Effects of methyl parathion on the cellular immune responses in giant black tiger shrimp, Penaeus monodon

Nantarika Bodhipaksha

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Effects of methyl parathion on the cellular immune responses in giant black tiger shrimp, *Penaeus monodon*

Bodhipaksha, Nantarika, Ph.D.
The College of William and Mary, 1994

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EFFECTS OF METHYL PARATHION ON THE CELLULAR IMMUNE RESPONSES IN GIANT BLACK TIGER SHRIMP, Penaeus monodon

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

by
Nantarika Bodhipaksha
1994
APPROVAL SHEET

This dissertation is submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Nantarika Bodhipaksha

Approved December, 1993

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ABSTRACT

The effects of an organophosphorus pesticide, methyl parathion, on cellular immune defense mechanisms of the giant black tiger shrimp (Penaeus monodon) were studied. Animals were exposed for 96 hours at concentrations equal to, above and below the LC₉₀ (3 ppb). Phagocytes were obtained from the heart and circulating hemolymph. Cellular immune responses, including chemotaxis, phagocytosis and the respiratory burst were examined. Chemotactic activity was determined by means of a modified Boyden double chamber technique. There was a dose-dependent decrease in the chemotactic activities of both circulating hemocytes and cardiac phagocytes. Phagocytic activity (percent phagocytosis) was examined by the microscopic enumeration of phagocytes which had internalized yeast cells. For both circulating and cardiac phagocytes, there was a decreasing trend in the phagocytosis of yeast cells as the concentration of methyl parathion was increased. Exposure to methyl parathion at and above 2 ppb resulted in a significant reduction in phagocytic activity for both cell types as compared to the control group. The phagocytic index for circulating and cardiac cells showed a decreasing trend with increasing concentrations with significant differences at and above 6 ppb. Since the measurement of superoxide has been accepted as an accurate way to quantify the intensity of the respiratory burst, superoxide production by hemocytes was measured by the reduction of the redox dye nitroblue tetrazolium (NBT). Both circulating and cardiac phagocytes exhibited significant increases in superoxide production at 6 and 10 ppb, as well as at 3 ppb in the case of cardiac phagocytes. Histopathological changes of the cells were observed in the gill, hepatopancreas, heart, muscle and ventral nerve, with the hepatopancreas containing the most pronounced changes. Electron microscopy revealed granular damage of the circulating hemocytes at 3, 6 and 10 ppb. These experiments indicate that methyl parathion alters cellular immune responses of Penaeus monodon in a dose-dependent manner. Further investigation of these immunological mechanisms is needed to explain the phenomenon of shrimp survival in contaminated environments.
EFFECTS OF METHYL PARATHION ON
CELLULAR IMMUNE RESPONSES IN GIANT BLACK TIGER SHRIMP,

PENAEUS MONODON
INTRODUCTION

Organophosphorus pesticide usage has increased since the ban on chlorinated hydrocarbons such as DDT. The advantage of organophosphorus pesticides is that, unlike some of the chlorinated hydrocarbons, they do not bioaccumulate in the environment. An estimated 40% of all crops have this type of pesticide applied to them (Wagner, 1983). Methyl parathion, a member of the parathion family, has been used extensively throughout the world. It is less toxic and less persistent in the environment than other parathions. Because the aquatic environment can become the reservoir for industrial and agricultural wastes, the organisms present in this environment are frequently found to be affected by these toxicants. A considerable amount of literature is available on the effects of various toxic chemicals on marine crustaceans (Tatem, 1977; Hale, 1989; Rao et al., 1988). However, very little information is available on the effects of methyl parathion on the immunological responses of the giant black tiger shrimp, Penaeus monodon, an important species in shrimp aquaculture.

Due to rapid growth of the shrimp culture industry around the world, maximum yield can only be obtained by minimizing mortality rates during the production period. This would be impossible if the immunity of the shrimp is impaired. The present study was undertaken to obtain data concerning the
effects of methyl parathion on the cellular immune system of shrimp. Phagocytosis processes which include chemotaxis, engulfment of foreign agents, and superoxide ion production were investigated using circulating hemocytes and cardiac phagocytes as representatives of the phagocytic cells in shrimp. Histopathologic and electron microscopic studies of cells and organ tissues were conducted to demonstrate the toxic effects of methyl parathion on exposed shrimp.

The results of these studies should benefit shrimp farm managers in their assessment of the effects of a contaminated environment on their crop. Shrimp farmers could prevent loss of animals prior to the onset of devastating disease. In addition, these results may be used to help set up environmental standards using shrimp as indicators of pollution.
I. INVERTEBRATE IMMUNITY

1.1 Introduction

The invertebrates include a vast array of animals. The challenge in working with invertebrate immune systems include diversities existing in five or six basic cell types in over 750,000 species (Ratcliffe et al., 1985). The diversity within the same species during different physiological and developmental states also makes techniques such as cell separation very difficult to perform in invertebrates. Despite this, the importance of invertebrate immunity is slowly being recognized since many molluscs and crustaceans are now being farmed extensively. An understanding of their host defense mechanisms could assist in avoiding and overcoming disastrous outbreaks of disease which may occur under the stressful, high density conditions of commercial culture.

The most important and fundamental aspect of the invertebrate cellular immune response is its function in combating harmful agents. Vertebrates, in contrast, are capable of both anticipatory and non-anticipatory immune responses (Horton, 1990). In mammals, the anticipatory responses are executed by T-and B-lymphocytes and antibodies while the non-anticipatory responses are carried out by macrophages, neutrophils, natural killer cells, eosinophils,
basophils, proteins of the coagulation system, lymphokines and many other elements. In the invertebrates, it would appear that the distinction between self and non-self is dependent on the combination of antibody-like substances with foreign material (McKay, et al., 1969). However, it is generally accepted that invertebrates are capable of non-anticipatory responses (Klein, 1989b). Many studies have shown that the defense mechanisms of invertebrates are extremely efficient in utilizing cellular and humoral components. Therefore, the belief that invertebrate immunity is affected simply by non-specific interaction of phagocytic cells with invading organisms, together with a number of apparently unrelated humoral factors such as lysozyme, is no longer tenable (Ratcliffe et al., 1985).

The so called "determinants" of infections in invertebrates are of prime importance in governing the outcome of infection or parasitism (Whitcomb, et al., 1974). These determinants include host genotype, anatomy, physiology, age, diet, behavior, degree of infection of the host and the nature of the infecting agent. For example, the infectivity of the snail, Biomphalaria glabrata by Schistosoma mansoni is due to a single dominant gene. Also, the resistance to infection is greatly influenced by the strain of the parasite, the age of the host and the presence of simultaneous infection caused by other parasites. Different parasites may cause a synergistic relationship and interfere with host defense (Bayne, 1983).
The mechanisms of invertebrate defense may be summarized as follows:

- Clotting, coagulation and wound healing
- Immunorecognition
- Opsonization
- Prophenoloxidase system and complement-like factors
- Humoral immunity
- Cellular immunity

1.2 Clotting, coagulation and wound healing

Invertebrate immune defense includes a range of cellular and humoral components. The clotting processes are of great importance in homeostasis (wound healing) and in the immune response. The homeostatic mechanisms in invertebrates work extremely rapidly and effectively. The rapid healing of a wound can be attributed mainly to the features of hemocyte coagulogen (Bohn, 1986). Wound closure with gelation of plasma is found in arthropods and has been reported in horseshoe crabs, arachnids, crustaceans and insects (Ratcliffe et al., 1985). Loeb (1903) developed the concept of two phases in the coagulation process of crustacea. According to this concept, a "cell coagulum" which is an agglutination of the blood corpuscles is formed in the first phase of clotting. The features of the first phase have been described as the formation of meshwork by the blood corpuscles as well as the
agglutination and fusion of hyaline exoplasmic components of these cells into masses of fibrin-like substances (Florkin, 1960). All these cells are involved in the formation of "cell fibrin" and in supplying substances such as blood coagulins which induce the second phase of clotting known as "plasma coagulation".

