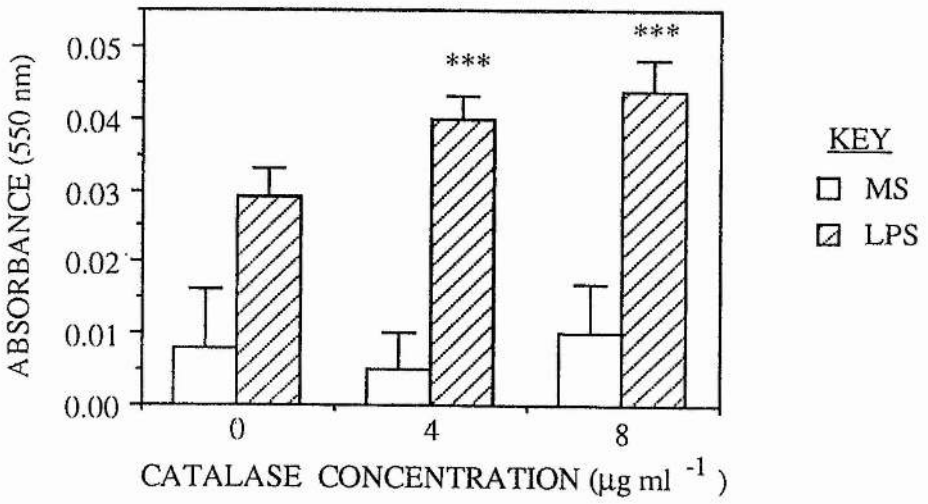
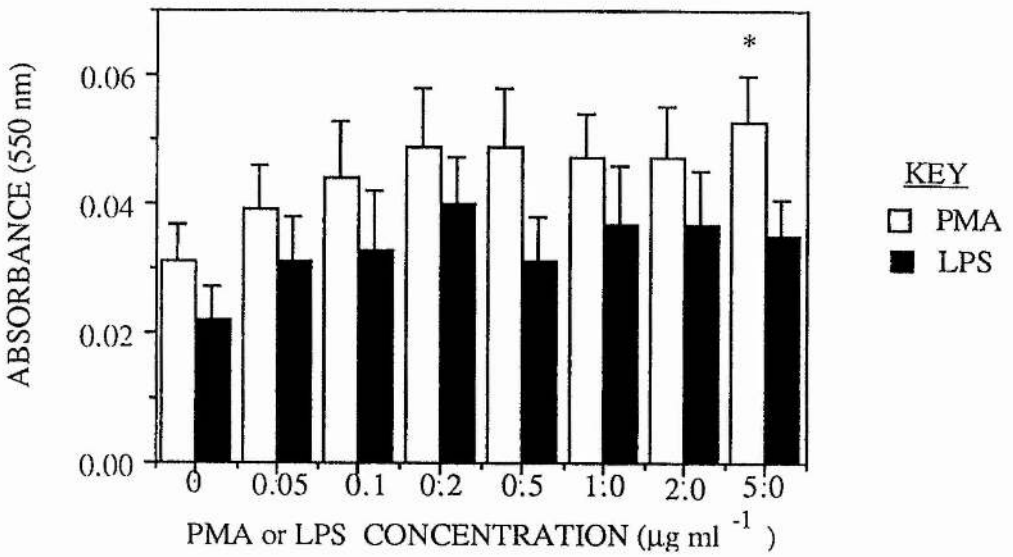

ABSORBANCE (550 nm)
0.05
0.04
0.03
0.02
0.01
0.00
***
***
0
4
8
CATALASE CONCENTRATION (µg ml -1 )
KEY
MS
LPS

Figure 4.3.

Figure 4.3. Reduction of ferricytochrome c at 550 nm by separated cells of *C. maenas* incubated with various concentrations of PMA or LPS *in vitro*.

Twenty-five microlitres of cells (1 - 9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM ferricytochrome c were challenged with 25 µl of PMA or LPS at a range of final concentrations (0 - 5 µg $ml^{-1}$) or, for the controls with 25 µl of 0.2 µm filtered MS. Values are means in absorbance at 550 nm ± S.E.M. after 30 min incubation at 20°C. n = 5 animals.

* Differences between 5 µg $ml^{-1}$ and 1 µg $ml^{-1}$ or 2 µg $ml^{-1}$ PMA concentrations are significant at $p \leq 0.05$.


ABSORBANCE (550 nm)
0.06
0.04
0.02
0.00
*
0
0.05
0.1
0.2
0.5
1.0
2.0
5.0
PMA or LPS CONCENTRATION (μg ml -1 )
KEY
PMA
LPS

an increase in absorbance to ca 0.070 (Fig. 4.4). This was significantly different from the MS-treated controls which gave a value of ca 0.050 ($p \leq 0.001$) (Fig. 4.4). In contrast, the semi-granular and granular cells yielded absorbance values of ca 0.064 and 0.063, respectively, following stimulation with PMA (Fig. 4.4) whereas the controls treated with MS gave absorbance values of ca 0.050 for the semi-granular cells and 0.055 for the granular (Fig. 4.4). These were not significantly different from the experimental treatments ($p = 0.062$ and $p = 0.071$, respectively).

### 4.3.4. Effect of Incubation Temperature

Figure 4.5 shows the effect of incubating the reaction mixtures at a range of temperatures from 4 - 37°C on the kinetics of the response. All the absorbance readings were taken after 30 min. For all the temperatures, the difference between the control (MS-treated) and experimental wells (con A stimulated) was significant (at 4°C $p = 0.011$, $n = 7$; at 10°C $p = 0.013$, $n = 7$; at 18°C $p = 0.008$, $n = 7$; at 25°C $p = 0.008$, $n = 7$; at 37°C $p = 0.003$, $n = 7$). However, comparison of the different temperatures showed that there was no significant difference in the reaction kinetics of the response. Consequently, all the plates were incubated at 20°C.

### 4.3.5. Determination of Energy Requirements

Haemocytes harvested into normal, glucose-containing AC produced an increase in absorbance to ca 0.050 at 550 nm after 30 min incubation with PMA. This was significantly higher than the MS-treated controls where the absorbance was ca 0.039 ($p = 0.003$, $n = 3$). Inclusion of 10 or 100 mM glucose in both experimental and control wells produced no further increase in the absorbance values (i.e. the

Figure 4.4.

Figure 4.4. Reduction of ferricytochrome c at 550 nm by each cell type of *C. maenas in vitro*.

Twenty-five microlitres of each cell type (1-9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM ferricytochrome c were challenged with 25 µl of PMA (5 µg $ml^{-1}$, final concentration) or, for controls with 25 µl of 0.2 µm filtered MS. Values are means in absorbance at 550 nm ± S.E.M. after 30 min incubation at 20°C. n = 5 animals.

*** Differences between the experimental (PMA-incubated) and control (MS-treated) group are significant at $p \leq 0.001$.

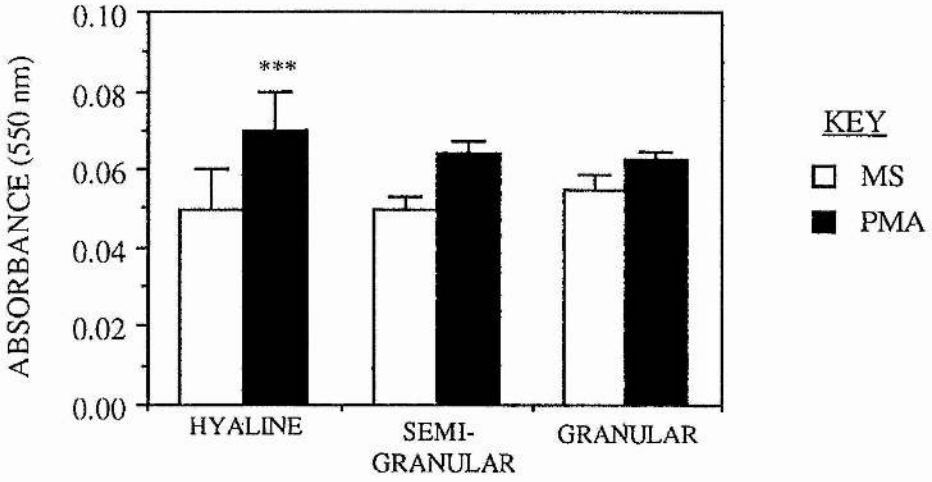

0.10
0.08
0.06
0.04
0.02
0.00
ABSORBANCE (550 nm)
***
HYALINE
SEMI-
GRANULAR
GRANULAR
KEY
MS
PMA

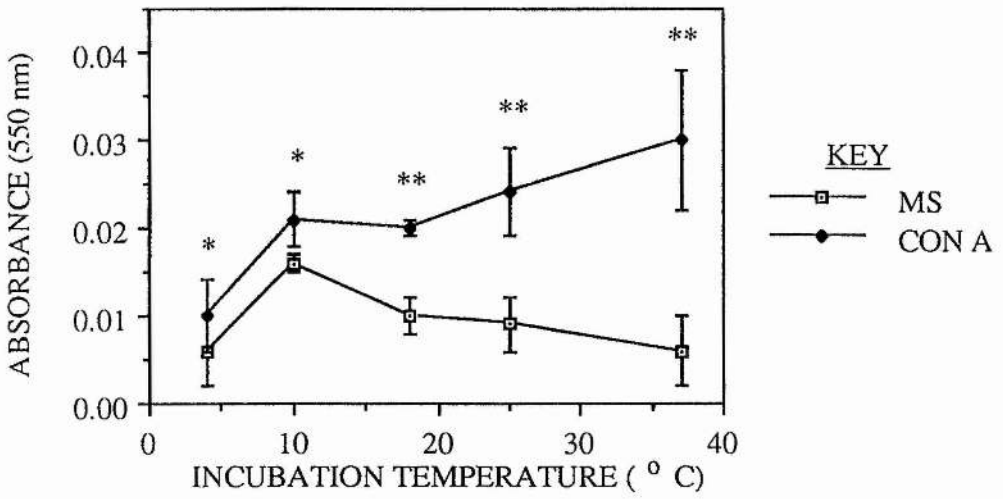
Figure 4.5.

Figure 4.5. Reduction of ferricytochrome c at 550 nm by separated hyaline cells of *C. maenas* at different incubation temperatures *in vitro*.

Twenty-five microlitres of cells (1-9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM ferricytochrome c were either challenged with 25 µl of con A (5 µg $ml^{-1}$, final concentration) or, for the controls with 25 µl of 0.2 µm filtered MS. Values are means in absorbance at 550 nm ± S.E.M. after 30 min incubation at 20°C. n = 8 animals.

* Differences between the experimental (con A-incubated) and control (MS-treated) group are significant at $p \leq 0.05$.

** Differences between the experimental (con A-incubated) and control (MS-treated) group are significant at $p \leq 0.01$.


ABSORBANCE (550 nm)
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
INCUBATION TEMPERATURE ( ° C)
*
*
**
**
**
KEY
MS
CON A

absorbance remained at ca 0.050 for the experimentals and ca 0.039 for the controls). Cells bled into glucose-free AC, however, did not produce a significant reduction in ferricytochrome c when treated with PMA. In this case, the absorbance value was ca 0.035, which was similar to the MS-incubated samples where the absorbance was ca 0.043 ($p = 0.130$, $n = 3$). Again, inclusion of glucose (10 mM) did not produce a significant change in absorbance values following incubation of the cells with PMA ($p = 0.192$, compared with MS-treated controls, $n = 3$).

### 4.3.6. Confirmation of Superoxide Production

Addition of exogenous SOD to PMA-stimulated cells resulted in lower absorbance values (ca 0.047) than the PMA-stimulated cells alone, for which the absorbance was ca 0.057 after 30 min (Table 4.1) ($p = 0.011$, $n = 6$). Inactivation of SOD by boiling for 30 min resulted in the loss of inhibition of ferricytochrome c reduction confirming that SOD acts specifically to scavenge superoxide ions.

### 4.3.7. Effect of Different Elicitors

Table 4.2 compares the effect of different elicitors on the hyaline cells. All the absorbance readings were taken after 30 min. Incubation of the cells in LPS produced a significant reduction in ferricytochrome c ($p = 0.046$, compared with the MS-treated controls, $n = 5$) whereas laminarin failed to elicit a change in absorbance ($p = 0.439$, compared with the MS-treated controls, $n = 6$) (Table 4.2). Occasionally some animals did not respond to LPS stimulation and further examination of these animals showed that the cells contained intracellular bacteria, possibly because the host had received a natural infection. The data from these specimens were not included in the statistical analysis. Concanavalin A ($n = 7$) and PHA-P ($n = 6$) always stimulated a

reduction in ferricytochrome c ($p < 0.001$ and $p < 0.001$, respectively) in contrast to the cells treated with 0.2 μm filtered MS (Table 4.2).

Table 4.1.

Table 4.1. Effect of SOD on ferricytochrome c reduction at 550 nm by separated hyaline cells of *C. maenas in vitro*.

[a] Twenty-five microlitres of hyaline cells (1 - 9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM of ferricytochrome c were challenged with 25 µl of PMA (5 µg $ml^{-1}$, final concentration) and 20 µl of either SOD (100 units $ml^{-1}$, final concentration) or 0.2 µm MS.

[b] Readings given are the mean absorbance values at 550 nm ± S.E.M. after 30 min incubation at 20°C. n = 6 animals.

[c] Cells treated with 25 µl of 0.2 µm filtered MS to act as buffer control.

[d] Cells incubated with PMA

[e] Cells incubated with PMA and SOD

[f] Cells incubated with PMA and boiled SOD

[g] Cells incubated without the PMA and only the SOD.

| Treatment[a] | Absorbance at 550 nm[b] |
|---|---|
| MS + MS[c] | 0.045 ± 0.005 |
| PMA + MS[d] | 0.057 ± 0.005 |
| PMA + SOD[e] | 0.047 ± 0.005 |
| PMA + SOD[f] | 0.056 ± 0.004 |
| MS + SOD[g] | 0.047 ± 0.005 |

Table 4.2.

Table 4.2. Effect of different elicitors on ferricytochrome c reduction at 550 nm by separated hyaline cells of *C. maenas in vitro*.

[a] Twenty-five microlitres of hyaline cells (1 - 9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM of ferricytochrome c were challenged with 25 µl of the appropriate elicitor (5 µg $ml^{-1}$, final concentration) at 20°C.

[b] Cells treated with 25 µl of 0.2 µm filtered MS.

[c] Cells challenged with 25 µl of elicitor (5 µg $ml^{-1}$, final concentration).

[d] number of animals.

[e] Values are the means in absorbance at 550 nm ± S.E.M.

[f] Phytohaemagglutinin.

* Differences between the experimental (elicitor-incubated) and control (MS-treated) group are significant at $p \leq 0.05$.

** Differences between the experimental (elicitor-incubated) and control (MS-treated) group are significant at $p \leq 0.01$.

*** Differences between the experimental (elicitor-incubated) and control (MS-treated) group are significant at $p \leq 0.001$.

| Treatment[a] | Con[b] | Exp[c] | n[d] |
|---|---|---|---|
| PMA (5 μg $ml^{-1}$) | 0.030 ± 0.01[e] | 0.050 ± 0.01** | 6 |
| CON A (5 μg $ml^{-1}$) | 0.030 ± 0.01 | 0.055 ± 0.01*** | 7 |
| PHA-P[f] (5 μg $ml^{-1}$) | 0.027 ± 0.002 | 0.036 ± 0.001*** | 6 |
| LPS (5 μg $ml^{-1}$) | 0.030 ± 0.010 | 0.036 ± 0.007* | 5 |
| Laminarin (5 μg $ml^{-1}$) | 0.030 ± 0.010 | 0.030 ± 0.010 | 6 |

## 4.4. DISCUSSION

The results of this set of experiments demonstrate that reduction of ferricytochrome c occurs when the hyaline cells of the shore crab, *Carcinus maenas*, are stimulated by PMA and other elicitors including PHA-P, con A and LPS. Laminarin failed to elicit this response.

The effect of SOD on the response of the crab cells to PMA was also determined. Superoxide dismutase is known to convert $O_2^-$ to molecular oxygen and $H_2O_2$ (Fridovich, 1978). Removal of the $O_2^-$ ions decreases the amount of ferricytochrome c reduction occurring in the wells. The results described in the present chapter show that when the cells are incubated with exogenous SOD and PMA, the absorbance values are less than when the haemocytes are incubated without SOD. This confirms that the observed reduction of ferricytochrome was due, at least in part, to $O_2^-$. Furthermore, the failure of the cells to produce $O_2^-$ in the absence of glucose indicates that the respiratory burst is dependent upon the HMS, in agreement with results obtained from fish (Secombes *et al.*, 1988) and mammals (Sbarra and Karnovsky, 1959). However, the reason why the addition of glucose to cells bled into glucose-free AC failed to restore the response is as yet unknown.

The effect of the spontaneous dismutation of $O_2^-$ to $H_2O_2$ on absorbance values was studied by Vandewalle and Petersen (1987). From spectrophotometric studies of a cell-free system, these authors proposed that $H_2O_2$, the major product of the spontaneous dismutation of the superoxide radicals, is capable of oxidising ferrocytochrome c (Fe II) thus counteracting the increase in optical density due to $O_2^-$ production. Turrens and McCord (1988), however, thought that the amount of $H_2O_2$ produced is not enough to affect ferricytochrome c (Fe III) reduction. In these experiments with *C. maenas* haemocytes, it was found that the absorbance values

were generally higher in the presence of catalase (Fig. 4.2). Catalase was, therefore, routinely included in the reaction mixtures to counteract the oxidation of the ferrocytochrome c (Fe II).

For all the experiments undertaken in the present study there was a decline in the optical density over the 30 min incubation period despite the presence of catalase. In contrast, studies of the respiratory burst in fish and mammals have revealed that the optical density tends to increase as the reaction proceeds. At present it is still unknown why crab hyaline cells produce a decrease in absorbance. It is possible the haemocytes produce such large quantities of $H_2O_2$ that optical density declines rather than increases (see Chapter 5 for further investigation into this matter).

The change in absorbance observed in *C. maenas* was also consistently lower than those observed in fish and mammals (Bayne and Levy, 1991; Johnston *et al.*, 1975). This may be partly accounted for by the relatively low rates of uptake that crab haemocytes exhibit, *in vitro* (Smith and Ratcliffe, 1978; Söderhäll *et al.*, 1986). Uptake levels of opsonised bacteria are only approximately 15% (Smith and Ratcliffe, 1978; see also Chapter 2). With vertebrates, uniform cell cultures are used routinely for experimental analyses and the cells are likely, therefore, to respond in the same way; i.e. with all the cells exhibiting a burst, the magnitude of the response will be greater. In crabs, the population of hyaline cells is heterogeneous (Bauchau, 1981) so it is possible that a proportion of the cells do not respond to stimulation, even with an elicitor as potent as PMA.