The coagulation of crustacean blood is a continuous process which begins around blood cells since these elements supply the substances which induce plasma coagulation (Hardy, 1982). Studies of hemocyte coagulogen and gel formation in arthropods have shown that in the absence of plasma proteins, a massive coagulum forms if the hemocytes are induced to disintegrate. This coagulogen is superficially similar to a true clot and resists treatment with highly concentrated solutions of denaturing agents, such as urea and guanidinium chloride as well as detergents or mixtures of both. The resistance of the coagulum allows for its crude purification. Nuclei, cell membranes and other cell remnants can be washed out with a solution containing 2 M urea and 2% sodium dodecyl sulphate. The remaining material will be a homologous gel-like structure which is called "hemocyte gel". The substance making up the gel is the "hemocyte coagulant" (Gupta, 1979).

The hemocyte coagulant is a very dark, densely packed material contained in the granules within the hemocytes. During an explosion-like ejection of the material, it swells considerably, presumably by hydration forming the gel (Bohn
and Barwig, 1984). During gel formation or soon thereafter, new disulfide bridges are formed, giving the gel its final stability (Bohn and Saks, 1986).

In arthropods, the coagulation process involves a complex enzyme cascade (Jackson and Nemerson, 1980). Generally, coagulogen is converted into the insoluble gel, coagulin, after activation by an elicitor such as a microbial extract, or a change in calcium ion concentration which activates several serine proteases. There are also protease inhibitors in the blood to prevent widespread activation of the coagulation system which would be fatal to the animal (Soderhall and Smith, 1984).

The interaction of coagulogens during clotting can be regarded as an adaptation of the open hemolymph system of arthropods. This restricts the clot formation to the wound site where the hemocytes disintegrate.

In arthropods, the clotting mechanism is very important not only for the closure of wounds, but also for the animal to survive by assisting in various kinds of immune reactions such as phagocytosis, nodule formation and encapsulation (Ratcliffe and Rowley, 1979).

1.3 Immunorecognition

It is now generally accepted that invertebrates do not express immunoglobulins and do not contain T- or B-lymphocytes as part of their hemograms, but that their recognition factors
are the agglutinins (Vasta and Marchalornis, 1983). These hemagglutinins or lectins have been detected in a wide range of organisms and several have been purified and characterized (Yeaton, 1981). The phagocytosis and elimination of erythrocytes and/or bacteria by the hemocytes of the crayfish, *Cherax destructor*, in vitro or in vivo, was dependent upon recognition factors present in the hemolymph or on the cell membranes of the reticuloendothelial network (Tyson and Jenkin, 1973). Lectins comprised of a heterogenous group of proteins or glycoproteins that are heat labile with polyvalent configurations bind specifically to cell surface polysaccharides (Yeaton, 1981) and in some cases have been shown to be calcium dependent (Marchalornis and Edelman, 1968). In the American lobster, *Homarus americanus*, Stewart and Zwicker (1972) and Cornick and Stewart (1973a, 1973b) considered serum recognition factors to be important in cellular responsiveness. However, it is still unclear how the lectins operate in vivo. The nature and extent of any regulatory mechanisms which modulate their reactivity in cellular resistance is unknown (Soderhall et al., 1986). Ofek and Sharon (1988) demonstrated that recognition may be indirect, depending on soluble factors that bind to foreign or damaged surfaces and mark them as targets for phagocytosis. These soluble factors could have actions similar to plasma antibodies and complement in vertebrates and could be called "opsonins".
Since the complex induced antibody responses based on rearranging genes have not been described in invertebrates (Marchalornis and Schluter, 1990), it could be presumed that the recognition molecules are found on the surfaces of cells involved in cell to cell recognition in development (Edelman, 1973) or in allore cognition phenomena. In response to challenge with bacteria or bacterial vaccines, invertebrates and vertebrates both exhibit the defense mechanisms involving lysozyme production (Boman et al., 1986). There is strong evidence for cellular recognition and aggressive reactions against foreign cells in invertebrates (Cooper, 1982). The recognition mechanisms in invertebrates for phagocytes to recognize non-self are generally mediated by protein molecules such as lectins (Brehe'lin, 1986).

Lectins are not antibodies or enzymes and are defined by their ability to bind specifically to sugar moieties. These sugar binding molecules are ubiquitous and can be found in all invertebrates (Gold and Balding, 1975). Considerable effort has been directed toward isolating and characterizing these molecules. The lectin specific for horse erythrocytes has been purified to homogeneity and is distinct from immunoglobulin (Liu et al., 1982). It is, however, clearly homologous to another vertebrate protein, the C-reactive protein (Liu et al., 1982). The C-reactive proteins have the capacity to react with cells and molecules of the immune system, and although they are unrelated to immunoglobulins,
they carry out many of the same general functions (Mold et al., 1982; Pypys, 1982). Therefore, although the available evidence suggests that invertebrates lack the adaptive immune responses characteristic of all vertebrates, many invertebrates, even ancient arachnids and insects, possess recognition molecules homologous to vertebrate defense molecules. Persuasive evidence based on protein sequencing data established the presence of a recognition family of molecules which corresponds to vertebrate hepatic lectins in the echinoderm (Giga et al., 1987).

In crustaceans, a lectin that agglutinated rabbit erythrocytes was purified from the American lobster (*Homarus americanus*) (Cornick and Stewart, 1973a). Lectins are carbohydrate binding, non-immunoglobulin proteins that agglutinate cells and/or precipitate glycoconjugates through interaction with glycoproteins or glycolipids (Boyd and Shapleigh, 1954). Lectins obtained from lobster hemolymph and hemocyte lysate demonstrated two peaks of activity by electrophoresis, one peak, LAg-1 reacted with N-acetylneuraminic acid residues and the other, LAg-2 reacted with N-acetyl-D-galactosamine residues. Heterogeneity of binding sites was demonstrated by Hall and Rowlands (1974). Although the carbohydrate binding specificity was limited to binding by lectins, secondary forces such as hydrophobicity and charge interaction, possibly operating through multivalent binding, may also stabilize the bond (Gold and Balding, 1975).
The uptake of erythrocytes by phagocytic cells from the crayfish \((Cherax\ destructor)\) was dependent on hemolymph factors (McKay and Jenkin, 1970). The elimination of bacteria from the hemolymph was promoted by opsonins which were depleted during this process, leading to a much slower secondary clearance. Crayfish phagocytes also ingested bacteria in the absence of hemolymph but at a much slower rate than in the presence of hemolymph (Tyson and Jenkin, 1974).

**OPSONIZATION**

In arthropods, there have been a number of debates as to whether or not there is specificity in opsonic activities in their sera. Studies with crustaceans were accepted as providing strong evidence for the presence of opsonins (Ratner and Vinson, 1983), but many studies using insects failed to reveal opsonins. Ratcliffe and Rowley (1983) found that 3 of 85 species of insects had strong titers of hemagglutinin activity against sheep red blood cells, but in no case were the hemagglutinins found to be opsonic. Pendland et al. (1988) showed that fungal spores that have galactose on their surfaces become opsonized by a galactose-binding lectin in the hemolymph of beet armyworm larvae. The lectin they isolated from hemolymph by affinity chromatography binds to the spores and significantly increases the numbers of spores that associate with granulocytes at optimal lectin concentrations. The process of opsonization is detectable in a very short
period of time, and is dependent on the presence of calcium. In some arthropods, divalent cation-dependent lectins do serve as opsonins. The observations of non-self recognition in arthropods (Vasta and Marchalornis, 1983; Komano and Natori, 1985) suggest that some serum lectins might contribute to a carbohydrate based system for non-self recognition. However, more studies using different species should be evaluated before a definitive statement can be made (Ratcliffe et al., 1985).

1.4 Prophenoloxidase (proPO) and complement-like factors

The role of the opsonization system in non-self recognition has been described in crustaceans (Soderhall and Smith, 1986). There are probably several phenoloxidases present in the blood, some of which are activated by a serine protease from the cuticle which interacts with a tyrosine derivative to form quinones which then form hard insoluble sclerotin and melanin granules (Ashida and Soderhall, 1984). There is a possible involvement of the prophenoloxidase (proPO) system in hemolymph coagulation by conversion of coagulogen to coagulin. Thus, small amounts of microbial cell products, such as beta-1,3-glucan and endotoxin, activate the proPO cascade and convert it to phenol oxidase. During the conversion, serine protease not only induces hemolymph coagulation but also generates "sticky proteins", some of which attach to foreign surfaces and act as non-self
recognition markers (Soderhall and Ajaxon, 1982). This often results in melanin deposition during immune reactivity. The melanization involved in cellular immunity in arthropods has been shown to accompany the host cellular defense response to wound healing and to microbial or parasitic invasion (Taylor, 1969). Unestam and Soderhall (1977) demonstrated that yeast cells or their constituent beta-1,3-glucans initiated melanization reactions in arthropod hemolymph by activating opsonization. It has also been established that bacterial cell walls or their lipopolysaccharides will specifically trigger activation of the proPO system (Ashida and Soderhall, 1984; Soderhall, 1981).