The differential effect of the elicitors on the hyaline cells, in this study, may indicate a variety of surface receptors on the phagocyte membrane. Other researchers have also found a differential effect on oxygen radical production with a range of

elicitors (Nakamura *et al.*, 1985; Ito *et al.*, 1992). Nakamura *et al.* (1985) demonstrated that scallop, *P. yessoensis*, amoebocytes produce a greater amount of $H_2O_2$ when stimulated by con A than when the cells were stimulated by the bacteria *Micrococcus luteus*, *Escherichia coli* or *Arthrobacter* sp. Similarly, Ito *et al.* (1992) showed that the phagocytes of the sea urchin, *S. nudus*, produce a different amount of $H_2O_2$ when stimulated by sheep red blood cells compared to human red blood cells. Further research on crustacean cells may provide more information on the type of receptors that are present on the haemocyte membrane.

The results of this chapter show that crustacean haemocytes do possess the ability to produce a respiratory burst when stimulated by exogenous materials. This metabolic process probably plays a part in the antimicrobial defense of the organism in a similar way to that reported for mammals (Gabig and Babior, 1981). However, it needs to be proven that a respiratory burst occurs when the hyaline cells actually phagocytose and that $O_2^-$ and $H_2O_2$ play a part in the antimicrobial activity in crustacean host defence. Adema *et al.* (1991a) have established that in the snail, *Lymnaea stagnalis*, the generation of oxyradicals can be detected extracellularly until the ingestion stage of phagocytosis is complete. Following internalization extracellular oxygen radical generation declines but production remains high intracellularly which suggests a possible role for oxygen radicals in the removal of ingested non-self material (Adema *et al.*, 1991a). It would be interesting to determine if the same pattern of $O_2^-$ production is observed in crustacean haemocytes. Experiments also need to be carried out to determine further the biochemical details of this response in the crab. In the following chapter, a comparison of the reaction kinetics of the respiratory burst response of crab hyaline cells and the phagocytes of other marine invertebrates will be made. In addition $H_2O_2$ production by *C. maenas* hyaline cells will be quantified.

# Chapter 5

## Superoxide production by the phagocytes of a range of marine invertebrate species *in vitro*

# 5.1. INTRODUCTION

The reduction of ferricytochrome c (Fe III) by $O_2^-$ ions has been used in the study of the respiratory burst in mammalian (Pick and Mizel, 1981) and fish blood cells (Secombes *et al.*, 1988). More recently, this procedure has been applied to the snail, *Lymnaea stagnalis*, (Adema *et al.*, 1991a) and the shore crab, *Carcinus maenas*, (Chapter 4), but has not been utilised extensively thus far for other invertebrate groups.

With *C. maenas*, the phagocytic hyaline cells are capable of a respiratory burst *in vitro* after stimulation by a range of soluble elicitors (Chapter 4). The inclusion of SOD in the reaction mixture confirmed that superoxide ions are produced by the stimulated haemocytes (Chapter 4). Despite the production of $O_2^-$ ions by the crab hyaline cells and the inclusion of catalase in the assay mixtures there is a decline in the optical density at 550 nm over the 30 min incubation period (Chapter 4), whereas in fish and mammals, the optical density of the reaction mixtures at 550 nm increases as the reaction proceeds (Pick and Mizel, 1981; Secombes *et al.*, 1988). This difference in kinetics could be accounted for by variable levels of $H_2O_2$ production by the phagocytes of different species.

The present set of experiments were carried out firstly to determine whether the generation of a respiratory burst by circulating phagocytes is a general phenomenon for invertebrates; secondly to compare the kinetics of the reaction in different species, and thirdly, to examine $H_2O_2$ production in those species showing different patterns of response following stimulation. Although the respiratory burst phenomenon in *C. maenas* was described in Chapter 4, this species is included in the present investigation as a control and as the basis for the $H_2O_2$ production analyses.

## 5.2. MATERIALS AND METHODS

### 5.2.1. Animals

Specimens of *C. maenas* ; the squat lobster, *Galathea strigosa*; the Norway lobster, *Nephrops norvegicus*; the edible sea urchin, *Echinus esculentus*; and the lugworm, *Arenicola marina*, were collected from St. Andrews Bay, Scotland. The tunicate, *Ciona intestinalis*, and the common mussel, *Mytilus edulis*, were obtained from the west coast of Scotland. All the animals were maintained in tanks with a flow through seawater system (32 ± 2‰; 10 ± 2°C) for two weeks prior to use and only healthy animals were subsequently used for experimental purposes.

### 5.2.2. Harvesting and Separation of Phagocytes

Blood samples were collected from all species, except the tunicate, using syringes and needles appropriate to the size of the host. In every case the area to be sampled was surface sterilized with 96% ethanol and care was taken to avoid contamination of the blood with pyrogens.

With the crustaceans, haemolymph removal and cell separation were carried out as previously described (Smith and Söderhäll, 1991; see also Chapter 2 sections 2.2.3. and 2.2.7.). With *C. intestinalis*, both bleeding and cell separation were carried out as described by Smith and Peddie (1992). Briefly, the tunicates were blotted dry, the tunics removed and the animals bled by cardiac puncture. The mussels were narcotised by placing them in 7.5% $MgCl_2.6H_2O$ for 20 min prior to the removal of the haemolymph from the adductor muscle (Smith and Söderhäll, 1991). Approximately 0.5 ml of haemolymph was collected from each mussel into 0.5 ml of 0.2 µm filtered marine anticoagulant (MAC) (0.1 M glucose; 15 mM trisodium citrate; 13 mM citric acid; 10 mM

EDTA; 0.45 M sodium chloride; pH 7.0) (Smith and Peddie, 1992). The sea urchins were bled through the peristomial membrane (Wardlaw and Unkles, 1978) and the fluid immediately diluted 1:1 in 0.2 μm filtered MAC. The sea urchin cells were spun at 1,900 g for 5 min at 4°C and resuspended in 1 ml of 0.2 μm filtered MAC to concentrate the number of cells prior to separation. With *A. marina*, approximately 1 ml of coelomic fluid was removed per animal from the coelomic cavity at the midlateral region (Smith and Söderhäll, 1991). Table 5.1 shows the osmolality of the blood of the different species and of the buffers in which the cells were suspended.

Wherever possible experiments were carried out on separated phagocytic cell fractions from individual animals. To separate the cells, mixed cell suspensions (ca 2 ml) were loaded onto individual 60% preformed Percoll (Pharmacia) continuous gradients in 0.2 μm filtered 3.2% NaCl and spun at 1,900 g for 10 min at 4°C (Söderhäll and Smith, 1983).

In each case identification of the separated phagocytes was carried out under phase contrast optics using the criteria given for crustaceans by Bauchau (1981), for *C. intestinalis* by Rowley (1981), for echinoderms by Smith (1981) and for polychaetes by Dales and Dixon (1981). After separation, the phagocytic cell fractions from the crustaceans, tunicate and echinoderm species were harvested and diluted with freshly made 0.2 μm filtered MS to a final concentration of ca 1 - 9 x $10^7$ cells $ml^{-1}$. Due to the low yield of phagocytic coelomocytes from *A. marina*, these cells were resuspended in 0.2 μm filtered MS at a concentration of ca 1 - 9 x $10^5$ cells $ml^{-1}$. For *M. edulis*, insufficient quantities of haemocytes from individual animals could be harvested for the cell separation, so the analyses were made on unseparated cell populations obtained from individual animals.

Table 5.1.

Table 5.1. Osmolality values for the blood of the experimental animals and the buffers. The osmolality values were measured by freezing point depression (Robling Automatic, Camlab, Cambridge) which had been calibrated against a standard (300 mOsm $kg^{-1}$ $H_2O$) (Camlab).

| Sample | Osmolality (mOsm $kg^{-1}$) |
|---|---|
| Aquarium seawater | 980 |
| Marine anticoagulant | 1020 |
| Marine Saline | 1090 |
| *C. maenas* haemolymph | ca 1026 |
| *G. strigosa* haemolymph | ca 1018 |
| *N. norvegicus* haemolymph | ca 1013 |
| *C. intestinalis* blood | ca 990 |
| *M. edulis* haemolymph | ca 985 |
| *E. esculentus* coelomic fluid | ca 980 |
| *A. marina* coleomic fluid | ca 990 |

## 5.2.3. Superoxide Anion Assay

The assay was carried out as described in Chapter 4. The elicitors used were PMA or con A and the final concentration of both (5 $\mu g\ ml^{-1}$) was selected on the basis of the results of the dose response experiments that had been previously carried out (Chapter 4, Section 4.2.3.b).

A parallel series of experiments were run in which the enzyme, SOD, was included in the experimental (ie PMA-treated) wells. Superoxide dismutase converts $O_2^-$ ions to hydrogen peroxide and molecular oxygen (Fridovich, 1978). The removal of the $O_2^-$ ions would lower the amount of ferricytochrome c reduction in the wells leading to smaller changes in absorbance. The assay was carried out as before (Chapter 4, Section 4.2.3.f).

## 5.2.4. Hydrogen Peroxide Assay

Quantification of $H_2O_2$ production was carried out with modifications according to the method of Pick and Mizel (1981).

The assay was carried out in microtitre plates as described for the superoxide assay. Twenty-five microlitres of cell suspension (1-9 x $10^7$) were pipetted into each well and the cells were allowed to settle and attach for 15 min. Excess liquid was then aspirated with a pipette. The experimental wells consisted of the attached cells, 100 µl of phenol red solution (PR$) (0.45 M sodium chloride; 10 mM potassium phosphate buffer - pH 7.0; 5.5 mM glucose; 0.2 g $l^{-1}$ phenol red; 19 U $ml^{-1}$ horseradish peroxidase, Type II, Sigma) and 50 µl of con A (5 $\mu g\ ml^{-1}$, final concentration in MS). Concanavalin A was selected in favour of PMA as previous studies of $H_2O_2$ production by the phagocytes of marine invertebrates utilised con A as the elicitor (Nakamura *et al.*, 1985). For the

control, 0.2 μm filtered MS was substituted for con A. All mixtures were then incubated for 30 min at 20°C. Ten microlitres of 1 N sodium hydroxide were then added to each well to induce cell death and terminate the production of $H_2O_2$. The plate was centrifuged at 500 g for 5 min at 4°C to remove any cell debris. The absorbance of the cell-free supernatants was then measured at 630 nm on a microplate reader (Dynatech MR5000) and the mean absorbance values for the quadruplicate wells determined for each treatment. The wells were read against quadruplicate blank wells containing PRS, sodium hydroxide and MS in the appropriate proportions.

Standard solutions of $H_2O_2$ were prepared by diluting a 10 mM stock solution of $H_2O_2$ with MS prior to use. The actual concentration of the stock solution was determined from its absorption at 240 nm, using an extinction coefficient 43.6 $M^{-1}$ $cm^{-1}$ (Claiborne, 1985). The change in absorbance was then standardised by adding 50 μl of known amounts of $H_2O_2$ to the assay mixture. Hydrogen peroxide production was calculated as nmoles $10^7$ $cells^{-1}$ 30 $min^{-1}$.

### 5.2.5. Statistical Analysis

Differences between treatments were statistically analysed by paired Student's t-test on combined data from a minimum of 3 - 5 animals from each species. Differences were considered significant when $p \leq 0.05$ (Sokal and Rohlf, 1981).

## 5.3. RESULTS

Prior to the addition of PMA, con A or for the controls, MS, the absorbance values of all the wells for each species were stable and there were no significant differences between the experimental and control treatments (Fig. 5.1 and 5.2) ($p > 0.05$ for all species).

### 5.3.1. Superoxide Anion Assay

The addition of the various reagents resulted in a rapid change in absorbance although the pattern of the response varied according to the treatment and species (Fig. 5.1). In each case, the inclusion of PMA or con A in the reaction mixtures caused an increase in absorbance within 5 min from the time zero value, although with *C. maenas*, the stimulatory effect of con A was much less than that of PMA (Fig. 5.1A). The absorbance values for *C. maenas* and *G. strigosa* remained elevated over the 30 min incubation period (Fig. 5.1A,B) but gradually declined with *N. norvegicus* (Fig. 5.1C). With the controls, addition of filtered MS to *C. maenas* and *N. norvegicus* cells also resulted in an increase in absorbance within 5 min, although the magnitude of the response was less than that induced by the two elicitors. For both species, the absorbance values in the control (MS-treated) wells then steadily decreased over the next 25 min to below that of their respective time zero values (Fig. 5.1A,C). For the controls, MS produced no marked change in the absorbance at any time point for *G. strigosa.* (Fig. 5.1B).

Variations in the individual baseline values were found although the underlying trends remained consistent. Equivalent variations between individual animals have been noted in other studies (see review by Adema *et al.*, 1991b). Calculation of the mean values for each treatment and species thus tended to produce large standard errors which complicated the interpretation of the data for replicate experiments. Statistical analysis using paired t-

Figure 5.1. A & B.

Figure 5.1 A, B. Reduction of ferricytochrome c at 550 nm by separated phagocytes from two crustacean species *in vitro*.

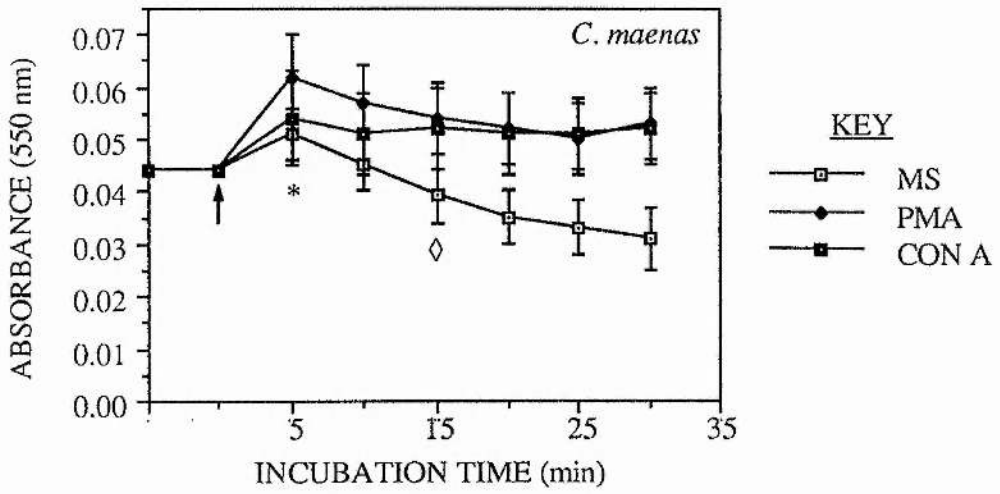
Twenty-five microlitres of cells (1 - 9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM ferricytochrome c were challenged with 25 µl of PMA (5 µg $ml^{-1}$, final concentration) or, with 25 µl of con A (5 µg $ml^{-1}$, final concentration) or, for the controls, with 25 µl of 0.2 µm filtered MS. The change in absorbance was measured at 5 min intervals at 550 nm for 30 min after stimulation. Values shown are the mean absorbance at 550 nm ± S.E.M. from a minimum of 5 animals per treatment for each species.

* Differences between the PMA-incubated and buffer-treated controls are significant at $p \leq 0.05$.

◊ Differences between the con A-incubated and buffer-treated controls are significant at $p \leq 0.05$.

↓ point of addition of either PMA or con A for the experimental or MS for the control groups.

5.1 A.


C. maenas
ABSORBANCE (550 nm)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
5
15
25
35
INCUBATION TIME (min)
*
◊
KEY
MS
PMA
CON A

5.1 B.

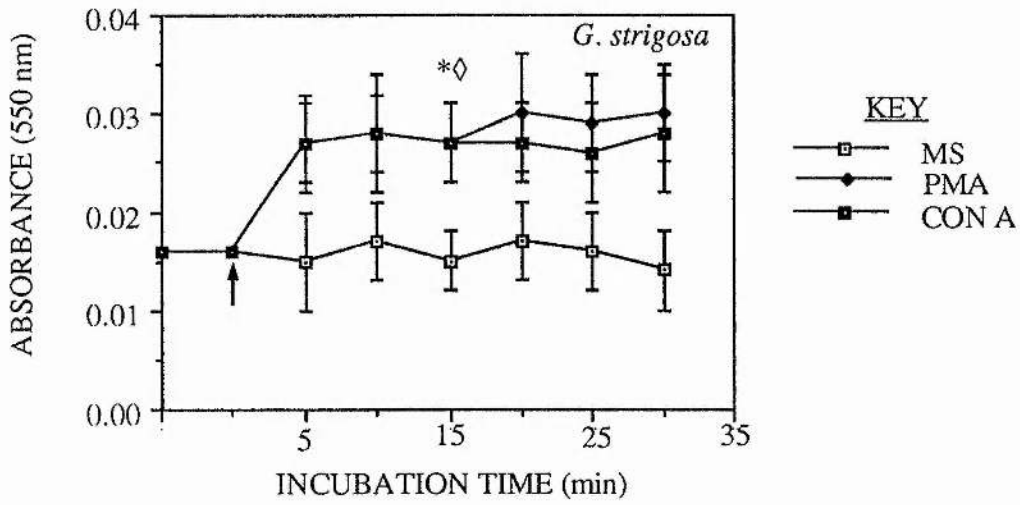

G. strigosa
ABSORBANCE (550 nm)
0.04
0.03
0.02
0.01
0.00
5
15
25
35
INCUBATION TIME (min)
*◊
KEY
MS
PMA
CON A

Figure 5.1. C.

Figure 5.1 C. Reduction of ferricytochrome c at 550 nm by separated phagocytes from *N. norvegicus in vitro*.

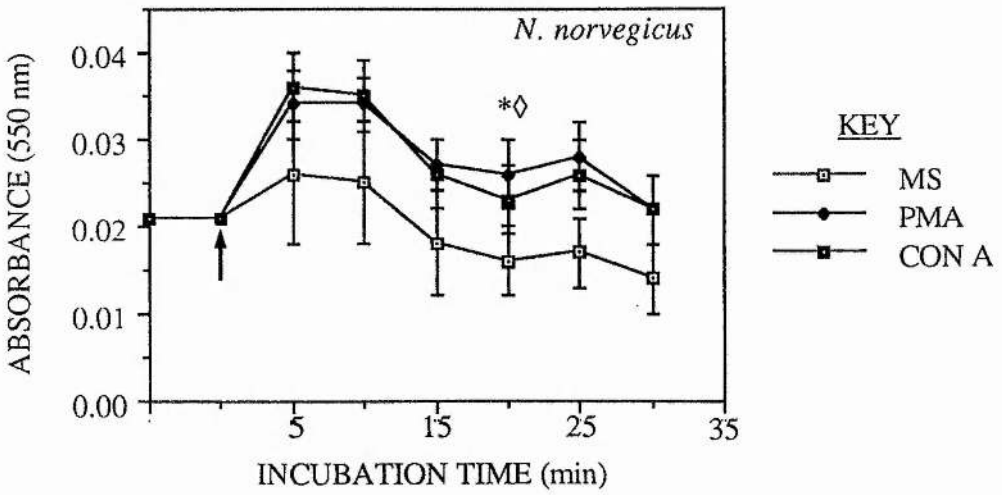
Twenty-five microlitres of cells (1 - 9 x $10^7$ cells $ml^{-1}$) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM ferricytochrome c were challenged with 25 µl of PMA (5 µg $ml^{-1}$, final concentration) or, with 25 µl of con A (5 µg $ml^{-1}$, final concentration) or, for the controls, with 25 µl of 0.2 µm filtered MS. The change in absorbance was measured at 5 min intervals at 550 nm for 30 min after stimulation. Values shown are the mean absorbance at 550 nm ± S.E.M. from a minimum of 5 animals per treatment for each species.

* Differences between the PMA-incubated and buffer-treated controls are significant at $p \leq 0.05$.

◊ Differences between the con A-incubated and buffer-treated controls are significant at $p \leq 0.05$.

↟ point of addition of either PMA or con A for the experimental or MS for the control groups.

5.1 C.


N. norvegicus
ABSORBANCE (550 nm)
0.04
0.03
0.02
0.01
0.00
*◊
5
15
25
35
INCUBATION TIME (min)
KEY
MS
PMA
CON A

tests allowed the control and experimental data for individual animals to be compared pairwise, so the true effects of different treatments on the cells could be determined.

These analyses show that, with *C. maenas*, differences between the experimental and control wells became significant at 5 min with PMA ($p = 0.044$, $n = 5$) and 15 min with con A ($p = 0.027$, $n = 5$) (Fig. 5.1A). In contrast, with *G. strigosa*, both PMA and con A produced a significantly higher absorbance than the MS control at 15 min ($p = 0.022$ for PMA; $p = 0.010$ for con A, $n = 5$) (Fig. 5.1B), while with *N. norvegicus*, differences between experimentals and controls became apparent at 20 min for both elicitors ($p = 0.015$ for PMA; $p = 0.039$ for con A, $n = 5$) (Fig. 5.1C). For these three crustaceans, the differences in the absorbance values between the experimental and control groups were statistically significant at all subsequent incubation times.

With the non-crustacean invertebrates, increases in absorbance at 550 nm were also observed after the addition of PMA or con A and, again, the magnitude of the responses varied according to the species and elicitor used (Fig. 5.2). For *C. intestinalis* and *M. edulis*, this increase in absorbance commenced within 5 min of exposure to the elicitor and the absorbance values continued to increase over the next 25 min (Fig. 5.2A,B). The phagocytes of *E. esculentus* and *A marina*, in contrast, showed an initial increase in absorbance at 5 min but thereafter remained constant at or near this elevated level for the duration of the assay (Fig. 5.2C,D).

Both con A and PMA were effective in eliciting an increase in absorbance at 550 nm for the blood cells of the non-crustacean invertebrates, although, in general, the response to PMA was stronger than to con A (Fig. 5.2A-D). The elicitors also stimulated a greater increase in absorbance than the MS-treated controls. In all cases, MS produced a slight rise in absorbance at 550 nm over the 30 min incubation period (Fig. 5.2). *A. marina*

Figure 5.2. A & B.

Figure 5.2 A, B. Reduction of ferricytochrome c at 550 nm by separated phagocytes from a tunicate and molluscan species *in vitro*.

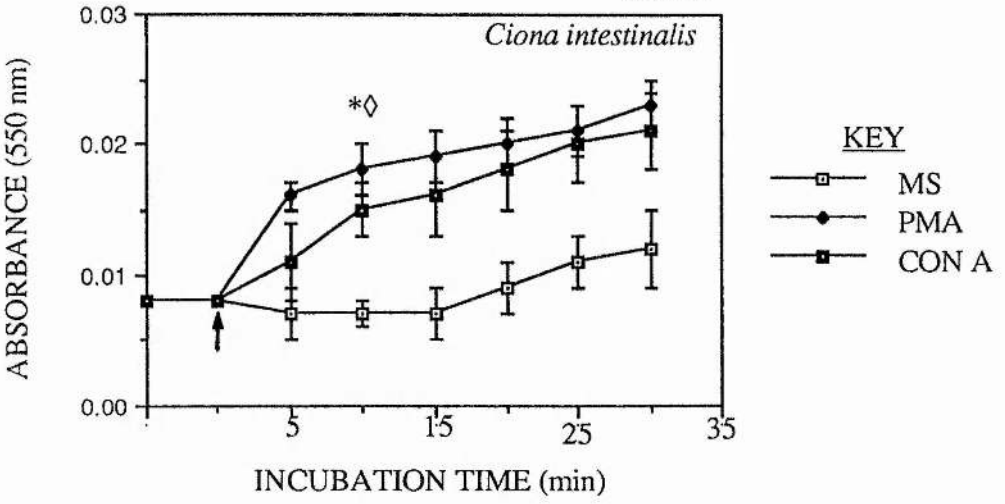
Twenty-five microlitres of cells (See Materials and Methods for concentration) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM ferricytochrome c were challenged with 25 µl of PMA (5 µg $ml^{-1}$, final concentration) or, with 25 µl of con A (5 µg $ml^{-1}$, final concentration) or, for the controls, with 25 µl of 0.2 µm filtered MS. The change in absorbance was measured at 5 min intervals at 550 nm for 30 min after stimulation. Values shown are means in absorbance at 550 nm ± S.E.M. from a minimum of 4 animals per treatment for each species.

* Differences between the PMA-incubated and buffer-treated controls are significant at $p \leq 0.05$.

◊ Differences between the con A-incubated and buffer-treated controls are significant at $p \leq 0.05$.

↑ point of addition of either PMA or con A for the experimental or MS for the control groups.

5.2. A.


Ciona intestinalis
*◊
ABSORBANCE (550 nm)
0.03
0.02
0.01
0.00
5
15
25
35
INCUBATION TIME (min)
KEY
MS
PMA
CON A

5.2. B.

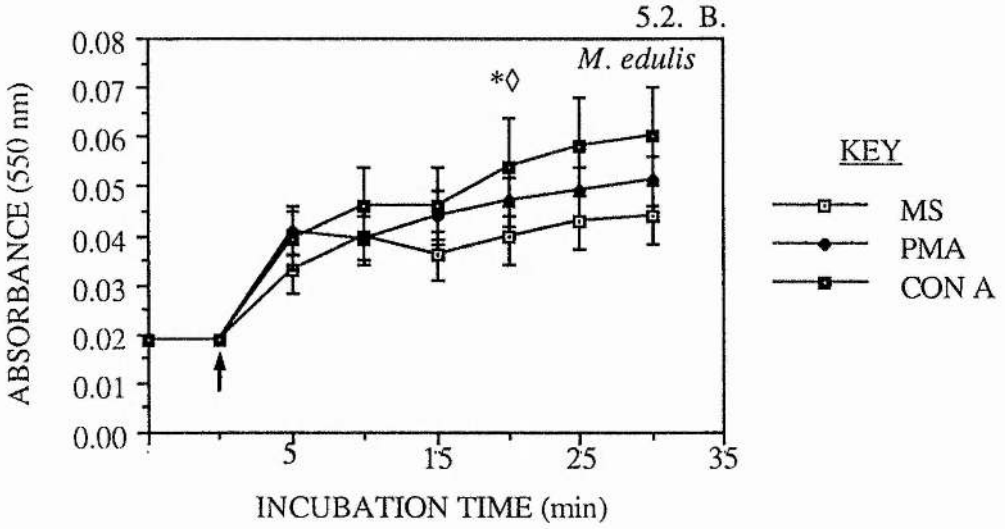

M. edulis
*◊
ABSORBANCE (550 nm)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
5
15
25
35
INCUBATION TIME (min)
KEY
MS
PMA
CON A

Figure 5.2. C & D.

Figure 5.2 C, D. Reduction of ferricytochrome c at 550 nm by separated phagocytes from an echinoderm and an annelid species *in vitro*.

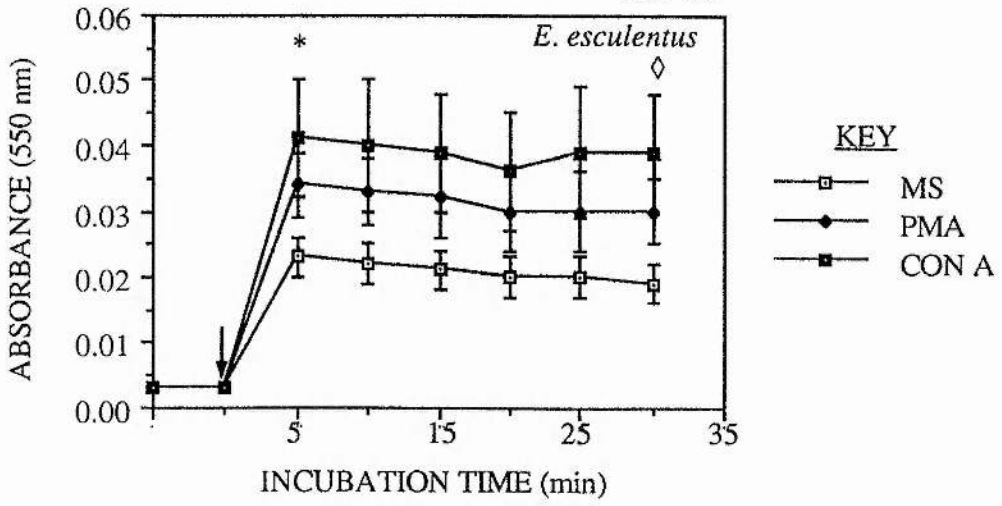
Twenty-five microlitres of cells (See Materials and Methods for concentration) incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration) and 50 µl of 160 µM ferricytochrome c were challenged with 25 µl of PMA (5 µg $ml^{-1}$, final concentration) or, with 25 µl of con A (5 µg $ml^{-1}$, final concentration) or, for the controls, with 25 µl of 0.2 µm filtered MS. The change in absorbance was measured at 5 min intervals at 550 nm for 30 min after stimulation. Values shown are means in absorbance at 550 nm ± S.E.M. from a minimum of 4 animals per treatment for each species.

* Differences between the PMA-incubated and buffer-treated controls are significant at $p \leq 0.05$.

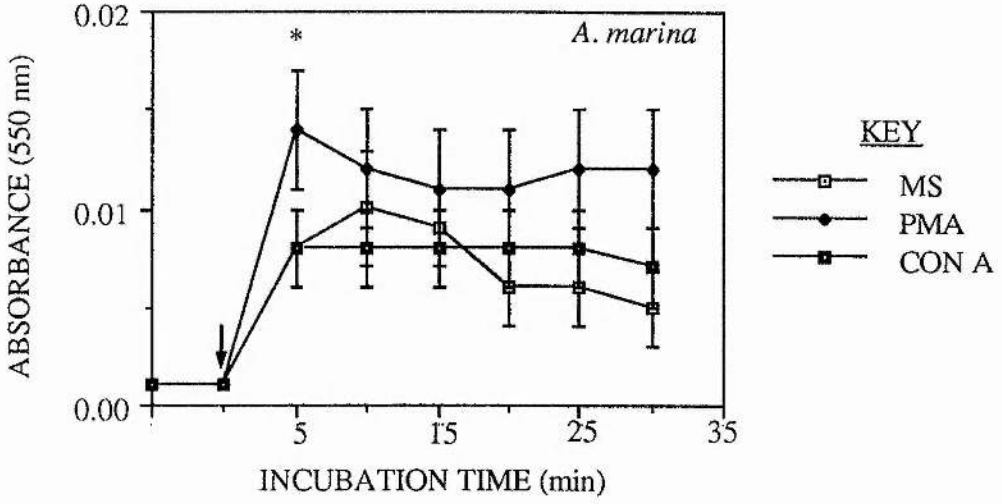
◊ Differences between the con A-incubated and buffer-treated controls are significant at $p \leq 0.05$.

↓ point of addition of either PMA or con A for the experimental or MS for the control groups.

5.2. C.


E. esculentus
*
◊
ABSORBANCE (550 nm)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
5
15
25
35
INCUBATION TIME (min)
KEY
MS
PMA
CON A

5.2. D.


A. marina
*
ABSORBANCE (550 nm)
0.02
0.01
0.00
5
15
25
35
INCUBATION TIME (min)
KEY
MS
PMA
CON A

was unusual in that there was a higher absorbance in the MS-treated wells after 10 min than in the con A-incubated samples but it was not a significant difference ($p > 0.05$) (Fig. 5.2D). However, after this time the absorbance of the control wells declined steadily to a value below that of the two elicitors by the end of the experimental period. As seen with the crustaceans, wide variations occurred in the values obtained for individual animals, although the pattern of the response was always the same for replicates within each species.

Analysis of the data by paired t-tests showed that the difference in the absorbance at 550 nm between the experimental and control (MS-treated) wells became significant after 10 min for *C. intestinalis* ($p = 0.034$ for PMA; $p = 0.040$ for con A; $n = 4$; after 15 min $p = 0.009$ for PMA and $p = 0.005$ for con A; $n = 4$) and 20 min for *M. edulis* ($p = 0.003$ for PMA; $p = 0.028$ for con A, $n = 6$). With *E. esculentus* phagocytes, the difference in the absorbance values between the PMA and MS-treated wells became significant after 5 min ($p = 0.018$, $n = 5$), but the difference between con A and the respective control incubated samples only became significant after 30 min ($p = 0.043$, $n = 5$). At this time, the level of significance is very close to the critical level, so the effect of con A on sea urchin phagocytes must be regarded as equivocal. Certainly, statistical analysis shows that, whereas PMA produced a significant increase in absorbance after 5 min ($p = 0.012$, $n = 7$ compared to the MS-treated controls), con A did not exert a significant response in *A. marina* phagocytes compared to the MS-treated controls at any of the time points. The difference in absorbance between experimental and control groups was statistically significant for all species for the remainder of the incubation period.

Table 5.2 shows the effect of including SOD in the experimental mixtures. A loss or decline in the observed stimulatory effect of PMA when incubated with SOD provides confirmation that $O_2^-$ ions are produced. The strongest effects of SOD in the presence of

Table 5.2.

Table 5.2. Effect of SOD on ferricytochrome c reduction at 550 nm by separated phagocytes, *in vitro*, from different species.

[a] Readings are the absorbance values at 550 nm after 30 min incubation with the treatment. Values shown are the means ± S.E.M. for each species. Each treatment was repeated 4 - 14 times with different animals for each species.

[b] Twenty-five microlitres of cells challenged with 25 µl of PMA (5 µg $ml^{-1}$, final concentration) were incubated with 20 µl of catalase (4 µg $ml^{-1}$, final concentration), 50 µl of 160 µM of ferricytochrome c, and 20 µl of either SOD (100 units $ml^{-1}$, final concentration) or MS at 20°C.

[c] Cells treated with 25 µl of MS instead of PMA.

[d] Cells incubated with SOD and MS in place of PMA.

| Species | Absorbance at 550 nm[a] | | | |
|---|---|---|---|---|
| | PMA[b] | PMA + SOD[b] | MS (Control)[c] | SOD[d] |
| *E. esculentus* n=4 | 0.019 (±0.003) | 0.011 (±0.001) | 0.009 (±0.002) | 0.009 (±0.004) |
| *N. norvegicus* n=4 | 0.019 (±0.001) | 0.011 (±0.002) | 0.015 (±0.002) | 0.014 (±0.002) |
| *C. intestinalis* n=7 | 0.031 (±0.005) | 0.025 (±0.005) | 0.019 (±0.004) | 0.018 (±0.004) |
| *M. edulis* n=6 | 0.049 (±0.005) | 0.044 (±0.005) | 0.041 (±0.005) | 0.037 (±0.006) |
| *A. marina* n=5 | 0.008 (±0.001) | 0.003 (±0.0006) | 0.005 (±0.001) | 0.003 (±0.001) |
| *G. strigosa* n=14 | 0.028 (±0.004) | 0.024 (±0.003) | 0.017 (±0.003) | 0.017 (±0.002) |

PMA were seen with *E. esculentus* and *N. norvegicus* (Table 5.2). For both species the absorbance values of the wells treated with PMA and SOD were significantly lower than for those treated with PMA alone ($p = 0.033$ for *E. esculentus* and $p = 0.002$ for *N. norvegicus*). The cells from *C. intestinalis*, *M. edulis* and *A. marina* showed smaller differences in absorbance between the two treatments (Table 5.2), but statistical analysis by paired t-tests revealed that the values derived for the PMA/SOD wells were still significantly below those containing PMA/MS ($p = 0.004$, $p = 0.008$ and $p = 0.003$, respectively). The PMA-stimulated cells from *G. strigosa* exhibited the smallest change in absorbance upon incubation with SOD (Table 5.2), but analysis of the data by paired t-test confirmed that this difference was significant at the 5% level ($p = 0.002$). In nearly every case, SOD alone had no measurable effect on the cells except for *M. edulis* which had a slight decline in the absorbance values (Table 5.2). Comparison of the wells treated with PMA and MS to those incubated with MS confirmed that for all species PMA stimulated the phagocytes under the present assay conditions (Table 5.2). These results indicate that PMA induces the generation of superoxide by the blood cells of marine invertebrates, although, because SOD in combination with PMA did not always abolish the effect of the elicitor, the reduction in ferricytochrome c must be due only in part to $O_2^-$ ions.

### 5.3.2. Hydrogen Peroxide Assay

Finally, in the experiments comparing $H_2O_2$ production by the hyaline cells of *C. maenas* with the phagocytic amoebocytes of *C. intestinalis*, it was found that control (MS-treated) cells from both species produced low, but detectable, amounts of $H_2O_2$ *in vitro* (Table 5.3). There was no significant difference in the levels of $H_2O_2$ production between the two species ($p = 0.669$, $n = 6$). Treatment with con A, resulted in an increase in $H_2O_2$ production by the cells of both species although the amount varied

Table 5.3.