PATHWAY OF OPSONIZATION SYSTEM

It has been reported that in most arthropods, the enzyme phenol oxidase, which is the terminal component of the proPO system, occurs in the form of an inactive proenzyme in opsonization (Soderhall, 1981). The constituent factors of the proPO enzyme cascade include a number of serine proteases (Soderhall, 1983). An activating enzyme is one of these proteases that requires preactivation by proteolysis for activity. The enzyme is highly specific in action and is dependent on calcium. It hydrolyzes only peptides with the structure R1-Gly-Arg-R2 (Soderhall, 1983). The enzyme is very sensitive to stimulation by non-self molecules. Glucans or
lipopolysaccharides (LPS) in very low concentrations can also activate the system (Soderhall and Hall, 1984).

Another serine protease is called protease S and is triggered by low calcium concentrations (less than 5 μM). The mechanism for the generation of small peptides after proteolytic cleavage is still unclear. It differs from the "alternate pathway" in that it does not require LPS or beta-1,3-glucans for activation. However, it does demonstrate the same hydrolytic specificity as the activating enzyme to convert to phenol oxidase (Soderhall and Hall, 1984).

In the crayfish, Parachaeraps bicarinus, receptors exist for the non-self carbohydrates to trigger the proPO cascade. These receptors for glucans and LPS however, exist within the system as hemocyte lysate supernatants. If hemolymph lysate preparations collected from glucan-treated crayfish are incubated for 60 minutes at 37° C or left at room temperature for 4-5 hours, the activity of this protease is lost (Soderhall, 1983).

Other constituents of the proPO pathway are the coagulation proteins. Soderhall (1981) demonstrated the connection between coagulation and the proPO activating system by exposing hemocyte lysate supernatants to beta-1,3-glucans. The gelation or clotting that occurred is calcium dependent and mediated by proteolysis (Soderhall, 1981). The proPO system is linked to this reaction by proteases that exhibit specificity identical to the activating enzymes.
The proPO system in crustaceans possesses two separate routes of activation. The first route, the "alternate pathway," depends on the induction of non-self molecules and involves activating enzymes and other serine proteases (Soderhall and Hall, 1984). The second pathway, the "classical pathway," is initiated by low calcium levels and operates independently of foreign molecules (Soderhall and Hall, 1984). The "alternate pathway" is used when microbial invasion threatens the homeostatic integrity of the host. The "classical pathway" is used to "prime" the blood cells and promote or induce coagulation when the host sustains injury or loss of blood through the cuticle (Figure 1.1). The system is comprised of serine proteases which cascade together with a coagulogen and other factors that are specifically activated by non-self molecules (beta-1,3-glucan or lipopolysaccharide [LPS]). Functionally, the proPO cascade provides opsonins, initiates capsule/nodule formation, participates in coagulation, facilitates microbial killing and mediates communication/cooperation between different hemocyte populations.

1.5 Humoral immunity in invertebrates

Humoral immunity in invertebrates involves both natural and induced biological molecules that react against foreign cells or heterologous erythrocytes (Dale, 1979). There are many important structural differences between invertebrate humoral substances and vertebrate antibodies, but both groups
Figure 1.1 The crustacean prophenoloxidase system. The system is comprised of a serine protease cascade together with a coagulogen and other factors that are specifically activated by non-self molecules. Functionally, the proPO cascade provides opsonins, initiates capsule/nodule formation, participates in coagulation, facilitates microbial killing and mediates communication between different hemocyte populations.
Lipopolysaccharide
LPS receptor

Beta 1,3 glucan
Beta 1,3 glucan receptor

spontaneous activation
(e.g. low calcium conc.)

protease S → active protease S

ProPO

Heat or detergents

serine protease

active serine protease

PO
of molecules possess common properties and provide a means of inactivating invading pathogens (Dall, 1964).

The body fluids of many invertebrates contain humoral factors that are capable of agglutinating bacteria, protozoans, vertebrate erythrocytes and other cells (Ratcliffe, 1985). The agglutinins in invertebrates often have opsonic properties and are regarded as recognition molecules. Hemagglutinins in the body fluid of arthropods are generally proteins or glycoproteins. Their activation often requires calcium ions or other divalent cations. They bind to carbohydrates such as sialic acid, N-acetyl galactosamine, glucosamine, galactose and related compounds. They are distinct from the immunoglobulins of vertebrates in terms of their structures, binding specificities, physiochemical properties and inducibility (Yeaton, 1981). Komano et al. (1981) found that in the fly, Sarcophaga peregrina, wounding can induce an agglutinin that is composed of alpha and beta subunits. The alpha subunit exists in normal larvae and is induced in response to injury. Some of the alpha subunits are converted to beta subunits which leads in turn to the production of active $\alpha,4-\beta,2$-hemagglutinin. This, together with the elimination from the tissues of carbohydrate moieties to which the agglutinins bind, may explain how many invertebrates contain high agglutinin titers without massive interactions with self tissue.
Natural agglutinin in the hemolymph of the American lobster, *Homarus americanus*, has been characterized by Cornick and Stewart (1973b). The inactivation temperature is between 56 and 65°C which is similar to that reported for the spiny lobster (Tyler and Scheer, 1945), the American oyster (Tripp, 1966) and the crayfish (Miller et al., 1972). The stability of the agglutinin activity increases with either acidic or basic pH changes from the physiological norm in the absence of calcium. In addition, heating in the absence of calcium results in reduced activity, probably due to denaturation of the agglutinin (Cornick and Stewart, 1973b). The pH changes may alter the molecular structure of the macromolecule, resulting in a higher proportion of the more active form. Marchalornis and Edelman (1968) observed the reversibility of the reaction in the crab, *Callinectes sapidus*, and suggested that the bonding of macromolecules is noncovalent. McKay and Jenkin (1970) found that in the crayfish, *Cherax destructor*, hemocytes could be stimulated to phagocytize erythrocytes in the presence of an opsonic agglutinin *in vitro*; but some hemocytes did not require opsonization for phagocytosis. The number of these hemocytes could be increased by injection of endotoxin. The authors suggest that some of these hemocytes might produce opsonic agglutinins.

The heterogeneity of hemagglutinins within species and within individuals of the American lobster was provided by Hall and Rowland (1974). This is immunologically important.
because of the implications for antigen-binding activity of different hemagglutinins. Using the techniques of purification by ammonium sulphate precipitation, electrophoresis and gel chromatography, lobster hemagglutinins have been categorized into 2 independent agglutinins, LAg-1 and LAg-2. The differences include molecular size and binding specificity and are as follows:

<table>
<thead>
<tr>
<th></th>
<th>LAg-1</th>
<th>LAg-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular size</td>
<td>&gt;19S antibody</td>
<td>11S antibody</td>
</tr>
<tr>
<td>Specificity</td>
<td>human and mouse RBC</td>
<td>mouse RBC</td>
</tr>
</tbody>
</table>

Both agglutinins are heat labile at 56°C for 15 minutes and become inactive after trypsin treatment, reduction or alkylation. They are calcium dependent and dissociate into subunits of 55,000 MW in 6M urea, indicating that they have noncovalent bonds in their structural subunits. The binding properties of LAg-2 contain a site which binds to \( N \)-acetylgalactosamine residues present on mouse and hamster erythrocytes. LAg-1 binds to \( N \)-acetylneuraminic acid residues on human and mouse erythrocytes (Cooper, 1982). The agglutinins were found on the surfaces of blood cells after synthesis and prior to their release into the hemolymph.
The humoral factors or activities in the crustacean immune system have been summarized by Smith and Chrisholm, (1992) and are as follows:

1) Agglutinins which aggregate foreign particles. These include bacterial agglutinins, hemagglutinins and/or lectins. They are present in nearly all species and appear to aid in the sequestration of infective agents; but there is little evidence to show that they have a role in recognition.