Table 5.3. Comparison of *in vitro* $H_2O_2$ production by the phagocytes of *C. maenas* and *C. intestinalis*.

Twenty-five microlitres of cells (ca 1-9 x $10^7$ cells $ml^{-1}$) were preincubated for 15 min to allow cells to settle and attach to the microtitre tray wells. Excess liquid and unattached cells were removed, and the remaining cells were incubated with 100 µl of PRS and 50 µl of con A (5 µg $ml^{-1}$, final concentration). Controls received 100 µl of PRS and 50 µl of MS instead of con A. All samples were incubated for 30 min at 20°C. Ten microlitres of 1 N sodium hydroxide were then added to each well to terminate the reaction. The cells was spun at 500 g at 4°C for 5 min to remove cell debris and the absorbance of the cell-free supernatant was measured at 630 nm.

[a] Values shown are the mean $H_2O_2$ production ± S.E.M. Each treatment was repeated six times with different animals from each species.

| Species | $H_2O_2$ production (nmoles $10^7$ cells$^{-1}$ 30 min$^{-1}$)[a] | |
|---|---|---|
| | Con A | MS (Control) |
| *Carcinus maenas* | 47.1 ± 10.7 | 15.3 ± 9.8 |
| *Ciona intestinalis* | 29.0 ± 7.0 | 18.5 ± 8.8 |

between individual animals (Table 5.3). Statistical analysis confirmed that the amount of $H_2O_2$ produced was significantly higher for the con A-treated cells than the MS controls for *C. maenas* ($p = 0.011$, $n = 6$) but not for *C. intestinalis* ($p = 0.118$, $n = 6$). Importantly, the amount of $H_2O_2$ produced by the hyaline cells of *C. maenas* after con A incubation was significantly higher than that of the con A-treated *C. intestinalis* amoebocytes (Table 5.3) ($p = 0.003$, $n = 6$).

## 5.4. DISCUSSION

This set of experiments demonstrate that stimulation of the phagocytes from a wide range of marine invertebrate species, including representatives of Crustacea, Tunicata, Mollusca, Echinodermata and Annelida, with PMA or con A results in an increase in absorbance at 550 nm compared to the MS-treated controls. The change in absorbance demonstrates that ferricytochrome c was reduced to ferrocytochrome c under the assay conditions.

A slight elevation in absorbance was also induced by MS for some species, although this was always less pronounced than the increase obtained with con A or PMA. Possibly, this change in absorbance may have been caused by endotoxin contamination in the MS as previous studies have already demonstrated the sensitivity of crustacean and tunicate cells to very low levels of LPS (Smith and Söderhäll, 1991; Jackson *et al.*, 1993; Chapter 4). While every reasonable precaution was taken to minimize the presence of endotoxin in the reagents, low levels may have been unavoidably present in the microtitre trays or have affected the samples during reading or incubation. Such extraneous endotoxin would have contaminated both control and experimental wells. Importantly in this study, the experimental (PMA or con A) wells always showed a higher absorbance than the controls, confirming that there was a positive response to the elicitors irrespective of the presence of any endogenous LPS.

Another possible explanation for the change in absorbance following the addition of MS may be osmotic influences. Table 5.1. is a summary of the osmolality of the blood of these animals and MS. The osmolality of MS is much higher that the osmolality of the blood of non-crustacean species. The buffer, MS, was designed for working with crustacean, in particular *C. maenas*, haemocytes, hence it is probably not the most appropriate buffer to have used for the non-crustacean species. However, the aim of this

work was to compare the responses of these other animals to *C. maenas*, so MS was used in this particular set of experiments. It would be pertinent for oxygen radical production by the phagocytes of these non-crustacean species to be measured using a buffer closer in osmolality to the blood.

Although the kinetics of the response differed between species, in each case the phagocytes produced $O_2^-$ following stimulation, as shown by the experiments where SOD was included in the reaction mixture. It is concluded that the phagocytes of the species examined are capable of producing a respiratory burst.

Previously, studies of the respiratory burst have been largely confined to commercially important groups such as bivalve molluscs (Nakamura *et al.*, 1985; Nottage and Birkbeck, 1990; Pipe, 1992) or crustaceans (Chapter 4). A limited number of studies have been carried out on gastropod molluscs (Dikkeboom *et al.*, 1988; Adema *et al.*, 1991a) and also on the sea urchin *Strongylocentrotus nudus* (Ito *et al.* 1992). The present study shows tunicate and annelid phagocytes also produce a respiratory burst upon appropriate stimulation.

The differences in the magnitude of the response to the elicitors observed between species may be a reflection of the phagocytic activity of the cells. For example, *in vitro* uptake in *C. maenas* and *C. intestinalis* has been reported to be ca 5 - 15% (Smith and Ratcliffe, 1978) and ca 5 - 40% (Smith and Peddie, 1992), respectively. Similarly in *M. edulis*, uptake levels of ca 65% have been reported (Renwrantz and Stahmer, 1983) and Fitzgerald and Ratcliffe (1982) recorded levels of ca 2 - 4% phagocytic uptake by *A. marina* coelomocytes. However, the response observed for the sea urchin phagocytes does not appear to follow this trend. There is very little information available for sea urchins but, *Ito et al.* (1992) reported uptake of red blood cells *in vitro* by the phagocytes

of *S. nudus* of ca 10%. In contrast, the magnitude of the respiratory burst response in *E. esculentus* was as high as the response observed for the more phagocytically avid molluscs.

Previously in chapter 4, the difference in the kinetics of the respiratory burst response between fish and mammals and the shore crab, *C. maenas* was noted. The results of these experiments show that only the phagocytes of *C. intestinalis* and *M. edulis* resemble those of fish and mammalian phagocytes by displaying a continuous rise in absorbance from time zero to 30 min. In contrast, the phagocytes of *G. strigosa* and *N. norvegicus* resemble the phagocytes of *C. maenas* by producing a fall in optical density after the first 5 min of the incubation period, despite the presence of catalase in the assay mixtures. Since these opposing results were obtained using the same experimental method, there must be a fundamental difference in the kinetics of the response elicited from the phagocytes of these species. It was previously suggested in Chapter 4 that gross $H_2O_2$ production by the crustacean haemocytes might account for the observed decline, over time in optical density of the assay mixtures (Chapter 4) and in the present chapter, the levels of $H_2O_2$ production by the cell from *C. maenas* and *C. intestinalis* were compared. The results clearly demonstrate that *C. maenas* phagocytes produce a greater amount of $H_2O_2$ compared to the phagocytes of the tunicate. It is proposed, therefore, that the observed decline in optical density during the incubation period for the ferricytochrome c assay with *C. maenas* hyaline cells may be due, at least in part, to the $H_2O_2$-mediated oxidation of ferrocytochrome c.

To conclude, the ability of the phagocytic hyaline cells of the shore crab, *Carcinus maenas*, to generate $O_2^-$ and $H_2O_2$ following stimulation has been established. These highly reactive oxygen intermediates would have severe deleterious effects on the host tissues if their production and location were not controlled. However, the nature and

presence of antioxidant protective mechanisms has not been demonstrated for the cells and tissues of *C. maenas*. The next group of experiments will be carried out with the aim of determining the type of antioxidant defences that are present in *C. maenas* and their location.

# Chapter 6

## Antioxidant enzyme activity and localisation in the haemocytes and plasma of *Carcinus maenas in vitro*

# 6.1. INTRODUCTION

The ability of the phagocytic hyaline cells of *C. maenas* to produce $O_2^-$ and $H_2O_2$ following stimulation is described in Chapters 4 and 5, respectively. Highly reactive oxygen metabolites, however, must be regulated so as to avoid potential cell and tissue damage such as protein degradation, lipid peroxidation, nucleic acid damage and enzyme degradation (Borg and Schaich, 1984). To minimise the potentially damaging effects of these oxygen intermediates on the host cells and tissues, the host animals or plants possess protective antioxidant defence mechanisms. Vertebrates have three classes of defence mechanisms:- 1) water soluble reductants (e.g. glutathione, ascorbate, urate); 2) fat soluble vitamins (e.g. ß-carotene, alpha-tocopherol) and 3) enzymes (e.g. catalase, superoxide dismutase - SOD, glutathione peroxidase - GPX). In the latter class, the enzymes act specifically:

Catalase (EC 1.11.1.6) reduces $H_2O_2$ to water;

$$2H_2O_2 = 2H_2O + O_2 ;$$

Glutathione peroxidase (GPX; EC 1.11.1.9.) reduces $H_2O_2$ (or organic peroxides, ROOH) to water (or alcohols, ROH). Reduced glutathione - GSH oxidised glutathione - GSSG.

$$2GSH + H_2O_2 \text{ (or ROOH)} = GSSG + H_2O \text{ (or ROH)} + H_2O.$$

and SOD (EC 1.15.1.1) reduces $O_2^-$ to $H_2O_2$

$$2O_2^- + 2H^+ = H_2O_2 + O_2$$

Recently, various studies have quantified the levels of antioxidant enzymes present in the haemocytes, tissues and plasma of some marine invertebrates (see reviews by Di Giulio *et al.*, 1989; Winston, 1991). The majority of studies have been concerned with molluscan hosts and marked differences in the the range of enzyme activity levels have

been noted (see review by Winston, 1991). Catalase activity has been detected in the mussel, *Mytilus californianus* (Marks and Fox, 1937), the clam, *Mercenaria mercenaria* (Blum and Fridovich, 1984) and *M. edulis* (Pipe *et al.*, 1993). Glutathione peroxidase activity is present in *Calyptogena magnifica, M. mercenaria* (Blum and Fridovich, 1984) and *M. edulis* (Pipe *et al.*, 1993). Superoxide dismutase activity has been found in the digestive gland (Winston *et al.*, 1990; Livingstone *et al.*, 1992) and the haemocytes (Pipe *et al.*, 1993) of the mussel, *M. edulis* and in tissue extracts of large, *C. magnifica*, and hard, *M. mercenaria*, clams (Blum and Fridovich, 1984). In addition, reduced glutathione (GSH) activity has been quantified in ribbed mussels, *Geukensia demissa*, and wedge clams, *Rangia cuneata*, (Wenning and Di Giulio, 1988) and glutathione reductase has been detected in *M. edulis* (Ramos-Martinez *et al.*, 1983).

For other invertebrate groups even less is known. Blum and Fridovich (1984) detected catalase, GPX and SOD activity in tissue extracts of giant tube worms, *Riftia pachyptila*. Glutathione peroxidase activity has also been detected in tissue extracts of the crayfish, *Oronectes limosus* (Smith and Shrift, 1979). However, only one enzyme was assayed for using crude extracts of the crayfish tissues (Smith and Shrift, 1979). Hence, very little is known as to the range of antioxidant defences present in crustaceans and their location either cellular or humoral has not been determined.

The aim of the present chapter is to:

1) determine if the antioxidant enzymes catalase, GPX and SOD are present in the haemocytes or the plasma of the shore crab, *C. maenas*;

2) quantify the level of enzymatic activity,

and 3) establish the localisation of these enzymes intracellularly using the technique of immunocytochemistry to detect the enzymes under light and confocal microscopy.

# 6.2. MATERIALS AND METHODS

## 6.2.1. Harvesting and Separation of Cells

Harvesting of the haemocytes and cell separations were carried out according to the method described in Chapter 2 (Sections 2.2.3. and 2.2.7., respectively).

## 6.2.2. Preparation of Individual Cell Type Lysate Supernatants

Pooled samples of either hyaline, semi-granular or granular cells were collected from 60% Percoll continuous density gradients for 3 animals. The haemocytes were washed once in 3.2% NaCl. Each cell pellet was then homogenised and lysate supernatants prepared as described in Chapter 2 (Section 2.2.4.). The haemocyte lysate supernatants were made up in the appropriate buffer for each enzyme assay. Hyaline, semi-granular and granular cell lysate supernatants are designated HyLS, SgLS and GLS, respectively. Quantification of the protein levels were carried out according to the method in Chapter 2 (Section 2.2.5.). Protein levels were adjusted to 1.0 mg $ml^{-1}$ for all the samples.

## 6.2.3. Preparation of Plasma

Approximately 1 ml of haemolymph, per animal, was withdrawn into ice-cold syringes without anticoagulant. The haemolymph was then centrifuged in sterile tubes at 600 g for 15 min at 4°C. The plasma samples was then kept on ice until use. Protein levels were determined as above and adjusted to 1.0 mg $ml^{-1}$ for all the samples.

## 6.2.5. Enzyme Assays

All assays were carried out on freshly prepared HyLS, SgLS, GLS and plasma. Measurements were made on three batches of HLS for each cell type and six plasma samples from individual animals.

a) Catalase

Catalase activity was measured by the decrease in $H_2O_2$ at 240 nm due to $H_2O_2$ consumption (extinction coefficient 43.6 $M^{-1}$ $cm^{-1}$; Claiborne, 1985). The assay was carried out at 25°C at pH 7.0 and measured at 240 nm on a 4053 kinetics spectrophotometer (LKB, Sweden). The test cuvettes contained 2.9 ml of 0.036% $H_2O_2$ solution and the assay was initiated by the addition of 100 µl of sample, either HLS or plasma, prepared in 50 mM potassium phosphate monobasic buffer (PB). The readings were read against a blank containing 3.0 ml of 50 mM PB. For comparison with the results for the HLS and plasma samples, this assay was also performed with known quantities of catalase. These were 5, 10, 20 and 50 units $mg^{-1}$ protein. Quantification of the catalase activity was carried out using the following equation:

$$\text{Units mg}^{-1}\text{ protein} = \frac{3.45}{\text{(min.) (mg protein/ml RM)}}$$

Unit = One unit will decompose 1.0 micromole of $H_2O_2$/min at pH 7.0 at 25°C, while the $H_2O_2$ concentration falls from 10.3 mM to 9.2 mM.

3.45 = Corresponds to the decomposition of 3.45 micromoles of $H_2O_2$ in a 3.0 ml reaction mixture producing a decrease in the $A_{240\ nm}$ from 0.45 to 0.40.

min = Time in minutes required for the $A_{240\ nm}$ to decrease from 0.45 to 0.40.

RM = Reaction Mix.

b) Glutathione peroxidase (GPX)

Glutathione peroxidase activity was assayed essentially as described in Wendel (1980). The assay was carried out at 25°C at pH 7.0 and read at 340 nm on a multiwell plate reader (Anthos HTII, Köln, Germany). Triplicate wells were prepared. The reaction cocktail on which the assay is based consists of 9.2 ml of 50 mM sodium phosphate and 0.40 mM EDTA with 1.0 mM sodium azide, 0.1 ml of 100 units $ml^{-1}$ glutathione reductase and 0.05 ml of 200 mM reduced glutathione in a 1.0 mg vial of ß-nicotinamide adenine dinucleotide phosphate (ß-NADPH, reduced form, Sigma). The test wells contained 300 µl of reaction cocktail, 5 µl of sample prepared in 10 mM sodium phosphate buffer with 1.0 mM dithiothreitol (buffer w/DTT) and 5 µl of 12.4 mM $H_2O_2$. All wells were read against triplicate blanks containing 300 µl of reaction cocktail, 5 µl of buffer w/DTT and 5 µl of 12.4 mM $H_2O_2$. In 310 µl of reaction mix, final concentrations were 47.8 mM sodium phosphate, 0.38 mM EDTA, 0.12 mM ß-NADPH, 0.95 mM sodium azide, 3.21 units of glutathione reductase, 1.04 mM glutathione, 0.16 mM dithiothreitol and 0.2 mM $H_2O_2$. For comparison the assay was carried out with known quantities of glutathione peroxidase of 1, 2, 5 and 10 units $mg^{-1}$ protein. The plates were shaken and the decrease in $A_{340\ nm}$ for approximately 5 min recorded. The $\Delta A_{340\ nm}$/min was determined using the maximum linear rate for both the test and blank wells. Glutathione peroxidase activity was then calculated using the following equation:

$$\text{Units mg}^{-1}\text{ protein} = \frac{(\Delta A_{340\text{ nm}}/\text{min Test} - \Delta A_{340\text{ nm}}/\text{min Blank})\ (2)}{(6.22)\ (\text{mg protein/ml RM})}$$

Unit = One unit will catalyse the oxidation by $H_2O_2$ of 1.0 micromole of reduced glutathione to oxidised glutathione/min at pH 7.0 at 25°C.

2 = 2 micromoles of GSH produced per micromole of ß-NADPH oxidised.

6.22 = Millimolar extinction coefficient of ß-NADPH at 340 nm.

RM = Reaction Mix.

c) Superoxide dismutase (SOD)

Superoxide dismutase activity was measured by the inhibition of xanthine oxidase-generated $O_2^-$ reduction of cytochrome c at 550 nm using modifications of the assay by McCord and Fridovich (1969). The assay was carried out at 25°C at pH 7.8 on a multiwell spectrophotometer (Dynatech MR5000) with quadruplicate wells prepared for each sample. The reaction cocktail on which the assay is based consists of 2.3 ml of deionised water, 2.5 ml of 216 mM potassium phosphate, 1.0 ml of 10.7 mM EDTA, 1 ml of 1.1 mM cytochrome c and 5 ml of 0.108 mM xanthine sodium salt. The test wells contained 280 µl of reaction cocktail, 10 µl of xanthine oxidase enzyme solution (XOD, 0.05 units $ml^{-1}$) and 10 µl of sample either HLS or plasma prepared in 216 mM PB. For comparison, wells containing no SOD (uninhibited) or a known quantity of SOD (inhibited) were prepared in parallel. These contained 280 µl reaction cocktail, 10 µl deionised water and 10 µl XOD or 10 units/ml SOD, respectively. All readings were read against quadruplicate blanks containing 280 µl of reaction cocktail and 20 µl of deionised water. For the wells containing SOD, the final concentrations were 50 mM PB, 0.1 mM EDTA, 0.01 mM cytochrome c, 0.05 mM xanthine, 0.005 units XOD and

1 unit SOD. The plates were shaken for 5 seconds and the increase in $\Delta_{550\ nm}$ recorded over 5 min. Quantification of SOD was then made using the maximum linear rate of increasing absorbance for the uninhibited, inhibited and blank systems. One unit of SOD will inhibit the rate of reduction of cytochrome c by 50% in a coupled system using xanthine and XOD at pH 7.8 at 25°C.

### 6.2.6. Immunocytochemistry

Monolayers of separated hyaline or granular cells were prepared as before (Chapter 2 Section 2.2.8.). Only hyaline and granular monolayers were prepared as semi-granular cells are more labile and tended to degranulate during attachment to the glass coverslips. The immunocytochemistry assays were modified from Pipe *et al.*, (1993).

The cells were fixed in 10% seawater/formalin for 15 min before washing in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (PBS-X). The cells were then incubated for 20 min with PBS containing 0.02% glycine, to block free aldehyde groups, followed by a further 20 min incubation with donkey blocking serum (Scottish Antibody Production Unit (SAPU), Carluke, Scotland). The monolayers were overlaid with 100 µl of primary antibody (1:50 dilution) and incubated overnight (15 h) at 4°C in a humid chamber. The following antibodies were investigated: a) anti-catalase; b) anti-GPX; c) anti-SOD (Cu/Zn form), and d) anti-SOD (Mn form). The anti-catalase antibody was raised against bovine antigen whereas anti-SOD and GPX were raised against human antigens (The Binding Site, Birmingham, UK). They were all presented as sheep immunoglobulin fraction diluted in PBS-X. Following incubation with the primary antibody, the cells were washed twice in PBS-X and then incubated for 1 h in

fluorescein isothiocyanate (FITC) conjugated anti-sheep/goat IgG (donkey) (SAPU) diluted 1:20 in PBS-X. The cells were washed in PBS and mounted on slides with Permafluor, a permanent aqueous mounting gel (Biogenesis). To confirm the specificity of the antiserum a set of negative controls were also prepared for each experimental run. For one monolayer, the primary antisera was substituted with normal (non-immune) serum from the same animal species as the primary antibody i.e. in this case sheep serum (SAPU) was used. Incubation with the non-immune serum ensured that positive staining was not due to any other factors in the antiserum. The antiserum has all the factors of the primary antibody serum except the primary antibody. Another control monolayer was also incubated overnight with PBS.

### 6.2.7. Microscopy

Quantification of the number of positive staining cells following incubation with each antibody was made. For each monolayer, 200 cells were counted under phase contrast optics of a Leitz Diaplan microscope using oil immersion. The number of positive staining cells was then expressed as a percentage.

Monolayers were also examined using a laser scanning confocal microscope (Biorad, Hemel Hempstead, U.K.) to determine the localisation of the antibody within the cells.

# 6.3. RESULTS

## 6.3.1. Levels of Enzyme Activity

Separated haemocyte lysate supernatants and plasma samples were assayed for antioxidant enzyme activities (Table 6.1). Catalase activity of ca 19.5 units $mg^{-1}$ protein was detected in the HyLS (Table 6.1). There was no catalase activity in the SgLS, GLS or plasma samples (Table 6.1). Only the HyLS, GLS and plasma had detectable levels of GPX and the levels of activity were similar for all three samples (ca 1 - 1.29 units $mg^{-1}$ protein) (Table 6.1). In contrast, SOD activity was present in all of the HLS and plasma samples (Table 6.1). The SOD activity in the plasma of ca 8.62 units $mg^{-1}$ protein was approximately four times the level found in the SgLS and GLS (ca 2.86 and 2.19 units $mg^{-1}$ protein, respectively) and approximately eight times the level of activity in the HyLS (Table 6.1).

## 6.3.2. Proportion of Positively Stained Cells

To determine the proportion of cells that contain the enzymes, the percentage of positively stained haemocytes for each monolayer was estimated by counting 200 cells (Table 6.2). Following incubation with the various antibodies, approximately 15% of the hyaline cells were stained positively for catalase compared with only 1% of the granular cells (Table 6.2). Catalase activity was not detected in the GLS by biochemical assay (Table 6.1). Positive staining for GPX occured in approximately 13% of the hyaline and only ca 8% of the granular cells (Table 6.2). For both the hyaline and granular cells, approximately 12 - 13% of the cells stained for the Cu/Zn form of SOD (Table 6.2).

Table 6.1.

Table 6.1. Antioxidant enzyme activities in separated haemocyte lysate supernatants or plasma of *C. maenas*.

Enzyme assays were carried out as explained in Materials and Methods (Section 6.2.5.).

a Hyaline cell lysate supernatant.

b Semi-granular cell lysate supernatant.

c Granular cell lysate supernatant.

d Values are the mean activity levels ± S.E.M. for 3 samples of haemocyte lysate supernatant for each cell type.

e Not detectable.

f Values are the mean activity levels ± S.E.M. for 6 plasma samples from individual animals.

| Enzyme | HyLS[a] | SgLS[b] | GLS[c] | Plasma |
|---|---|---|---|---|
| Catalase (Units $mg^{-1}$ protein) | 19.56 ± 10.93[d] | N.D.[e] | N.D. | N.D. |
| GPX (Units $mg^{-1}$ protein) | 1.01 ± 0.16 | N.D. | 1.25 ± 0.22 | 1.29 ± 0.20[f] |
| SOD (Units $mg^{-1}$ protein) | 0.78 ± 0.42 | 2.86 ± 2.86 | 2.19 ± 0.58 | 8.62 ± 0.59 |

Table 6.2.

Table 6.2. Percentage of positively stained cells following incubation with specific antibodies against each of these enzymes - catalase, GPX or SOD (Cu/Zn or Mn form).

[a] Values are means ± S.E.M. n = 6 animals.

| Cell Type | Catalase | GPX | SOD (Cu/Zn) | SOD (Mn) |
|---|---|---|---|---|
| Hyaline | 16.26 ± 5.24[a] | 12.73 ± 3.56 | 13.30 ± 2.88 | 11.54 ± 1.23 |
| Granular | 1.96 ± 0.50 | 7.75 ± 1.39 | 12.37 ± 2.97 | 13.22 ± 2.36 |

Similarly when the hyaline and granular cells were incubated with the Mn form of SOD, ca 11.5% and ca 13.2% of the cells, respectively, were stained positively (Table 6.2).

### 6.3.3. Microscopy

Figure 6.1 A and B show hyaline and granular cells from *C. maenas* under phase contrast optics. The photomicrographs show the size and morphology of each cell type and gives an indication of the nucleus : cytoplasm ratio. Figure 6.1 A shows that hyaline cells spread extensively when they adhere to glass and have few granules present in the cytoplasm. In contrast, Figure 6.1 B reveals that granular cells have less cytoplasm and are densely packed with granules.

A proportion ca 11 - 16% of the hyaline cells stained positively following incubation with each antibody (Fig. 6.2. A-D). Positive staining did not occur when the cells were incubated with the antiserum not containing the antibody (Fig. 6.2. E). Similarly no staining was observed with the cells that were incubated overnight with PBS. Positive staining was very strong with all four antibodies (Fig. 6.2. A-D). With the catalase and GPX antibodies, labelling was distributed throughout the cytoplasm with spots of particularly dense staining (Fig. 6.2. A,B). For the two forms of SOD, the staining was more localised (Fig. 6.2. C,D). With both SOD (Cu/Zn) and (Mn), the antibodies tended to stain around the nucleus (Fig. 6.2. C,D). In addition, the SOD (Mn) antibody labelled discretely the cell membrane of the hyaline cells (Fig. 6.2. D).

The granular cells did not stain for the enzymes as strongly as the hyaline cells (Fig. 6.3. A-D). For catalase, GPX and SOD (Mn) forms of the enzyme, staining was

Figure 6.1. A & B.

Figure 6.1. A and B. Phase contrast photographs of hyaline and granular cells from the shore crab, *Carcinus maenas*.

A. Hyaline cells spread on a glass coverslip. Arrows indicate psudopodia. Note the absence of granules. Under oil immersion Magnification = x 100. Scale bar = 10 μm.

B. Granular cells attached to glass coverslips. Arrows indicate granules. Note the presence of little cytoplasm and the few psudopodia which have formed. Under oil immersion Magnification = x 100. Scale bar = 10 μm.

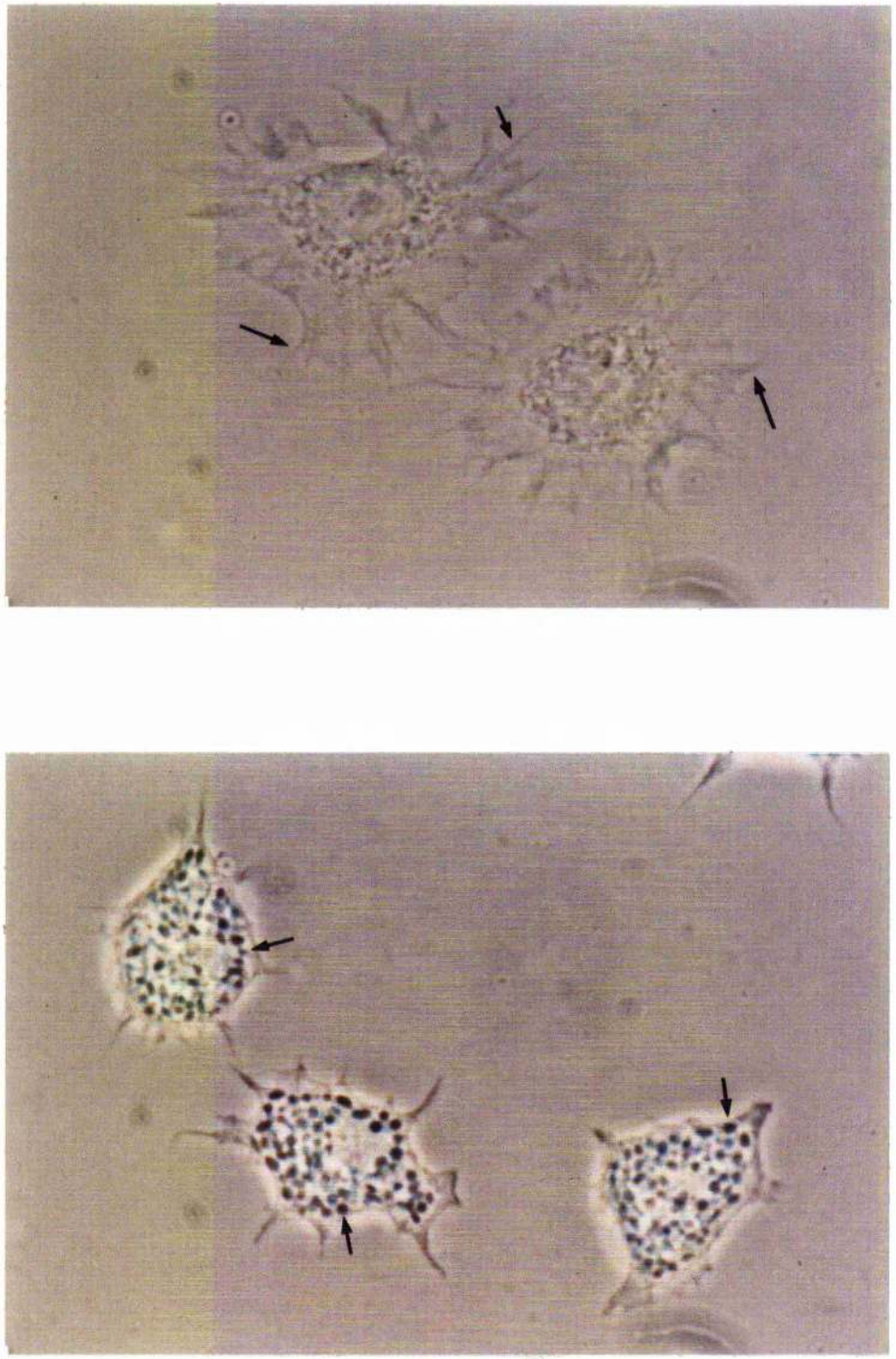
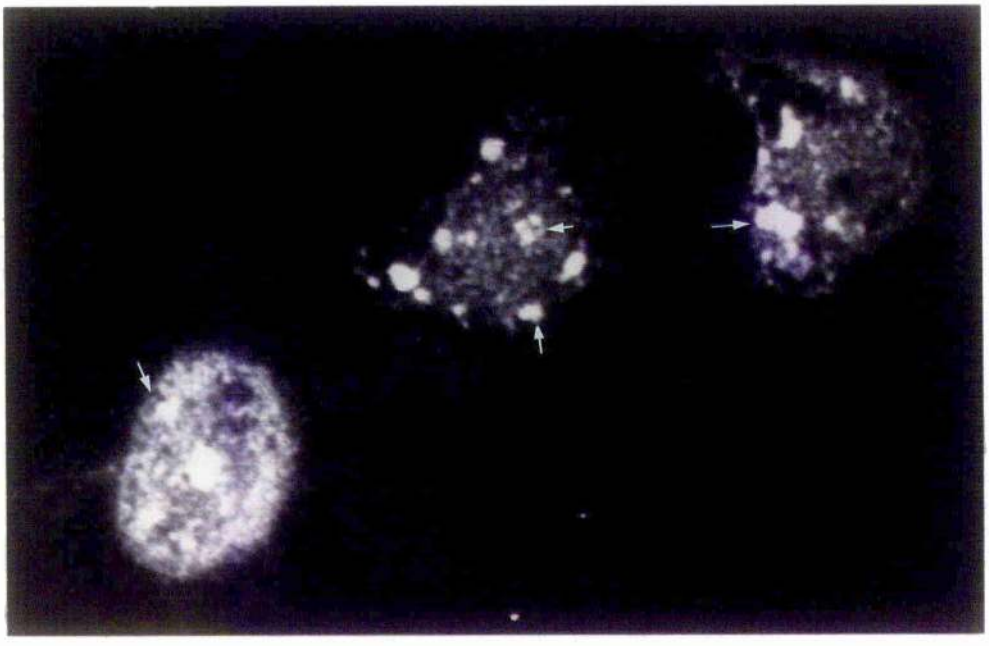


Figure 6.2. A & B.

Figure 6.2. A,B. Photomicrographs of *C. maenas* hyaline cells showing the immunocytochemical location of antioxidant enzymes.
Cells were incubated overnight (15 h) with primary antibody presented as sheep immunoglobulin fraction. The cells were then washed in PBS-X and incubated for 1 h in FITC-conjugated anti-sheep/goat IgG (donkey). Photomicrographs are optical sections of a fluorescence image under oil immersion at x 60.

A Hyaline cells incubated with anti-catalase. Arrows indicate areas of dense staining. Zoom 3.0. Bar = 10 μm.
B Hyaline cells incubated with anti-GPX. Arrows indicate areas of dense staining. Zoom 2.0. Bar = 10 μm.



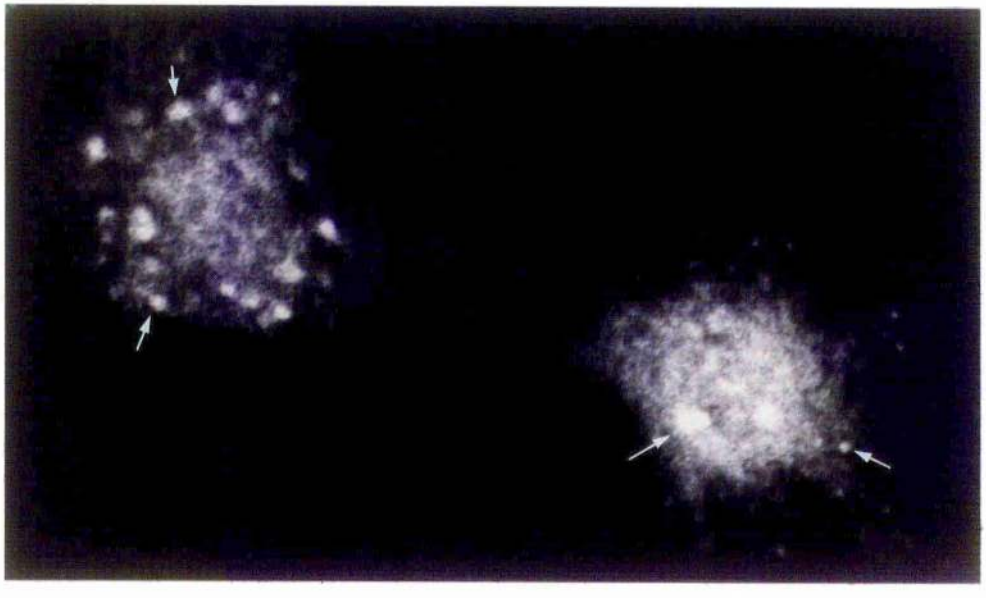
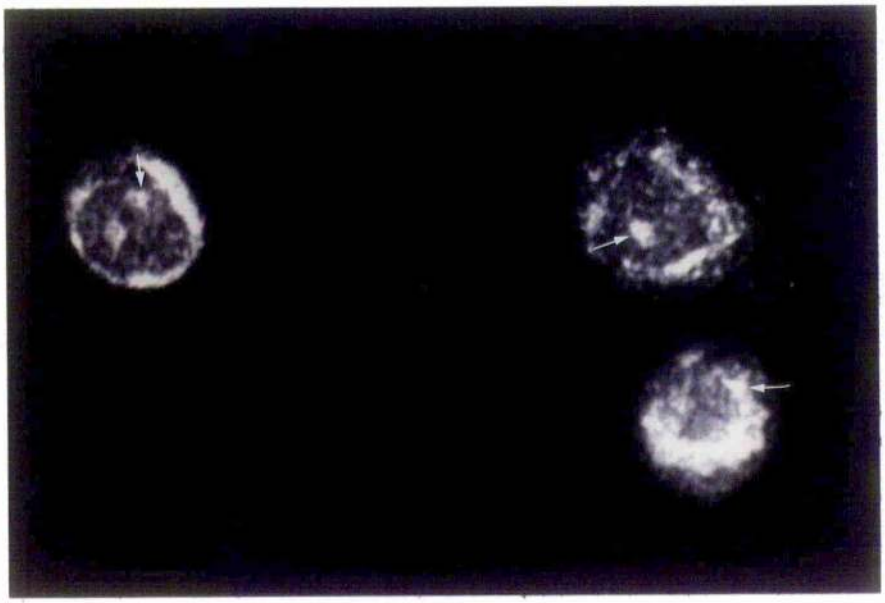


Figure 6.2. C & D.

Figure 6.2. C,D. Photomicrographs of *C. maenas* hyaline cells showing the immunocytochemical location of antioxidant enzymes.
Cells were incubated overnight (15 h) with primary antibody presented as sheep immunoglobulin fraction. The cells were then washed in PBS-X and incubated for 1 h in FITC-conjugated anti-sheep/goat IgG (donkey). Photomicrographs are optical sections of a fluorescence image under oil immersion at x 60.