2) Killing factors:
   a. Lytic agents which are bactericidal. Bacteriolysins and lysozyme are seldom reported. Hemolysins are present in the spiny lobster.
   b. Pigments which are microbicidal. Melanin and its precursors are fungicidal and bactericidal.
   c. Neutralizing factors which act as antiviral agents. There are reports of such factors in the crabs, Callinectes sapidus and Carcinus maenas.
   d. Cytotoxic agents which destroy cells. Crayfish granular cells produce substances which are cytotoxic for both normal and tumor vertebrate cells in vitro.

3) Clotting factors which prevent blood loss and seal wounds. These are found in all species and involve plasma gelation as well as cell aggregation.
4) Recognition factors which bind specifically to non-self molecules and trigger cell responses. For example, proPO factors are released from somatic cells. Beta-1,3 glucan binding factor is found in crayfish plasma.

1.6 Invertebrate cellular immunity

The invertebrate immune response to foreign agents is primarily cellular. A variety of immunocytes have been found among the primitive phagocytic amoebocytes of arthropods.

Phagocytic cells are found throughout the animal kingdom. They serve a nutritive function in lower invertebrates and become progressively more specialized toward internal defense mechanisms in the higher phyla. Objects too large to be phagocytized are sequestered within multicellular aggregates that show some similarities to mammalian granulomas, particularly when different classes of immunocytes are involved. In arthropods, the cores of the aggregates become melanized and, in nearly all the phyla examined such aggregates become fibrous and/or coated with glycosaminoglycan-like material. In shrimp, the cellular response may not protect the host against all bacterial invaders, in which case deposition of other protective material such as calcium around the granuloma may occur (Issarasak et al., 1991).
The ability of invertebrates to agglutinate foreign cells was demonstrated by Loeb (1903). Several hemagglutinins have been detected, purified and characterized.

In *Penaeus monodon*, hemocytes are separated into three types based on their ultrastructural features. These are called agranulocytes, small granular hemocytes and large granular hemocytes (Martin and Graves, 1985).

**AGRANULOCYTES**

The agranulocytes of shrimp as described by Martin and Graves (1985) have an ovoid shape, lack granules and appear non-refractile when examined by phase microscopy. The nucleus is ovoid and occupies much of the cell. When examined by transmission electron microscopy, the contents of the nucleus are almost equally divided between heterochromatin and euchromatin with at least one ovoid nucleus present. The nuclear envelope is smooth, and nuclear pores are common. The cytoplasm forms a thin layer around most of the nucleus and expands slightly at the two poles. The cytoplasm contains a small amount of rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER), and the membranes of these two organelles are often continuous with one another. Free ribosomes and mitochondria with lamellar cristae are also present, but no Golgi bodies are seen. Small vesicles (0.04 μm in diameter) are rare. These agranular hemocytes are comparable to hyaline cells which lack granules and in the
crab, *Callinectes sapidus*, hyaline cells which contain fine granules (Sawyer et al., 1970). Shrimp agranulocytes are also comparable to prohyalocytes which lack granules in lobsters (Cornick and Stewart, 1978). Bauchau (1981) states that hyaline cells contain either none or one Golgi body and none or only a few small granules. Martin and Graves (1985) suggest that the sharp division between the agranular cells and those that contain any number of granules must be maintained because if cells containing granules are included with agranular cells, the division between agranular and granular hemocytes becomes arbitrary. The ultrastructure of hemocytes with granules is also very different from the ultrastructure of agranular cells. Hemocytes with only a few granules possess Golgi bodies and abundant RER and SER and are more similar to hemocytes with a larger number of small granules than they are to agranular hemocytes.

In *Penaeus japonicus*, agranulocytes may be comparable to undifferentiated hemocytes (Tsing et al., 1989). Undifferentiated hemocytes are elongated and most of the nuclear chromatin is dispersed. In the cytoplasm, free ribosomes are numerous and the RER is moderately developed. Dictyosomes are few and the cells rarely contain small rounded cytoplasmic granules (less than 0.1 um in diameter) which are membrane bound and uniformly opaque. The reason for using the term "undifferentiated hemocyte" results from electron microscopic studies. Hemocytes without any granules are very
scant in the blood of *P. japonicus* and *P. monodon*, and even in rare cases where they are seen, it is not possible to confirm that these cells do not possess any granular inclusions. Therefore, the word "agranular" or "hyaline" cells may not be appropriate.

The ridgeback prawn, (*Sicyonia ingentis*) has been studied extensively as a model for decapod crustaceans. A hemocyte classification scheme based on morphological (Martin and Graves, 1985; Martin et al., 1987) and cytochemical (Hose et al., 1987) criteria has been established. The identification of hyaline cells in shrimp (comprising 50 to 60% of the circulating hemocytes) was made by selecting the cells which contain from zero to five granules. Shrimp hyaline cells have prominent round, electron-dense deposits filling the cytoplasm. The agranular hyaline hemocytes are rare (1 to 10% of the total hemocyte count), very fragile and their lysis initiates clotting (Omori et al., 1989).

**SMALL GRANULAR HEMOCYTES**

Small granular hemocytes (SGH) are the most abundant type of hemocyte, comprising approximately 75% of all hemocytes. The granules are approximately 0.4 um in diameter (Martin and Graves, 1985). SGH are ovoid, non-refractile and possess a band of microtubules that encircle the cells as in the agranular hemocytes. They are slightly longer and wider than agranular hemocytes. The presence of a variable number
of small, black granules in the cytoplasm makes them identifiable using light microscopy.

At the transmission electron microscope (TEM) level, SGH can be distinguished from agranular hemocytes by the following differences: 1) the presence of a few granules, 2) an increased number of mitochondria, 3) an increased amount of cytoplasm, 4) a small number of irregularly shaped, electron-lucent vesicles about the same size as the granules and 5) the more convoluted outline of the nucleus.

The granules seen by TEM are typically circular in section and only occasionally are elongated ones seen. The granules are electron-dense and about the same diameter as mitochondria (0.4 μm). The cytoplasm contains free ribosomes, RER, SER and vesicles, and several Golgi bodies are typically located within infoldings of the nuclear envelope. The cis-cisternae on the forming side of each Golgi body are typically swollen; whereas the trans-cisternae are filled with an electron-dense material which may also be seen in small vesicles (0.04 μm) in the vicinity of the Golgi bodies. There is a second class of vesicles 0.4 μm in diameter, the same size as the granules which are electron-lucent, but these are less common (Martin and Graves, 1985). Mix and Sparks (1980) identified crab (Cancer magister) hemocytes using light microscopy and Wright's stained smears. They found an intermediate cell category in crabs which corresponded to the small granular hemocytes in shrimp. In lobsters studied by
Cornick and Stewart (1978), the hyalinocytes and eosinophilic granulocytes are ovoid cells with fine granules that exhibit basophilic or eosinophilic staining using Giemsa stain. Martin and Graves (1985) refer to both these two types as small granular hemocytes. In P. japonicus (Tsing et al., 1989), the SGH are irregular in shape with numerous digitations. They are characterized by the presence of cytoplasmic granules which are much more plentiful than they are in agranulocytes. These granular inclusions are surrounded by membranes. The smallest ones are always in the vicinity of the Golgi complex and secrete small vesicles with electron-dense contents. Free ribosomes are abundant and the RER is developed in narrow but elongated cisternae. The mitochondria are rounded and numerous, and the nucleus is often in a horseshoe shape. Regions of very dense chromatin (heterochromatin) are numerous and packed around the inside of the nuclear envelope. In some SGH of P. adspersus, there are numerous cytoplasmic vesicles with weakly electron-dense granular contents. These vesicles sometimes merge to form vacuoles as large as the nucleus. The origin of these inclusions is unknown, and they do not seem to have any relationship to the RER, Golgi apparatus or dense granules. Since the amount of enzymes that exhibit lysosomal activity is most abundant in SGH, this could be related to the increased endocytotoxic capability of these cells (Brehe'lin and Arcier, 1985).
LARGE GRANULAR HEMOCYTES