C Hyaline cells incubated with anti-SOD (Cu/Zn). Arrows indicate areas of dense staining. Zoom = 2.0. Bar = 10 μm.
D Hyaline cells incubated with anti-SOD (Mn). Arrows indicate areas of dense staining. Zoom = 3.0. Bar = 10 μm.



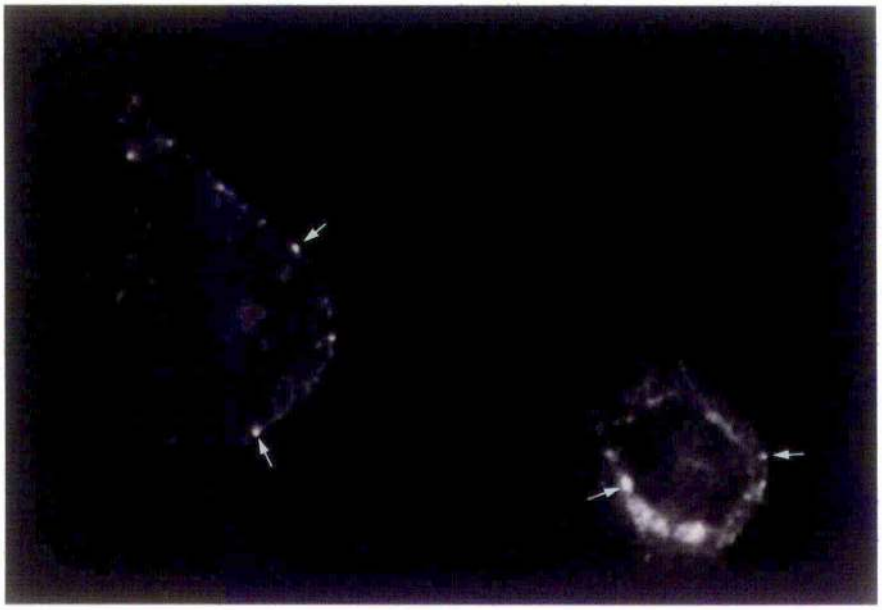


Figure 6.2. E.

Figure 6.2. E. Photomicrograph of *C. maenas* hyaline cells incubated in non-immune serum (control).

Cells were incubated overnight (15 h) with non-immune serum (sheep). The cells were then washed in PBS-X and incubated for 1 h in FITC-conjugated anti-sheep/goat IgG (donkey).

Note the absence of any positive staining. Under oil immersion at x 60. Bar = 10 μm.

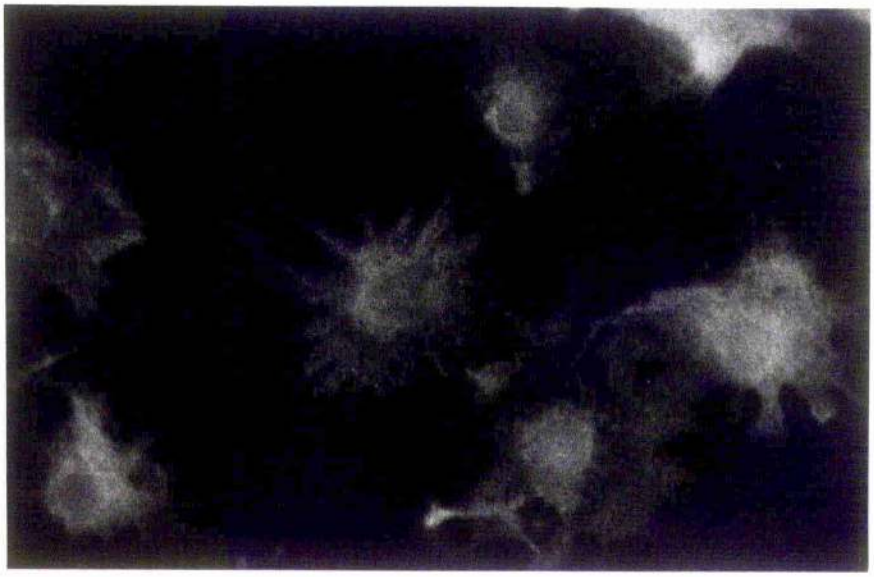
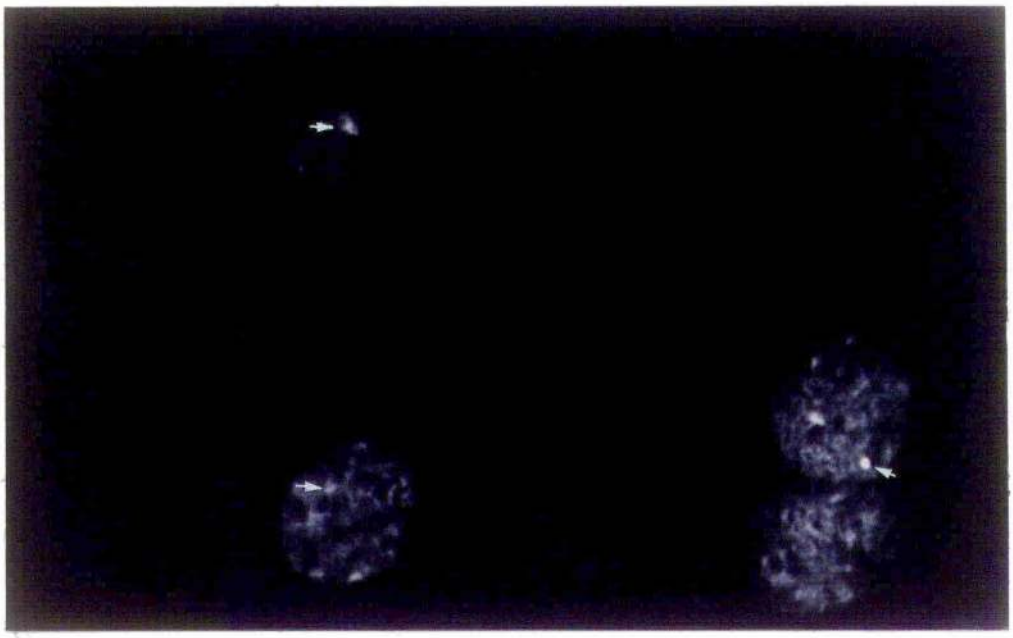


Figure 6.3. A & B.

Figure 6.3. A,B. Photomicrographs of *C. maenas* granular cells showing the immunocytochemical location of antioxidant enzymes.
Cells were incubated overnight with primary antibody presented as sheep immunoglobulin fraction. The cells were then washed in PBS-X and incubated for 1 h in FITC-conjugated anti-sheep/goat IgG (donkey). Photomicrographs are optical sections of a fluorescence image under oil immersion at x 60.

A Granular cells incubated with anti-catalase. Arrows indicate areas of dense staining. Zoom = 3.0. Bar = 10 μm.

B Granular cells incubated with anti-GPX. Arrows indicate areas of dense staining. Zoom = 2.0. Bar = 25 μm.



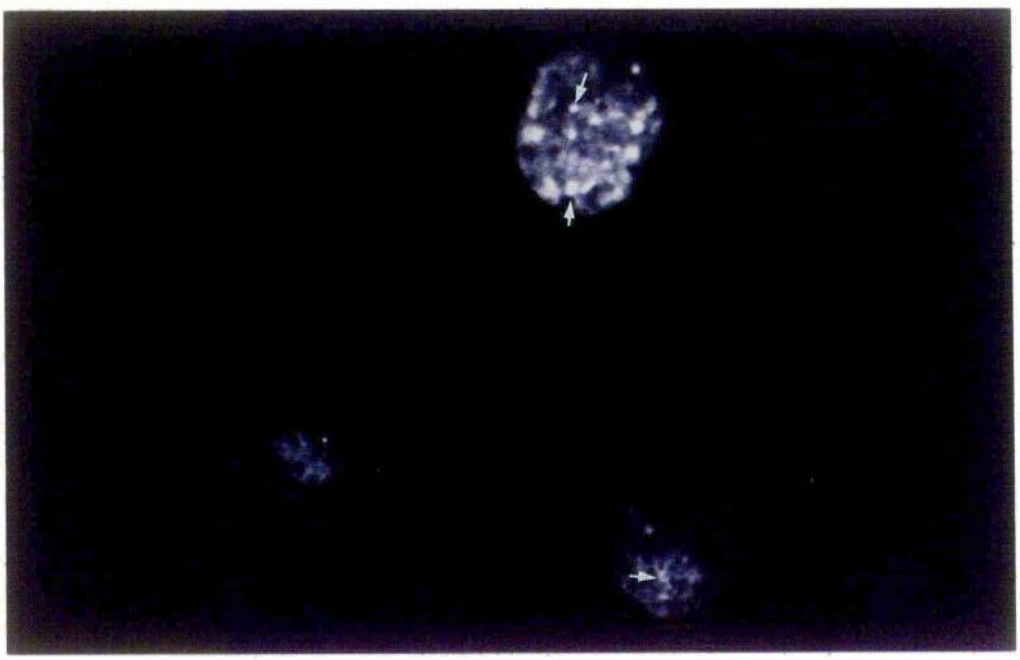
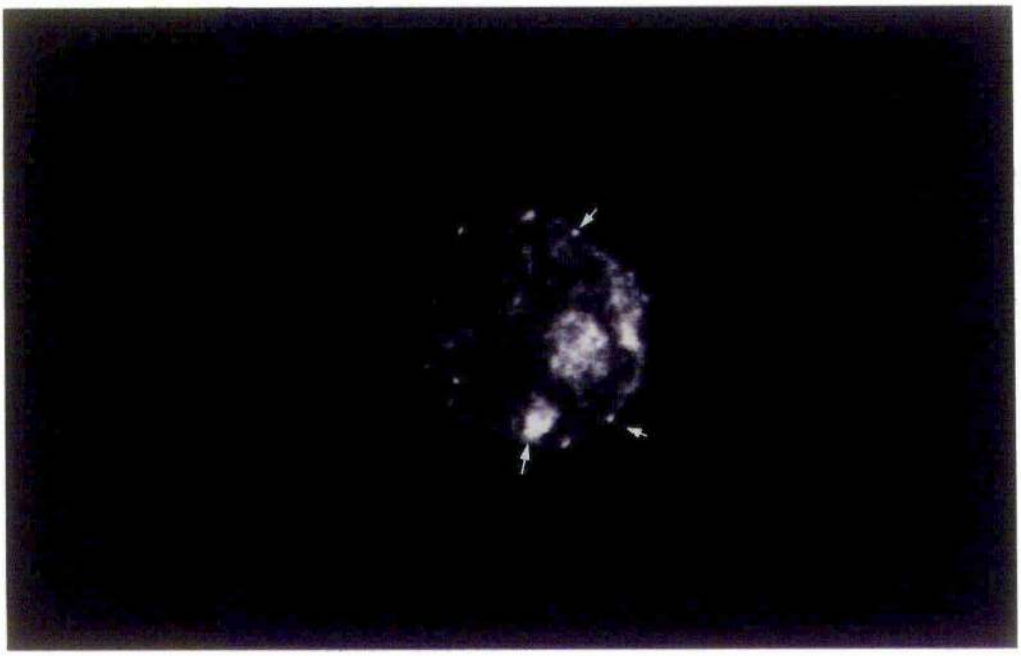


Figure 6.3. C & D.

Figure 6.3. C,D. Photomicrographs of *C. maenas* granular cells showing the immunocytochemical location of antioxidant enzymes.
Cells were incubated overnight with primary antibody presented as sheep immunoglobulin fraction. The cells were then washed in PBS-X and incubated for 1 h in FITC-conjugated anti-sheep/goat IgG (donkey). Photomicrographs are optical sections of a fluorescence image under oil immersion at x 60.

C Granular cells incubated with anti-SOD (Cu/Zn). Arrows indicate areas of dense staining. Zoom = 3.0. Bar = 10 μm.
D Granular cells incubated with anti-SOD (Mn). Arrows indicate areas of dense staining. Zoom = 3.0. Bar = 10 μm.



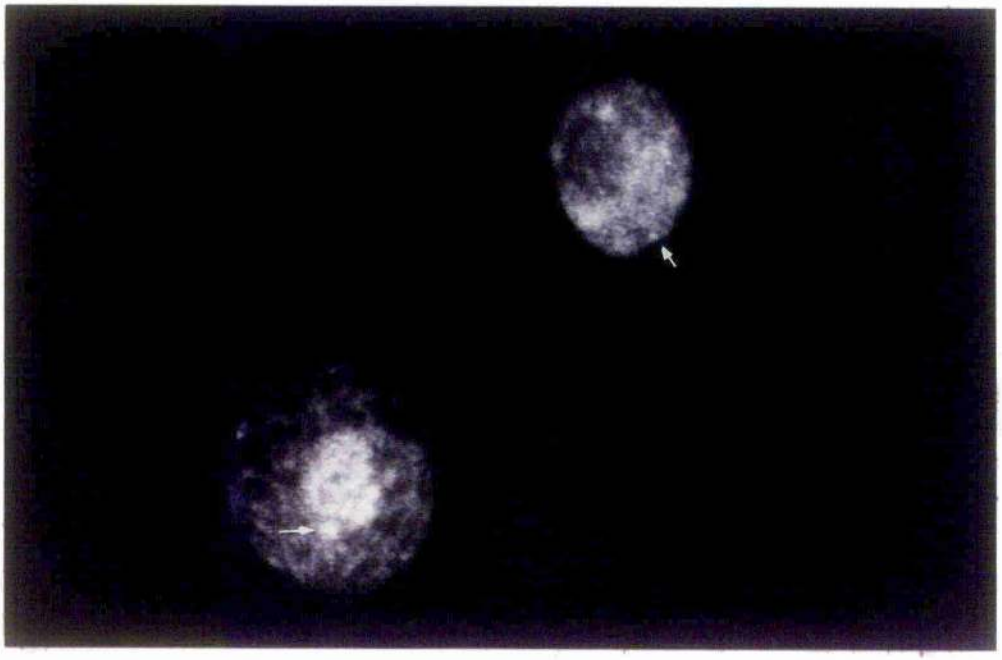
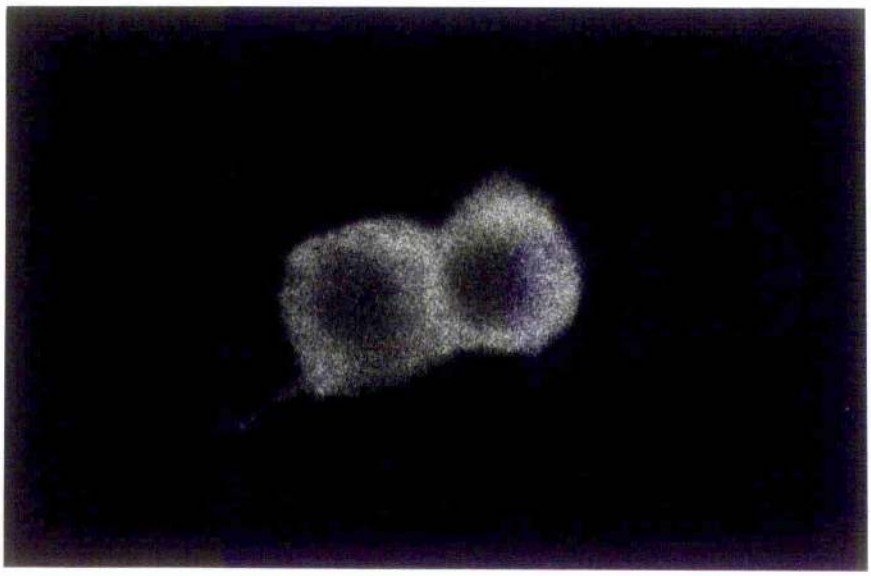


Figure 6.3. E.

Figure 6.3. E. Photomicrograph of *C. maenas* granular cells incubated in non-immune serum (control).

Cells were incubated overnight (15 h) with non-immune serum (sheep). The cells were then washed in PBS-X and incubated for 1 h in FITC-conjugated anti-sheep/goat IgG (donkey).

Note the absence of any positive staining. Under oil immersion at x 60. Bar = 10 μm.



distributed throughout the cells with some some localisation around the granules (Fig. 6.3 A, B and D, respectively). The SOD (Cu/Zn) antibody tended to label the cell membrane of the granular cells (Fig. 6.3. C). Incubation of the granular cells with non-immune serum produced no positive staining (Fig. 6.3. E). Similarly, no staining was observed when the granular cells were incubated with PBS.

## 6.4. DISCUSSION

From these preliminary studies, it is apparent that the antioxidant enzymes - catalase, GPX, SOD (Cu/Zn) and (Mn) forms, are located within the haemocytes and plasma of the shore crab, *Carcinus maenas*.

The results of this study are not in full agreement with findings from other studies, such as those on vertebrate and molluscan cells (see review by Halliwell and Gutteridge, 1985; Pipe *et al.*, 1993, respectively), as activity levels vary. However, there are some noteworthy similarities in the localisation of these enzymes. In the present study, catalase activity was only found within cells, in particular the hyaline, and not in the plasma. This is consistent with the findings for mammalian phaocytic leukocytes and macrophages (see review by Halliwell and Gutteridge, 1985) and for the mussel *Mytilus edulis* haemocytes (Pipe *et al.*, 1993). In animals and plants which contain catalase, it is commonly found to be located within subcellular organelles known as peroxisomes (see review by Halliwell and Gutteridge, 1985). Unfortunately from these results, the precise location of the catalase within the hyaline cells of *C. maenas* cannot be determined.

In the present study of *C. maenas*, GPX was found both in the plasma and the cells with slightly higher levels detected in the plasma. A similar pattern has been noted in the mussel *M. edulis* where considerably higher levels of GPX are present in the plasma than the cells (Pipe *et al.*, 1993). In mammals, a distinct form of GPX has been found in the plasma (Takahashi *et al.*, 1987). It would be interesting to purify and characterize GPX from invertebrate cells and plasma to determine if there is also more than one form of the enzyme in these animals. The present analysis of *C. maenas* haemocytes

demonstrates that GPX is distributed throughout the cytoplasm, although there is a degree of localisation around the granules of the granular cells. Similarly, GPX is localised within the cytoplasm of *M. edulis* haemocytes (Pipe *et al.*, 1993) and in mammals, intracellular forms of GPX have been shown to be located in the cytoplasm (Yoshimura *et al.*, 1980).

The two forms of SOD have different subcellular locations in animal tissues as the (Cu/Zn) form is considered to be a cytoplasmic enzyme (Geller and Winge, 1982) whereas SOD (Mn) is thought to be a mitochondrial enzyme (Weisiger and Fridovich, 1973). From the work carried out in the present study, it would appear that SOD (Mn) is not associated with mitochondria in hyaline cells, but instead is localised round the cell membrane. However, with the granular cells, SOD (Mn) is present throughout the cytoplasm. Biochemical analysis of subcellular fractions of SOD (Mn) is required before the localisation of this enzyme can be conclusively established. In contrast to SOD (Mn), SOD (Cu/Zn) is localised throughout the cytoplasm of the hyaline cells and associated with the cell membrane of the granular cells. The biochemical assay for SOD detected activity in semi-granular cells. Superoxide dismutase was the only enzyme to be detected in the semi-granular cells. Unfortunately, there was great variability in the results between samples, and one of the haemocytes lysate supernatant samples showed no activity at all. It is unlikely to be a reflection of the purity of the cell separation as for the other assays there was no cross contamination of the cell types. Further research is required to clarify the levels of SOD in semi-granular cells.

The presence of catalase, GPX and SOD within the hyaline cells is not surprising. These cells produce highly toxic oxygen metabolites following non-self stimulation (see

Chapters 4 and 5) and the activity of these enzymes converts potentially deleterious oxygen radicals to water and oxygen. Hence, the presence of these enzymes within these cells would minimise the risk of damage to the host tissues. The present study shows that the granular cells also contain GPX and SOD, although the granular cells do not produce oxyradicals through a respiratory burst (Chapter 4). Adema *et al.* (1991a) have demonstrated that oxygen radicals are released from the cells of the snail, *Lymnaea stagnalis*, during the respiratory burst. If the same scenario applies to *C. maenas* and oxygen radicals are released from crab hyaline cells, it would be likely that granular cells may come into contact with them and therefore require protective mechanisms.

In this study only three specific antioxidant enzymes have been detected. It would be interesting to assay HLS samples for other enzymes such as glutathione reductase to identify the full repertoire of enzymes that might be present in *C. maenas*. Biochemical analysis of subcellular fractions is also required to determine the precise organelle location of these enzymes. Research into the role of antioxidant enzymes in the proPO system in crustaceans is also required. Regulators of the proPO system have already been identified (Häll and Söderhäll, 1982; Söderhäll, 1983; Hergenhahn *et al.*, 1988). These protective enzymes may also be involved by protecting the host tissues from the oxygen radicals that are produced during the PO mediated oxidation of phenol to quinones (see review by Nappi and Vass, 1993).

In addition it would be interesting to look for non-specific defences. Recently some research has demonstrated the link between dietary vitamin intake and immunocompetence in fish (Hardie *et al.*, 1993). Vitamin C affects disease resistance in some fish species (Durve and Lovell, 1982) by affecting a range of immune responses

including complement activation (Hardie *et al.*, 1991) and phagocytosis (Li and Lovell, 1985). It would be pertinent to see if dietary supplementation with vitamins affects phagocytosis or the other immune responses in *C. maenas*.

Another potential area of research is to look at enzyme regulation. It is already recognised that anemones with algal endosymbionts can regulate the levels of antioxidant enzymes in the tissues (Dykens and Shick, 1982, 1984). These animals have the highest levels of the enzymes in tissues with the greatest concentration of symbionts (Dykens and Shick, 1984) and much higher levels than animals that do not have symbionts (Dykens and Shick, 1982). Crabs do not have algal symbionts, but work by Wenning *et al.* (1988) has demonstrated that, in *G. demissa*, pollution induced increases in $O_2^-$ production results in the enhancement of antioxidant enzyme levels. These results suggest that there is a self-regulating production of antioxidant enzymes associated with increasing oxidative stress (Wenning *et al.*, 1988). Regulation of the levels of antioxidant enzymes may also occur in response to infection. A link between several diseases and oxyradical production has been demonstrated (Nottage and Birkbeck, 1990; Anderson *et al.*, 1992b). It would be interesting to identify whether antioxidant enzyme levels in the crab could be regulated in response to environmental pollution or disease.

# Chapter 7

# General Discussion

## 7.1. Interaction between Hyaline Cells of *C. maenas* and Bacteria *in vitro*

In this thesis, using the shore crab, *Carcinus maenas*, as the experimental animal some of the parameters that influence phagocytosis and some of the metabolic responses of the phagocytic hyaline cells are presented. Previous work has indicated that crab haemocytes ingest both Gram-negative and Gram-positive bacteria *in vitro* (Smith and Ratcliffe, 1978). Subsequently, Smith and Söderhäll (1983a) and Söderhäll *et al.* (1986) suggested that uptake is enhanced by the activation of the proPO system. The present study confirms that treatment of the Gram-negative bacterium, *Psychrobacter immobilis* (formerly *Moraxella* sp.) with HLS containing an active proPO system results in enhanced uptake by the hyaline cells. Opsonisation only occurs if the proPO system is active (see Chapter 2), as evidenced by the abolition of enhanced uptake by incubating the HLS with serine protease inhibitors. This finding shows that recognition factors are generated as a result of the proteolytic activation of the proPO system (see Chapter 2) although the exact nature of the opsonic factor(s) still needs to be clarified. Experiments also confirm the work of Söderhäll *et al.* (1986) that PO, itself, is not responsible for opsonisation (see Chapter 2). Related studies of the metabolic processes associated with phagocytosis by hyaline cells, further show that energy, generated during electron transfer and oxidative phosphorylation, are essential for the ingestion stage of phagocytosis (see Chapter 2). In addition, an intact cytoskeleton is necessary for the engulfment of non-self materials (see Chapter 2).

This study also demonstrates that, following ingestion, ca 84% of the bacteria are killed within 3 h. To determine whether bactericidal action is due to oxygen radical formation, as has been established for mammals (Gabig and Babior, 1981) and fish

(Sharp and Secombes, 1993) some of the metabolic responses of crustacean cells following non-self stimulation were also investigated. Evidence is presented that PMA and con A stimulate oxygen radical production by the hyaline cells of *C. maenas* (see Chapters 4 and 5). Parallel assays on a range of invertebrate species, encompassing representatives of the Annelida, Mollusca, Echinodermata and Urochordata, show that production of a respiratory burst is a general phenomenon for invertebrate phagocytes (see Chapter 5). The oxygen moieties which are generated have potentially deleterious effects on the host tissues and cells and for *C. maenas* protective antioxidant enzymes; catalase, GPX and SOD, (Cu/Zn) and (Mn) forms, were found to be localised within the haemocytes and plasma (see Chapter 6). The activities of these enzymes probably serve to convert toxic oxygen intermediates to water and oxygen, thereby removing deleterious ions and ensuring protection of the host.

Phagocytosis is effective in rapidly clearing bacteria from the circulation of *C. maenas* (Smith and Ratcliffe, 1980a). Within 5 min, 75% of the bacteria are cleared and after 6 h, 90% are removed (Smith and Ratcliffe, 1980a). From the work carried out in this thesis, it was found that hyaline cells effectively kill bacteria *in vitro* (see Chapter 3). At a bacteria:hyaline cell ratio of 20:1, killing is 80% effective within 3 h, but at higher ratios (i.e. 200:1), killing is impaired and approximately 50% of the bacteria survive. It should be remembered that this is an *in vitro* situation with only one cell type present and therefore is unlikely to be a reflection of the consequences of a bacterial load of this size *in vivo*.

## 7.2. Overall Host Responses to Bacterial Infection *in vivo*

When bacteria infect the body fluids of *C. maenas*, phagocytosis and nodule formation eliminate or minimise the infection (Smith and Ratcliffe, 1980a,b). Previously, the importance of hyaline cells has been underestimated, as killing factors, opsonins and other "cell activators" are derived from granular cells (Söderhäll *et al.*, 1986; Chisholm and Smith, 1992). As the generation of oxygen radicals has been shown to occur in a whole range of animals from annelid phagocytes (Chapter 5) to vertebrate leukocytes (Babior *et al.*, 1973), it is possible that superoxide ion production is an ancient phenomenon. Possibly, in crustaceans, the metabolic activity of the hyaline cells in generating oxygen radicals evolved separately to the opsonins and antibacterial factors. Hence, the following scenario is proposed as a possible explanation of the interaction of the host responses to bacterial infection.

Once bacteria have gained access to the haemolymph they come into contact with the cells. The non-self signals of the bacteria, such as LPS, induce degranulation and lysis of the granular cells (Söderhäll *et al.*, 1986) effecting the release of opsonic, antibacterial and nodule promoting factor(s). The opsonic factor(s) serve to enhance phagocytic uptake by the hyaline cells, and following ingestion, the hyaline cells kill the bacteria. Antibacterial factors, which are found exclusively in the granular cells, are simultaneously released into the haemolymph (Chisholm and Smith, 1992). Bacteria which have not been ingested by the hyaline cells, will be destroyed by these antibacterial factors. Chisholm and Smith (1992) have shown that these factors are 90% effective within 1 h of challenge and are bacteriostatic and bacteriolytic in action (Chisholm,

1993). Killing may also occur, within the clumps or nodules, by antibacterial factors released from the granular cells. White *et al.* (1985) have shown that antibacterial activity is associated with haemocyte clumps within the gills of *C. maenas*. Some killing may also be achieved by the production of oxygen radicals by the hyaline cells. However, the link between oxygen radical production by crustacean cells and intra-nodule bactericidal activity still needs to be demonstrated. Oxygen radicals may further play a part in the oxidative process of melanization of the nodules (Nappi and Vass, 1993). The presence of antioxidant enzymes within the haemocytes or in the plasma probably ensure that the host tissues are not deleteriously affected by the oxygen radicals produced during phagocytosis or nodule formation. *In vivo*, phagocytosis and nodule formation triggered or augmented by factors released from the semi-granular and granular cells, result in the quick, efficient sequestration of bacteria from the circulation and minimises the deleterious consequences of a widespread infection.

## 7.3. Summary and Points of Further Study

The growth of aquaculture, the recognised role of invertebrate vectors in the transmission of disease and the growing need for ethically acceptable hosts for medical and scientific research have prompted the recent expansion in the field of comparative immunology. Much of this work has involved crustacean species, with investigations undertaken into both the humoral and cellular defence responses (see reviews by Smith and Chisholm, 1992; Söderhäll and Cerenius, 1992). Despite this, there are still many questions that have not been resolved, not least non-self recognition at the molecular level. Few cell surface receptors have been identified for crustaceans, and, although the

activation of the proPO system is thought to produce opsonic factor(s) (Söderhäll *et al.*, 1986), identification of the recognition molecule(s) has yet to be carried out. It is essential that opsonic factors are purified and characterized if functional studies of non-self recognition in crustaceans are to be informative at the molecular level. Characterization of the opsonic factor(s) will provide data on the repertoire of surface receptors that are present on crustacean haemocytes. Knowledge of these cell surface receptors may also provide clues as to why only ca 15% of hyaline cells are capable of uptake. Perhaps receptors are not present on the other 85%, and the haemocytes that are capable of phagocytosis are a subpopulation of the hyaline cells.

The measurement of toxic oxygen metabolite production, by the superoxide anion and phenol red assays, provides a simple, rapid, sensitive and quantitative method for investigating the metabolic events that underlie non-self stimulation of invertebrate phagocytes. Possible applications of these techniques include the assessment of the physiological role for $O_2^-$ and $H_2O_2$ in the host defence systems of marine invertebrates. It would also be pertinent to confirm that products of a respiratory burst are responsible for the bactericidal activity of hyaline cells.

The respiratory burst assays and the assays used to measure antioxidant enzyme activities may also provide tools for investigating the biological effects of environmental pollution. Utilisation of these assays, to monitor the haemocyte responses of biological marker species to xenobiotic pollution may prove to be useful (Di Giulio *et al.*, 1989). Many of the previous studies of the effect of environmental pollution have used the method of single species toxicity LC50 assays (see review by Cairns, 1989). More

recently, Smith and Johnston (1992) have examined the effects of environmental pollution on cellular responses in *Crangon crangon*. These authors demonstrated that sub-acute levels of PCBs significantly decrease haemocyte counts, haemolymph volume and PO activity (Smith and Johnston, 1992). Possibly oxygen radical generation may be another parameter which could be measured to evaluate the effects of pollutants on immunocompetence in invertebrates. A study by Chen *et al.* (1991) suggested that the earthworm, *Lumbricus terrestris*, would be a suitable marker for the assessment of nonspecific immunotoxicity of environmental xenobiotics. However, baseline values for nitroblue tetrazolium reducing activity of the coelomocytes will have to be determined (Chen *et al.*, 1991). It is important that baseline values of oxygen radical generation and antioxidant enzyme levels using a range of assays are established for healthy animals taking into account such factors as natural variation within populations and seasonality. Unfortunately, environments are often affected by more than one pollutant. Hence, the synergistic action of pollutants, such as phenols and nitroaromatics, on cellular responses also needs to be elucidated before the true effects of pollution on the immunocompetence of organisms can be established.

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# IN VITRO SUPEROXIDE PRODUCTION BY HYALINE CELLS OF THE SHORE CRAB *Carcinus maenas* (L.)

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☐Abstract—**This study examines the phagocytic hyaline cells of the shore crab, *Carcinus maenas*, for a respiratory burst in vitro. Following stimulation of the cells with phorbol myristate acetate (PMA), ferricytochrome c was reduced at 550 nm. Addition of superoxide dismutase (SOD) to the reaction mixture decreased this reduction, confirming that superoxide ions are produced by the stimulated hemocytes. Phytohemagglutinin, con A, and LPS were also shown to stimulate the cells although laminarin failed to elicit a burst. This is the first demonstration of a respiratory burst for crustacean hemocytes.**

☐Keywords—**Shore crab; *Carcinus maenas;* Respiratory burst; Ferricytochrome c; Hyaline cell; Superoxide anion; Phagocytes; Hemocyte.**

## Introduction

Phagocytosis is a process shown by certain blood cells of nearly all animals. For invertebrates that lack T lymphocytes and do not express specific immunoglobulins, phagocytosis plays a central role in the removal of microorganisms from the blood or body fluids. Although many workers have examined phagocytosis in invertebrates (1), most studies have been concerned with recording phagocytic indices and rates in vitro in different species, or monitoring the effects of variables such as temperature and opsonisation on levels of uptake (2,3). Fewer investigations have studied the metabolic events associated with phagocytosis in invertebrates or of the activation of their phagocytes by foreign materials. To further our understanding of phagocytosis, experiments need to be carried out on the underlying intracellular events that are associated with nonself stimulation of the phagocytic cells in invertebrate animals.

With the shore crab, *Carcinus maenas*, previous work has shown that the hemocytes are able to ingest both Gram positive and Gram negative bacteria in vitro (4). Subsequent experiments with separated cell populations (5) have established that uptake is achieved only by the hyaline cells and is enhanced by factors (opsonins) produced by the granular cells (6). As yet the biochemical nature of the opsonins in *C. maenas* and their mode of action are unknown. Our understanding of the biochemistry of the cell signals involved in phagocytosis in this animal is hampered by the paucity of knowledge of the intracellular processes involved in recognition and ingestion. The present study aims to examine some of the events associated with recognition by exploiting the cell separation technique of Söderhäll and Smith (5). This has the advantage of confirming purity of the hyaline cells and allows us to examine the effects of different treatments on individual cell types without complications caused by cellular interactions (6).

In mammals, stimulation of the phagocytic membrane is known to produce highly reactive oxidising agents

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with powerful microbicidal activity (7,8). This is known as the respiratory burst. The enzyme responsible for the reaction (NADPH oxidase) is membrane-associated and catalyses the conversion of oxygen to superoxide ions ($O_2^-$) (8). The $O_2^-$ ions generated dismutate spontaneously or are catalysed by the enzyme superoxide dismutase (SOD) to give hydrogen peroxide ($H_2O_2$) (9). The $H_2O_2$ provides additional microbicidal activity (8,10). In mammalian polymorphonuclear leukocytes, the respiratory burst has been studied using the reduction of cytochrome c (Fe III) by $O_2^-$ (8). Recently this technique has also been used to examine the biochemistry of the respiratory burst in fish blood cells (11,12) and it has also been applied to molluscs (13). Using the techniques of chemiluminescence and nitroblue tetrazolium reduction Anderson et al. (14) and Cheng (15) have reported that, in insects and molluscs respectively, the hemocytes appear to be incapable of producing superoxide and other reactive oxygen moieties. However, Nakamura et al. (16) have shown that hemocytes of the bivalve mollusc *Patinopecten yessoenis* produce $H_2O_2$, in vitro, while Shozawa (17) and Connors and Yoshino (18) have reported $O_2^-$ production by the hemocytes of the gastropod mollusc *Biomphalaria glabrata*. Dikkeboom et al. (19–23) have shown that the hemocytes of a variety of molluscan species produce $O_2^-$ and $H_2O_2$. Pipe (24) examined the generation of $O_2^-$ and $H_2O_2$ by hemocytes of the mussel *Mytilus edulis*. Lastly, Ito et al. (25) have reported the production of $H_2O_2$ by the phagocytes of the sea urchin *Strongylocentrotus nudus*. Because there are no equivalent studies in crustaceans, the present study uses a method proposed by Pick and Mizel (26) to examine the production of superoxide by hyaline cells of the shore crab, *Carcinus maenas*, and to study the basic features of this phenomenon in vitro.

## Materials and Methods

### *Animals*

Specimens of *Carcinus maenas* were collected from St. Andrews Bay, Scotland and were kept in a flowing seawater aquarium (ca. 10°C ± 2°C) for 2 weeks prior to use. Only healthy adult males at moult stage $C_4$ (27) were used in experiments.

### *Bleeding and Separation of Cells*

Bleeding was carried out as described by Söderhäll and Smith (5). The hemolymph was diluted 1:1 with citrate: EDTA buffer as anticoagulant (AC) (5). The diluted hemolymph was loaded immediately onto preformed 60% Percoll (LKB Pharmacia, Sweden) gradients in 3.2% NaCl and the cells spun at 2,000 *g* for 20 min at 4°C (5). The cells were harvested and diluted with 3.2% NaCl to approximately $1–9 \times 10^7$ cells $mL^{-1}$, as counted on a hemocytometer, with the different cell types in each band identified according to the criteria given in Bauchau (28). The crabs were usually bled at the same time of day to avoid possible variations in the hemocyte populations caused by endogenous rhythms.

### *Elicitors*

The following were tested as elicitors of the respiratory burst: (a) laminarin (β 1,3-glucan from *Laminaria digitata*, Calbiochem, La Jolla, CA); (b) lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4 phenolic extraction, Sigma, Poole, Dorset); (c) phytohemagglutinin (PHA-P, sterile filtered L9132, Sigma); (d) phorbol 12-myristate 13-acetate (PMA, Sigma); and (e) concanavalin A

(con A, Type VI specific affinity chromatographically purified, Sigma).