Large granular hemocytes (LGH) comprise 10-20% of all hemocytes, and they are filled with granules 0.8 μm in diameter. The shape of cells is ovoid to spherical and they are about the same size as the SGH. Clusters of microtubules have not been observed around the periphery of these cells. They are filled with large, highly refractile granules that often obscure the nucleus and cause the entire cell to appear bright when observed using phase contrast microscopy. When examined by TEM, each cell contains 25-40 granules that are twice as large as the SGH granules. The granules have an ovoid to irregular outline and their substructure varies. Most are homogenous, some display scattered electron dense areas, and a few display concentric rings of electron dense material embedded in a less dense matrix (Martin and Graves, 1985). The latter two types of granules are thought to be immature stages and their substructure may be lost by further deposition of electron dense material. The cytoplasm lying between the granules and the nucleus appears to be reduced relative to the SGH due to the large areas occupied by granules. Free ribosomes and RER are present but are not as abundant as in the SGH. Golgi bodies have not been observed. LGH in shrimp correspond to the coarsely granular granulocytes in the crab, *Callinectes sapidus* (Sawyer et al., 1970). If compared to lobster hemocytes, LGH correspond to the chromophobic granulocytes which are packed with large non-
staining or basophilic granules (Cornick and Stewart, 1978). A study by Bauchau (1981) concluded that granulocytes contain zero or one Golgi body and have abundant large granules. As described by Tsing et al. (1989), the LGH of P. japonicus are ovoid and about 10 μm in diameter. They possess masses of dense heterochromatin close to the nuclear envelope. The cytoplasmic granules are often numerous and an ovoid or spindle shaped and are rarely spherical. They are always membrane-bound and generally possess a homogeneous electron dense content. Sometimes they exhibit an internal structure consisting of alternating electron dense and electron lucent bands. The diameter of these inclusions varies from 0.3 to 1.5 μm and they are believed to have resulted from the fusion of dense vesicles synthesized by the Golgi apparatus. The cytoplasm of these cells contains few free ribosomes. Their RER is not very well developed and the mitochondria are scarce. The hyaloplasm often appears finely granular. LGH contain less acid phosphatase than SGH; therefore the frequency of bacterial killing by these cells is less. However, the beta-glucuronidase and esterase seem to be in equivalent amounts in LGH and SGH (Hose et al., 1987).

TOTAL HEMOCYTE AND DIFFERENTIAL COUNTS

Comparisons of total hemocyte counts and differential counts in many organisms provide a useful way of assessing the physiological state of the animal. Unfortunately, for most
crustaceans a wide range of values is not unusual and differences in the classification schemes used have prevented comparisons among different crustaceans. In addition, the work of Stewart et al. (1967) has shown that various parameters such as sex, diet, length of captivity and the stage in the molt cycle may affect the hemocyte count.

The total and differential hemocyte counts for shrimp such as Sicyonia sp. and Penaeus sp. showed large variations in values. In both species, the proportion of agranular hemocytes and large granular hemocytes was very low, but the number of small granular hemocytes was considerably higher (approximately 75% of all hemocytes) (Martin and Graves, 1985). The studies of hemogram modifications carried out on P. japonicus postlarvae (Tsing et al., 1989) showed that the number of cells per unit volume varied as a function of the developmental stage. The differential counts for agranular hemocytes, small granular hemocytes and large granular hemocytes which are the primary hemocytes after natural lysis of the molting process, increase with time. However, the relative percentage of each hemocyte cell type is fairly stable during the whole intermolt stage. It is difficult to determine if changes in total hemocyte counts are related to hemocyte production, hemocyte disappearance or changes in blood volume, since there are very few studies on total hemocyte enumerations. However, in decapod crustaceans it is possible to affirm that exuviation is preceded by a 50%
decrease in total circulating hemocytes (Tsing et al., 1989; Bauchau, 1981). In P. japonicus, the total hemocyte counts varied from 9,000 to 15,000 cells/ml (Tsing et al., 1989).

NONCIRCULATING HEMOCYTES

Other than the circulating hemocytes mentioned above, the immune system of invertebrates also involves non-circulating cells. In crustaceans, these non-circulating cells are found in the heart, gill and hepatopancreas. They participate in the surveillance and clearance of foreign substances (Foster, 1981). These cells are primarily involved in the phagocytosis and pinocytosis of foreign materials.

BRACHIAL PODOCYTES

The brachial podocytes occupy the stem of the decapod gill and depending on the species, may also be found in the lamellae. Podocytes carry out ultrafiltration and engage in specialized pinocytosis. These functions resemble those in the podocytes in the antennal gland (excretory organ) and the podocytes in Bowman's capsule in the vertebrates (Stangways-Dixon and Smith, 1970). Before the material is engulfed into the cytoplasm, it must first penetrate the basal lamina and pass through pores or slit diaphragms which span the spaces between the "foot processes". The basal lamina and pores limit the size of molecules that can be pinocytized (Johnson, 1987). Normally, the function of brachial podocytes is
considered to be the removal of substances from the hemolymph and the processing of various proteins produced during tissue maintenance, growth and molting. Their cytoplasm contains a large vacuole and a number of small satellite vacuoles (Wright, 1964). The materials and granules seen in the satellite vacuoles are too large to pass through the pore diaphragms and would also be produced during fixation or normal lysosome processing. These materials may remain in the podocytes permanently or are released into the hemolymph, particularly at molting time (Johnson, 1987). The aggregation of hemocytes in the gill can create emboli and infarcts which may cause all cells in the infarct including podocytes to become necrotic (Johnson, 1980b). Sloan et al. (1975) pointed out that antennal glands are of lesser importance in clearance than are the brachial podocytes.

The clearance of viral particles, which are less than 30 nm in diameter, is also performed by podocytes (Cooper-Willis, 1979). The role of bacterial elimination was described by Smith and Ratcliffe (1981) who described the ultrastructural appearance of brachial podocytes in the shore crab, Careinus maenus. After the crabs had been injected with heat-killed bacteria, many of the podocytes became disorganized and eventually necrotic. There were granulomas in the satellite vacuoles of the affected podocytes that seemed to resemble the cytoplasmic granules of hemocytes.
Podocytes are considered to be highly specialized cells. There is a possibility that brachial podocytes engage in phagocytosis of necrotic material produced during phagocytic responses to injected foreign material. This activity, however, warrants further investigation (Johnson, 1987).

PHAGOCYTIC RESERVE CELLS

In decapods, the phagocytic reserve cells are derived from hemopoietic tissue and are fixed to the linings of various hemal spaces. These reserve cells may be in the connective tissue or in the myofiber within the heart (Ghiretti-Magaldi et al., 1977; Johnson, 1980a). They are not surrounded by a visible basal lamina or cell coats in the same manner as hemocytes. These cells lie on the basal lamina of tissue facing hemal spaces. In mature cells, the material stored in the cells occupies a large membrane bound inclusion, and the cells are called the "reserve inclusion" (RI) cells (Johnson, 1980a). The RI cells contain hemocyanin, calcium, iron and other substances. Since there is extensive RER and strong evidence for the presence of a Golgi apparatus in these cells, RI cells are considered to be active synthesizers. The production and storage of hemocyanin also takes place within the RI cells (Ghiretti-Magaldi et al., 1977). Cellular responses to carmine in brown shrimp, Penaeus aztecus, indicate that fixed phagocytes in the heart are loosely attached to the basal lamina surrounding myocardial cells and
are weakly phagocytic for carmine particles. Following engulfment, the particles accumulate in large cytoplasmic vacuoles containing cellular debris and dense flocculent material (Foster, 1981).

The roles of phagocytic reserve cells have not been shown to be the same in all decapod species. There has been confusion between reserve cells and podocytes in the gill and antennal gland since reserve cells were mistakenly called "nephrophagocytes" (Johnson, 1980a). The reserve cells are responsible for the synthesis of hemocyanin, are derived from hemopoietic tissue and possess phagocytic activities.