Soluble elicitors were used in our investigation instead of bacteria and yeast as preliminary work had shown that particulate matter affected the optical density of the reaction mixtures (see below): soluble elicitors therefore give more reproducible results with the superoxide anion assay. PMA is a known activator of the respiratory burst response in fish (12) and con A has been shown to stimulate $H_2O_2$ production in scallop amoebocytes (16). In addition, LPS and laminarin were used as possible natural elicitors since these are derived from microbial cell walls and are known to induce cellular defense responses in crustaceans (29,30). Finally PHA-P was chosen as an elicitor because it is known to have a variety of bioactive effects (31).

### *Superoxide Anion Assay*

The reaction was carried out in 96-well, flat-bottomed microtitre plates, one per animal. For each treatment quadruplicate wells were prepared. All of the chemicals were made up in marine saline (MS) (0.5 *M* NaCl; 11 m*M* KCl; 12 m*M* $CaCl_2 \cdot 6H_2O$; 45 m*M* Tris; 0.1 *M* HCl; 26 m*M* $MgCl_2 \cdot 6H_2O$; pH = 7.4). The experimental mixture in each well consisted of 25 μL of cells, 25 μL of elicitor (1 mg $mL^{-1}$, stock solution) 20 μL catalase (1 mg $mL^{-1}$, stock solution) and 50 μL of 160 μ*M* ferricytochrome c. For the controls, MS was substituted for the elicitor. All reagents were independently incubated with ferricytochrome c to ensure that no interaction occurred between these compounds. The plates were incubated at 20°C and read at 550 nm at 5-min intervals on a microplate reader (Dynatech, MR5000). Two readings were made for each plate at 5-min intervals prior to the addition of the elicitor to ensure that the absorbance values of the wells were stable. Individual well values within treatments did not vary by more than 0.005 absorbance units. Each treatment was repeated for a minimum of five animals and the absorbance values for the quadruplicate wells were meaned for each treatment.

The assay was carried out with the inclusion of 0, 1, or 2 mg $mL^{-1}$, stock concentration, of catalase (from bovine liver EC 1.11.1.6, Sigma) with the elicitor LPS to ascertain the effect of $H_2O_2$ oxidation of ferrocytochrome c. To determine the optimum concentration for stimulation of the respiratory burst, PMA or LPS were used at final concentrations in the range of 0–5 μg $mL^{-1}$. A comparison was made of the ability of PMA to stimulate the hyaline, semigranular, or granulocyte cells.

To investigate whether $O_2^-$ production is dependent on the hexose monophosphate shunt (HMS) as in fish (12), the effect of glucose on ferricytochrome c reduction by crab hyaline hemocytes was investigated. Each animal was bled twice, once into AC and then into AC without glucose. Assays were also carried out for each hemocyte preparation with 0, 10, and 100 m*M* levels of glucose added to the MS in which the ferricytochrome c was dissolved.

Finally, to confirm that the reduction of ferricytochrome c was at least in part caused by $O_2^-$, SOD (Sigma EC 1.15.1.1 from bovine erythrocytes) was included in the reaction mixture. The SOD was rehydrated in distilled water and then made up in MS. Twenty microlitres were added to each well to give a final concentration of 100 units $mL^{-1}$. In the control wells, 20 μL of MS was substituted for the SOD. As an additional control to confirm the specific effect of SOD on ferricytochrome c reduction, parallel wells were set up containing hemocytes, PMA, ferricytochrome c, and SOD that had been heat-inactivated by boiling for 30 min. All samples were then incubated and read as above.

*Statistical Analysis*

Differences between treatments were analysed statistically by paired Student's *t*-test on combined data from five to seven animals depending on the experiment. Differences were considered significant when $p \leq 0.05$ (32).

## Results

Stimulation of the hyaline cells with PMA resulted in a reduction of ferricytochrome c to give an absorbance value of ca. 0.053. Cells stimulated with MS gave an absorbance of ca. 0.031 after 30 min. The difference between absorbance values for the experimentals and controls was significant with $p = 0.044$ after 10 min and $p = 0.005$ after 30 min. The time course of this reaction is shown in Figure 1.

Figure 2 shows the effect of 0, 1, and 2 mg $mL^{-1}$ of catalase on the reoxidation of ferrocytochrome c. Catalase significantly increased the absorbance values in the LPS treated wells from ca. 0.029 to ca. 0.040 for 1 mg $mL^{-1}$ and to ca. 0.044 for 2 mg $mL^{-1}$ ($p < 0.001$ for both concentrations compared to the catalase-free controls, Fig. 2).

To determine the optimum concentration of elicitor, the response to increasing doses of PMA or LPS was examined. Optimum stimulation by PMA occurred between 0.2–5 μg $mL^{-1}$, and by LPS between 0.1–5 μg $mL^{-1}$ (data not shown). Accordingly, 5 μg $mL^{-1}$ of elicitor was chosen for all further assays.

Comparison of the abilities of the three hemocyte types to produce a respiratory burst showed that for the hyaline cells, stimulation with PMA for 30 min resulted in an increase in absorbance to ca. 0.070 (Fig. 3). This was significantly different from the MS-treated controls that produced a value of ca. 0.050 ($p \leq 0.001$) (Fig. 3). By contrast, the semigranular cells and granulocytes yielded absorbance values of ca. 0.064 and 0.063, respectively, following stimulation with PMA (Fig. 3) whereas the controls treated with MS gave absorbance values of ca. 0.050 for the semigranular cells and 0.055 for the granulocytes (Fig. 3). These were not significantly different from the experimental treatments ($p = 0.062$ and $p = 0.071$, respectively).

Hemocytes harvested into normal, glucose-containing AC produced an increase in absorbance to ca. 0.050 at 550 nm after 30 min incubation with PMA. This was significantly higher than the

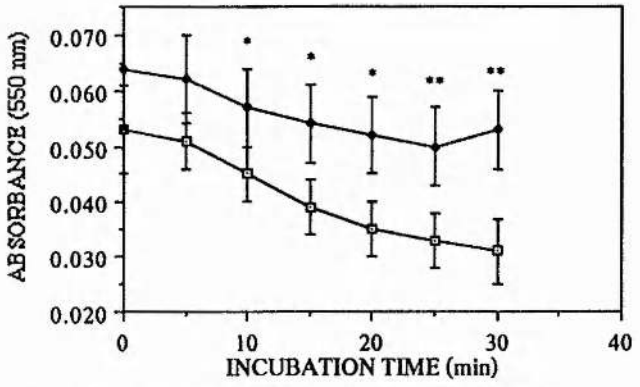


**Figure 1.** Reduction of ferricytochrome c at 550 nm by separated hyaline cells of *C. maenas* in vitro. Twenty-five microlitres of cells were incubated with 20 μL of catalase (4 μg $mL^{-1}$, final concentration); 50 μL of 160 μ*M* ferricytochrome c and challenged with 25 μL of 5 μg $mL^{-1}$ (final concentration) of PMA; or, for controls with 25 μL of MS. The change in absorbance was measured at 5-min intervals at 550 nm for the first 30 min after stimulation. Values are means in absorbance at 550 nm ± SE bars. $n = 5$. *$p \leq 0.05$; **$p \leq 0.01$; (–⊡–) MS; (–♦–) PMA.

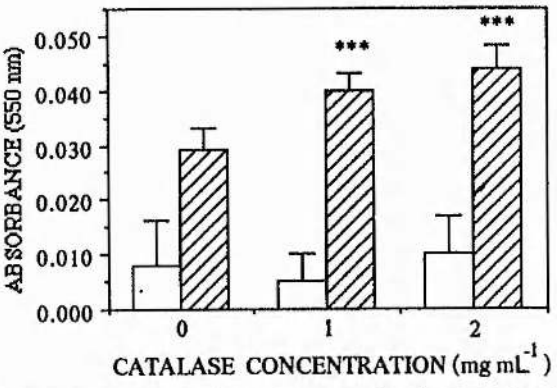


**Figure 2.** Effect of catalase on the LPS-elicited respiratory burst of *C. maenas* hyaline cells, in vitro. Twenty-five microlitres of cells were incubated with 20 μL of 0 (MS); 1 or 2 mg $mL^{-1}$ of catalase (stock solution); 50 μL of 160 μ*M* of ferricytochrome c and challenged with 25 μL of 5 μg $mL^{-1}$ LPS (final concentration); or, for controls with 25 μL of MS. Values are means in absorbance at 550 nm ± SE bars after 30-min incubation at 20°C. $n = 6$. ***Differences between the experimental (catalase-incubated) and buffer-treated controls are significant at $p \leq 0.001$. In every case, there is a significant difference ($p \leq 0.05$) between the LPS-incubated and MS-treated controls. (□) MS (▨) LPS

MS-treated controls where the absorbance was ca. 0.039 ($p = 0.003$). Inclusion of 10 or 100 m*M* glucose in both experimental and control wells produced no further increases in the absorbance values (i.e., the absorbance remained at ca. 0.050 for the experimentals and ca. 0.039 for the controls). Cells bled into glucose-free AC, however, did not produce a significant reduction in ferricytochrome c when treated with PMA. In this case, the absorbance value was ca. 0.035 similar to the MS-incubated samples where the absorbance was ca. 0.043 ($p = 0.130$). Again, inclusion of glucose (10 m*M*) did not produce a significant change in absorbance values following incubation of the cells with PMA ($p = 0.192$, compared with MS-treated controls).

Addition of exogenous SOD to PMA-stimulated cells resulted in lower absorbance values (ca. 0.052) than the PMA-stimulated cells alone, for which the absorbance was ca. 0.062 after 30 min (Table 1, $p = 0.011$, $n = 6$). Inactivation of SOD by boiling for 30 min resulted in the loss of inhibition of ferricytochrome c reduction (data not shown) confirming that SOD acts specifically to scavenge superoxide ions.

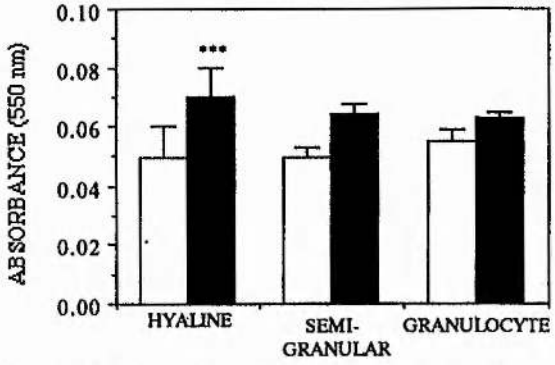


**Figure 3.** The different cellular response of separated crab hemocytes to PMA stimulation, in vitro. Twenty-five microlitres of each cell type ($1-9 \times 10^7$ $mL^{-1}$) were incubated with 20 μL of catalase (4 μg $mL^{-1}$, final concentration); 50 μL of 160 μ*M* ferricytochrome c; and either challenged with 25 μL of 5 μg $mL^{-1}$ of PMA (final concentration); or, for the controls, 25 μL of MS. The change in absorbance at 550 nm was recorded after 30-min incubation at 20°C. These values are means of absorbance readings from five animals with bars representing SE. ***$p \leq 0.001$. (□) MS; (■) PMA.

**Table 1. Effect of SOD on Ferricytochrome c Reduction by Separated Hyaline Cells of *C. maenas*, in vitro.**

| Treatment* | Absorbance† |
|---|---|
| Control‡ | 0.050 |
| PMA | 0.062 |
| PMA + SOD | 0.052 |
| SOD§ | 0.053 |

* Twenty-five microlitres of hyaline cells were challenged with 25 μL of PMA (5 μg $mL^{-1}$, final concentration); incubated with 20 μL of catalase (4 μg $mL^{-1}$, final concentration); 50 μL of 160 μ*M* of ferricytochrome c; and 20 μL of either SOD 100 units $mL^{-1}$ (final concentration) or MS at 20°C.

† Readings given are the absorbance values at 550 nm after 30-min incubation. Values are the results of a representative animal. Each treatment was repeated six times with different animals and similar trends were obtained on each occasion, although there were variations in baseline values.

‡ Cells treated with 25 μL of MS to act as buffer control.

§ Cells incubated without the PMA and only the SOD.

Table 2 compares the effect of different elicitors on the hyaline cells. All the absorbance readings were taken after 30 min. Incubation of the cells in LPS produced a significant reduction in ferricytochrome c ($p = 0.046$, compared with the MS-treated controls, $n = 5$) whereas laminarin failed to elicit a change in absorbance ($p = 0.439$, compared with the MS-treated controls, $n = 6$, Table 2). Occasionally some animals did not respond to LPS stimulation and further examination of these animals showed that the cells had a bacterial infection. The data from these specimens were not included in the statistical analysis. Con A ($n = 7$) and PHA-P ($n = 6$) always stimulated a reduction in ferricytochrome c ($p < 0.001$ and $p < 0.001$, respectively) in contrast to the cells stimulated with MS (Table 2).

## Discussion

Our results demonstrate that the hyaline cells of the shore crab, *Carcinus maenas*, produce superoxide anions when stimulated by PMA. These cells were also found to respond to PHA-P, con A, and LPS by producing superoxide ions that reduced the ferricytochrome c. Laminarin failed to elicit this response. The differential effect of the elicitors on the hyaline cells may indicate a repertoire of surface receptors on the phagocyte membrane. Soluble elicitors (LPS, laminarin, PHA-P, and con A) were used since particulate matter, such as bacteria or zymosan, tended to give less reproducible results. To further our understanding of this response in vitro, studies into the activation of crustacean phagocytes by bacteria and other particulate matter using fluorometric rather than spectrophotometric techniques are currently being carried out.

**Table 2. Effect of Different Elicitors on Ferricytochrome c Reduction by Separated Hyaline Cells of *C. maenas*, In vitro.**

| Treatment* | Con† | Exp‡ |
|---|---|---|
| PMA (5 μg mL$^{-1}$) | 0.023§ | 0.038¶ |
| con A (5 μg mL$^{-1}$) | 0.023 | 0.041# |
| PHA-P (5 μg mL$^{-1}$) | 0.027 | 0.035# |
| LPS (5 μg mL$^{-1}$) | 0.029 | 0.035‖ |
| Laminarin (5 μg mL$^{-1}$) | 0.035 | 0.038 |

* Twenty-five microlitres of hyaline cells were challenged with 25 μL of the appropriate elicitor (5 μg mL$^{-1}$, final concentration); incubated with 20 μL of catalase (4 μg mL$^{-1}$, final concentration); and 50 μL of 160 μ*M* of ferricytochrome c at 20°C.
† Cells treated with 25 μL of MS.
‡ Cells challenged with 25 μL of elicitor. All elicitors were made up in MS to give a final concentration of 5 μg mL$^{-1}$.
§ Readings given are the absorbance values at 550 nm after 30-min incubation. Values are the results of a representative animal. Each treatment was repeated a minimum of five times with different animals and similar trends were observed on each occasion although there were variations in baseline values.
¶# Differences between experimental and MS treated controls are significant. ‖$p \leq 0.05$; ¶$p \leq 0.01$; #$p \leq 0.001$.

The present study also considered the effect of SOD on the crab cells. Superoxide dismutase is known to convert $O_2^-$ to molecular oxygen and hydrogen peroxide (9). Removal of the $O_2^-$ ions would lower the amount of ferricytochrome c reduction in the wells. The results obtained in our study show that when cells are incubated with exogenous SOD and PMA, the absorbance values are less than when the cells are incubated without SOD. This confirms that the observed reduction of ferricytochrome was caused, at least in part, by $O_2^-$. Furthermore, the failure of the cells to produce $O_2^-$ in the absence of glucose indicates that the respiratory burst is dependent upon the hexose monophosphate shunt, as is the case with fish (12) and mammals (33). However, the reason why the addition of glucose to cells bled into glucose-free AC failed to restore the response is unknown.

The effect of the spontaneous dismutation of $O_2^-$ to $H_2O_2$ on absorbance values was studied by Vandewalle and Petersen (34). From spectrophotometric studies of a cell-free system, these authors proposed that hydrogen peroxide, the major product of the spontaneous dismutation of the superoxide radicals, is

capable of oxidising ferrocytochrome c (II) thus counteracting the increase in optical density caused by $O_2^-$. Turrens and McCord (35) disagreed, believing that the amount of hydrogen peroxide produced is not enough to affect ferricytochrome c reduction. In our experiments with *C. maenas* blood cells, it was found that the absorbance values were generally higher in the presence of catalase (Fig. 2). Catalase was, therefore, routinely included in the reaction mixtures to counteract the oxidation of the ferrocytochrome c.

In all our experiments there was a reduction in the optical density over the 30-min incubation period despite the presence of catalase. By contrast, studies of the respiratory burst in fish and mammals have revealed that the optical density tends to increase as the reaction proceeds. At present it is still unknown why crab hyaline cells produce a decrease in absorbance. Possibly the hemocytes produce such large quantities of $H_2O_2$ that optical density falls rather than rises. Further research is needed on the kinetics and quantification of $H_2O_2$ production in these cells to resolve this enigma.

The change in absorbance observed in *C. maenas* was also consistently lower than those observed in fish and mammals (36,37). This may be partly accounted for by the relatively low rates of uptake that crab hemocytes exhibit in vitro (4,6). With vertebrates, uniform cell cultures are used routinely for experimental analyses and the cells are likely, therefore, to respond in the same way: that is, with all the cells exhibiting a burst, the magnitude of the response will be greater. In crabs, the population of hyaline cells is heterogeneous (28) so it is possible that some of the cells do not respond to stimulation, even with an elicitor as potent as PMA.

To the best of our knowledge, this is the first study to show that crustacean hemocytes have the ability to produce a respiratory burst when stimulated by exogenous materials. This phenomenon probably plays a part in the antimicrobial defense of the organism in a similar way to that reported for mammals (38). However, the ability of superoxide and hydrogen peroxide radicals to act in an antimicrobial manner in crustacean host defense still needs to be elucidated. Experiments also need to be carried out to determine further biochemical details of this response in the crab. Analysis of the respiratory burst is a potentially valuable way of investigating phagocyte activation in invertebrates. Moreover the ferricytochrome c assay is a simple and rapid technique for objectively assessing this cellular phenomenon with respect to different soluble elicitors. Evaluation of the magnitude and duration of the response should reveal much about the range of cell-surface receptors and about the nature of signal transduction during nonspecific recognition.

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