**FIXED PHAGOCYTES**

In crustaceans, fixed phagocytes are the only fixed cells that play an important role in phagocytosis. In most decapods, they are found in the hepatopancreatic hemal sinus and in the arterioles of the hepatic artery. Fixed phagocytes are derived from hemopoietic tissue (Cooper-Willis, 1979), and fixed phagocytes and circulating hemocytes are the only true phagocytes to be formed in the hepatopancreas (Johnson, 1987). Hemolymph flow that allows for the greatest opportunity for contact between hemocytes and foreign particles, and the properties of the hemocytes and particles, are probably the most important factors in phagocytosis. The location where phagocytosis takes place depends on where the material is introduced (Merrille et al., 1979), the type of material, the...
effect of foreign intrusion on the heartbeat, etc. The place where phagocytosis might appear to be the most efficient is in the area where hemal spaces are narrow and/or intricate. In these areas, the flow rate is reduced because of a fall in hydrostatic pressure thereby allowing more opportunity for contact between hemocytes and particles (Johnson, 1987). Similar conditions possibly pertain to the heart for limited periods of time since phagocytosis of gram negative bacteria has been described in the heart of blue crabs (Johnson, 1976) and lobsters (Johnson et al., 1981). The presence or role of fixed phagocytes associated with blood vessels in locations other than the hemal sinuses of the hepatopancreas requires further investigation.

THE ROLE OF HEMOCYTES IN HOST DEFENSE MECHANISMS

Hose and Martin (1989) have divided shrimp hemocytes into two major categories i.e., deposit cells and granulocytes. The deposit cells are the hyaline cells or agranulocytes which initiate coagulation (Omori et al., 1989). The granulocytes are responsible for defense against invasion by foreign materials or microorganisms by phagocytosis and encapsulation. The small granular hemocytes are the primary phagocytic hemocytes, and the large granular hemocytes which phagocytize bacteria less frequently are the secondary phagocytic hemocytes (Hose et al., 1987). The cytoplasmic vesicles of granulocytes contain acid phosphatase, esterase.
and beta-glucuronidase which are the enzymes that degrade microorganisms within the phagosome (Hose and Martin, 1989). The vesicles, and possibly the granules, fuse with the phagosome during phagocytosis. Degranulation has been observed in both small and large granular hemocytes in response to the presence of gram negative bacteria in crayfish and crabs (Soderhall et al., 1986). Both of these types of hemocytes contain opsonization properties of the proPO system. The loss of granules from the hemocytes results from their fusion with the phagosome and the hydration of the contents of the vesicles. The release of proPO granules is thought to be the initial event in the recognition of non-self particles and activation of phagocytes (Soderhall et al., 1986). The capacity of hemocytes to phagocytize and bind to erythrocytes indicates that there are receptors for erythrocyte components as well as receptors for opsonins on the hemocyte plasma membrane (Goldenberg et al., 1984). The in vitro activation of hemocytes for phagocytosis and binding to erythrocytes suggests that hemocyte receptors for erythrocytes are endogenous (Goldenberg et al., 1984). Opsonization of bacteria with cell-free hemolymph significantly increased maximal phagocytic rates in small granular cells (Hose and Martin, 1989). Opsonic factors in crustaceans are thought to be either products of proPO activation (Smith and Soderhall, 1983; Soderhall et al., 1986) or agglutinins, specifically, lectins (Paterson and Stewart, 1974). Two lectin opsonins
have been purified from the American lobster (Hall and Rowlands, 1974). Studies of the opsonic properties of the proPO system have shown that phenol oxidase itself is not an opsonin but that an intermediate in its formation may be opsonic (Soderhall et al., 1986). Phagocytosis of bacteria by a hemocyte monolayer was enhanced in the presence of beta-1,3-glucan, a proPO activator (Ratcliffe et al., 1985). Soderhall et al. (1986) suggested that bacterial endotoxin or beta-1,3-glucan stimulates labile granulocytes to release proPO granules into the plasma. The intact granules become activated and coat the microorganism enhancing uptake by phagocytes. In shrimp, hyaline cells or agranulocytes lyse upon contact with seawater forming circular areas of clotted hemolymph. Bacteria along with granulocytes are forced into areas between adjacent expanding clots, thus increasing the chance for phagocytosis to occur (Hose and Martin, 1989).

In the circulatory systems of arthropods, the heart distributes hemolymph along arteries that empty into sinuses that constitute the body cavity thereby bathing the organs in hemolymph. The hemolymph can travel widely in the body cavity providing ample opportunity for hemocytes to encounter foreign invaders. Although chemotaxis is commonly considered to be the first of several events which leads to particle engulfment and killing of foreign agents, there are only a few confirmed reports of chemotaxis in invertebrates (Bayne, 1990). The evidence for chemotactic responses to foreign substances is

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the observation of phagocytes sending out finger-like pseudopods which engulf the foreign object. When the object is larger than the phagocyte, the phagocytes become extended over the foreign surface. As more phagocytic cells accumulate, the foreign agent becomes encapsulated. During encapsulation, phagocytes degranulate to release lysosomal contents over a large portion of the surface of the foreign agent which generally results in death of the organism.

In general, the phagocytic process of invertebrate hemocytes resembles that of vertebrate macrophages. Phagosome formation occurs when pseudopods extend around the object and internalize it or when there is direct contact of particles with hemocytes, inducing local membrane invagination. Phagocytic hemocytes are capable of amoeboid movement and are more easily examined microscopically when they adhere to a glass or plastic surface than when they are non-adherent. As the cell spreads, it changes from a roughly spherical shape to a more flattened shape and the membrane may be redistributed during the spreading process. As a result, putative receptors for foreign surfaces may accumulate on the side of the cell that faces the substrate which decreases the availability of receptors to bind foreign particles. This makes the microscopic observation of phagocytosis difficult. The process of phagocytosis normally induces lysosomes to move toward and fuse with the phagosomes. This fusion may begin before the particle is fully internalized, resulting in the
leakage of lysosomal enzymes into the fluid medium (Cheng, 1990). The phagosome-lysosome fusion generally occurs after the complete formation of phagocytic vacuoles and digestion of the engulfed material then ensues. The products of particle digestion can contribute to the nutrient supply of the animal. For example, when molluscan hemocytes digest bacteria, they accumulate glycogen (Cheng, 1977) and other carbon-rich compounds (Bayne, 1973). When indigestible particles are phagocytized, the engulfing cell may undergo diapedesis. Alternatively, sequestration may occur, and in this case the cell with its contents migrates to a site within the body where it becomes metabolically quiescent and adopts permanent residence (Bayne, 1990).
II. METHYL PARATHION

2.1 Introduction

Methyl parathion (0,0-dimethyl-O-p-nitrophenyl phosphorothioate) is an organophosphorus insecticide which was developed during the second World War. The concern about the persistence and pollution effects of organochlorine compounds has contributed to a significant increase in the use of organophosphates for crop production. Organophosphates have the distinct advantages of being less persistent in the environment, as well as being highly effective in controlling the population of aphids and similar soft-bodied small insects (Hassall, 1990). However, these chemicals are far more acutely toxic than the chlorinated hydrocarbons (Wagner, 1983). Methyl parathion is a member of a subgroup of organophosphorus insecticides with moderate to high chemical stability. It is a hydrophobic compound but has a high solubility in oil. With persistent contact, the compounds soak into leaves but do not travel around the plant. The sprayed substance is enzymatically activated before it reaches the site of action in the nervous system of the insect.

Methyl parathion is 3-4 times less toxic than parathion. At room temperature, methyl parathion is stable in both neutral and acidic environments (Sharmila et al., 1988). The
structure of methyl parathion is shown in Figure 2.1. There are two non-enzymatic reactions based on the chemical structure that should be noted. The first reaction is the susceptibility of methyl parathion to alkaline hydrolase due to the reaction of the hydroxyl ion (OH⁻) with the relatively positive phosphorus atom. A similar reaction occurs during acetylcholinesterase inhibition. The hydroxyl group of a serine amino acid residue at the active site of the enzyme attacks the phosphorus. It follows, therefore, that electron-withdrawing substituents which tend to make the phosphorus atom more positive, promote both hydrolytic instability and reactivity with acetylcholinesterase. Secondly, methyl parathion may undergo isomerization. The effect of the isomerization is to convert a P=S compound to one containing the P=O group. Since =O is more electronegative than =S, the phosphorus atom becomes electron deficient. This thiono conversion results in a compound which is more susceptible to hydrolysis and becomes a highly efficient inhibitor of acetylcholinesterase. Phosphorothionates are, therefore, latent or indirect inhibitors of that enzyme and only act directly on the acetylcholinesterase after its conversion to the P=O form. The hydrolysis of acetylcholine is demonstrated in Figure 2.2.

Chemical isomerization of methyl parathion is slow at room temperature. The reaction may, however, be enzymatically catalyzed in the organism to the point of lethal synthesis.
Figure 2.1 The molecular structure of methyl parathion.
The two nonenzymatic reaction sites are found at the relatively positive phosphorous (P) atom and the =S linkage.
Since methyl parathion is manufactured at elevated temperatures, phosphorothionates are likely to be contaminated with "thiolo" isomers which may cause direct inhibition of acetylcholinesterase (Corbett, 1975).

2.2 Mode of action

In the adult insect, methyl parathion acts on the enzyme acetylcholinesterase which is necessary for hydrolysis of the synaptic transmitter, acetylcholine. The reaction of acetylcholinesterase to hydrolyse acetylcholine into choline and acetic acid is shown in Figure 2.3.

Acetylcholinesterase is the principle enzyme responsible for the hydrolysis of acetylcholine in insect and vertebrate nervous systems (Issarasak, 1990). Acetylcholinesterase is located in the presynaptic and postsynaptic membranes of insects (Weber, 1976). There is typically one pair of fused ganglia per segment, joined to the ganglia in the adjacent two segments by bunched nerve fibers called "connectives". The autonomic nervous system is a single visceral system in insects. There are no synapses between the point of leaving the central nervous system and the effector cells in the viscera. The peripheral nerves of insects are not surrounded by myelin sheaths, an important fact in relation to the selective action of pesticides.

Organophosphorus compounds have some structural similarities to acetylcholine. The phosphate group is
Figure 2.2 Hydrolysis of acetylcholine. Acetylcholine is hydrolysed by acetylcholinesterase. This results in the splitting and deactivation of acetylcholine into choline and acetic acid. The estimated destruction rate of acetylcholine is approximately 300,000 molecules of acetylcholine per minute at 37°C (AChE, Acetylcholinesterase).
Acetylcholine

\[
\begin{align*}
\text{(CH}_3\text{)}_3\text{N}^+\text{CH}_2\text{CH}_2\text{OC}\cdot\text{CH}_3 & \quad + \quad \text{H}_2\text{O} \\
\text{Acetylcholine} & \\
\text{AChE} & \\
\text{(CH}_3\text{)}_3\text{N}\cdot\text{CH}_2\cdot\text{CH}_2\text{OH} & \quad + \quad \text{CH}_3\cdot\text{COOH} \\
\text{Choline} & \quad \text{Acetic acid}
\end{align*}
\]
attracted to the esteric site and the rest of the molecule is aligned by interacting with numerous side groups of amino acids. These form the total active area of the enzyme. Although most organophosphorus compounds have no positively charged groups to associate with the normal anionic site, they undergo a series of changes during which acetylcholine binds to cholinesterase at two attachment sites. The ester-forming site contains a serine residue in the protein chain. The negative or anionic site contains a glutamic acid residue. The carbon atom of the carbonyl group of the substrate carries a slight positive charge and makes an electrophilic attack on the hydroxyl group of the serine. This results in "acetylation" of the enzyme and in the splitting and deactivation of acetylcholine, and free choline readily leaves the enzyme surface. The esteric bond is weak and is rapidly hydrolyzed during the "recovery stage" of the enzyme. The hydrolysis is probably facilitated by a basic histidine residue nearby. The surface of the enzyme is then free to accept another molecule of acetylcholine (Figure 2.3). It has been estimated that about 300,000 molecules of acetylcholine are destroyed by one molecule of enzyme per minute at 37°C (Hassall, 1990).

Instead of acetylating the enzyme, organophosphorus compounds phosphorylate it (Figure 2.3). The esteric bond is relatively stable to hydrolysis. The stability of the bond
depends on the nature of the phosphorylating entity, which is frequently either dimethylphosphate or diethylphosphate. It takes approximately 80 minutes for half of the enzyme molecules to be dephosphorylated when dimethylphosphate is attached to them. It takes much longer for molecules of the enzyme to become operative again after this process than when the enzyme undergoes the natural process of acetylation.

2.3 Methyl parathion in the aquatic environment

The use, disposal and contamination of methyl parathion in aquatic environments can be summarized as follows (Edward, 1973):

1) direct application or spray into the water for mosquito or insect larvae control
2) direct application in the area near the water
3) run-off from agricultural areas
4) drainage from populated areas or pesticide manufacturing facilities
5) disposal of pesticide containers into aquatic systems

Methyl parathion contamination of water usually occurs in low concentrations and is adsorbed to small particles. Therefore, the majority of the pesticide is found in the sediment. During its degradation, some of the pesticide
Figure 2.3 Cholinesterase active sites. (Adapted from Hassal, 1990).

(A) Active site of cholinesterase with the natural substrate near its surface.

(B) Acetylation of the enzyme for a very short time after the splitting of acetylcholine.

(C) The stable phosphorylated enzyme remains after splitting an organophosphate.

SER, serine; GLU, glutamic acid; HIS, histidine; EB, esteric bond.
ESTER FORMING SITE

CH₂—CH₂

SER—O—H⁺

HIS

(A)

CH₃

CH₂—CH₂—N⁺(CH₃)₃

CHOLINE LEAVING SURFACE

H₂O—CH₂—CH₂—

SER—O

HIS

(B)

RO—P=O

LEAVING GROUP

H₂O

HIS

(C)
residue accumulates in benthic animals (Moelenberg and Kjoeboe, 1983).

Factors affecting the degradation of methyl parathion may be physical, chemical or biological. Physical and chemical factors include temperature, pH, salinity and oxygen concentration. The half-life of methyl parathion is shorter at higher temperatures (Virgil et al., 1979) and salinities (Weber, 1976) because of increased degradation. The biological factors affecting degradation involve primarily microorganisms and phytoplankton. Hill and Wright (1978) suggested that bacteria play a significant role in pesticide degradation and found that methyl parathion can be metabolized and degraded by organisms such as Bacillus subtilis and Flavobacterium sp.

Redox transformation of organic compounds can be an important degradation pathway in aquatic ecosystems (Wolfe et al., 1986). Failure to take into account redox processes can result in an overestimation of the exposure concentrations of certain organic compounds in ecosystems where strongly oxidizing or reducing conditions exist (Baughman and Burns, 1980). For methyl parathion, the calculated alkaline hydrolysis half-life at pH 9 and 25°C is 13 weeks. The microbial degradation half-life, assuming there are $1 \times 10^7$ organisms/L, is 9 weeks (Wolfe et al., 1986). In contrast, under anaerobic conditions that are typical in most sediments, half-lives of less than one hour have been observed (Munnecke,
The disappearance of methyl parathion under anaerobic conditions has been described by first order kinetics, and amino methyl parathion (o,o,dimethyl-o-p-aminophenyl phosphorothionate) has been identified as a product.

EFFECTS ON THE AQUATIC ENVIRONMENT

Pesticides such as methyl parathion in the aquatic environment have detrimental effects both on organisms and on the ecosystem.

Organismic effects

The potential adverse impact of a pesticide is based primarily on its direct toxicity to an organism or on its bioaccumulation within an organism to the extent that it renders the organism unsuitable for use as food for human consumption (Lee et al., 1982). The toxic effects depend upon many factors such as concentration, resistance of the animal to the pesticide and/or the antagonistic or synergistic effects of other chemicals that might also exist in the environment. The impact of toxic chemicals on aquatic animals can be divided into two categories, lethal and sublethal effects. Lethal effects occur when the concentration of the pesticide is high enough to cause acute mortality. The effects of sublethal concentrations which are chronic and are usually not as obvious as lethal effects can cause dysfunction of organs and/or systems of the animals.
Toxic effects of organophosphorus pesticides vary with the species, age and sensitivity of the animal. For methyl parathion, the 48 hour LC₅₀ in shrimp, Penaeus indicus, is 0.095 ppm, while in Metapenaeus monoceros the LC₅₀ is 1.2 ppm (Reddy and Rao, 1988).

There have been numerous studies on the effects of methyl parathion on enzymatic and biochemical reactions in crustaceans. In shrimp exposed to subacute concentrations of methyl parathion, there was an increase in ammonia, urea, glutamine and related enzymes in body fluids, and a reduction in glutamate dehydrogenase in the hepatopancreas. These changes in enzyme levels, especially the increases in AMP-deaminase and adenosine deaminase, induced higher catabolism of purines and other amino acids which results in higher ammoniagenesis. These changes may cause ammonia toxicity to hemocytes (Reddy et al., 1988).

The level of lipid-containing pesticide in the animal body decreases with increases in lipase, and at the same time new lipid production increases. This was hypothesized by Reddy and Rao (1988) to be a mechanism to reduce the stress caused by pesticide absorption. Respiration and oxygen consumption rates of shrimp exposed to methyl parathion increase in the first 12 hours due to a physical adaptation of the animal (Reddy et al., 1988). After that, the consumption of oxygen eventually declines because of asphyxiation; since the respiratory center in the brain has been disrupted.
Tissue enzymes which are reduced include succinate dehydrogenase, malate dehydrogenase and cytochrome C oxidase. These reductions confirm the impairment of oxidative metabolism and decreased ATP turnover at the mitochondrial level (Rao et al., 1988; Reddy et al., 1988). Cellular immunological effects may result from decreases in acid phosphatase which is an important degradative enzyme in granular hemocytes (Reddy et al., 1986).

Another effect of organophosphorus pesticides in shrimp is an alteration in the initiation and progress of limb regeneration. In Penaeus indicus, exposure to different sublethal concentrations of phosphamidon retards the regeneration of a severed fifth periopod. Inhibition of limb regeneration is dose dependent. These results suggest that crustacean limb regeneration, as well as immunoassays, could be used as indicators for assessing the toxic effects of environmental pollutants (Reddy and Rao, 1988).

Ecosystem effects

Contamination by pesticides in the aquatic environment influences all biological components of the ecosystem. In general, pesticides are harmful to most organisms except for some microorganisms that can utilize them as energy sources. Changes in mineral and energy movements in the ecosystem can occur (Edward, 1973). Pal and Konar (1990) concluded that the regular discharge of sublethal doses of methyl parathion into
the water is hazardous to phytoplankton, zooplankton and benthos. The effects of 200 ppm methyl parathion in the sediment on the behavior of a number of marine organisms showed that burrowing behavior was impaired in some species and highly significant avoidance of the contaminated sediment was demonstrated by the benthic invertebrates, \textit{Crangon crangon} and \textit{Solea solea} (Moelenberg and Kjoeboe, 1983).

These effects on aquatic animals may result in a decrease in fishery catches in a region because of abnormal migration and breeding behavior. The contamination of water and sediment can also cause a decrease in aquaculture production resulting in economic loss. The possibility of accumulations of pesticides to levels that could be dangerous for human consumption exists both in aquaculture and fisheries (Konrad et al., 1969). Small fish are generally more susceptible to pesticide poisoning than are larger fish of the same species which may greatly impact recruitment. This is due to larger gill surface areas relative to body mass, higher rates of metabolism and smaller lipid pools in the bodies of smaller fish (Horsberg et al., 1989).

\textbf{INHIBITION OF ACETYLCHOLINESTERASE}

The primary effect of organophosphorus pesticides in invertebrates is the inhibition of acetylcholinesterase (AChE) (Corbett, 1974). In the fish, \textit{Pleuronectes platessa}, a similar effect was observed. Only about 10\% of the AChE
activity was recovered after incubation with 1.3 ppb paraoxon (Galgani and Bocquene, 1990). Because of the damage to their nervous systems, fish exposed to organophosphorus compounds were lethargic and very sensitive to disturbances. A slight tap on the aquarium resulted in severe contraction of the trunk muscles. Fish exposed to the pesticide also appeared to lose equilibrium and direction of movement (Areechon and Plumb, 1990).

In penaeid shrimp exposed to methyl parathion, the activity of acetylcholinesterase in nervous tissue was significantly inhibited. When exposed to both lethal and sublethal concentrations for 48 hours, the degree of inhibition was dose-dependent. Considerable inhibition occurred even at low concentrations of pesticide (Reddy and Rao, 1988).

Methyl parathion and other phosphorothionate insecticides, are considered to be latent inhibitors in that they are converted to active AChE inhibitors by the microsomal oxidative system in the presence of NADH or NADPH (O'Brien, 1967). There is strong evidence that methyl parathion is metabolically converted to a more active AChE inhibitor by the oxidation of the thiono-sulphur atom (P=S) to an oxygen atom (P=O). The resulting oxygen analogue (methyl paraoxon) is several times more potent as an inhibitor of AChE than is methyl parathion (Benke et al., 1975). The high degree of AChE inhibition after 48 hours of methyl parathion exposure
suggests that it has been converted to methyl paraoxon (Figure 2.4). After transfer of pesticide-exposed penaeid shrimp to toxicant-free water, AChE activity in nervous tissue showed a progressive recovery. Virtually normal activity was obtained after a 7 day recovery period following exposure to sublethal concentrations. The recovery process is thought to be related to the dephosphorylation of the organophosphorus compound and resynthesis of the fresh enzyme. At near lethal concentrations, methyl parathion causes serious damage to nervous tissue through AChE inhibition (Reddy and Rao, 1988). After a short period of near lethal exposure, shrimp required a longer period of reclamation in pesticide-free media to overcome the toxic stress. In certain cases, hydrolysis, biodegradation and rapid excretion of toxic chemicals upon transfer to pesticide-free water may facilitate a quick recovery. Water changes may, therefore, be used as a measure to protect this economically important animal from potentially deleterious effects (Benke et al., 1975).

2.4 Immunological effects

Immune responses are important defense mechanisms against diseases in all organisms. Their impairment will allow an increased incidence of infection which may directly or indirectly influence the survival of individuals or of a species. In humans, many pesticides have been associated with allergic responses, especially certain types of allergic
Figure 2.4 Reaction between cholinesterase and methyl paraoxon. Methyl parathion is metabolized into methyl paraoxon which reacts with acetylcholinesterase at the esteric site and becomes a phosphorylated enzyme complex which acts as an irreversible cholinesterase inhibitor (AChE, Acetylcholinesterase).
AchE methylparaoxon intermediate

Phosphorylated AchE

Regenerated AchE
dermatitis (Street, 1981). Persons with a prolonged history of contact with organophosphorus insecticides may suffer from liver damage together with digestive and nervous system disorders (Street, 1981). These pesticides alter host defense activities by many different mechanisms. For humans exposed to 10 μM organophosphorus insecticide, there was an inhibition of stimulation of lymphocytes by phytohemagglutinin. Mice under dietary treatment with methyl parathion had enhanced sensitivity to bacterial infections in both naive and immunized animals (Fan et al., 1978). Bacterial clearance rates were decreased, and phagocytic as well as bactericidal activities were reduced. Reduced opsonin levels and a concomitant decrease in IgG2, which are likely to be involved in decreased opsonization and phagocytic processes in vertebrates, have been noted (Street, 1981).

The immunological effects of methyl parathion have been studied mainly in vertebrates (Descotes, 1988). In rabbits, oral exposure to methyl parathion for 4 weeks affected humoral immunity by lowering the T-dependent antibody response as shown by the anti-sheep red blood cell (sRBC) hemolysin and anti-sRBC hemagglutinin tests (Street and Sharma, 1975). Alterations in cellular immunity have also been demonstrated by the induction of delayed type hypersensitivity in rabbits (Street and Sharma, 1975) and altered T-lymphocyte proliferation in man. After being exposed to 10 μM methyl
parathion in vitro for 3 days, human lymphocytes showed a decreased proliferative response (Lee et al., 1979).

Some organophosphorus compounds such as malathion suppress the humoral immune response in channel catfish, *Ictalurus punctatus*, following chronic exposure (Plumb and Areechon, 1990). For another pesticide, endrin, a sublethal exposure concentration for rainbow trout resulted in significantly reduced responses as determined by the migration inhibition factor (MIF) assay, the plaque forming cell (PFC) assay and serum agglutination titer (SAT) assay (Bennett and Wolke, 1987). The effect of trichlorfon on the iron-specific immune response in carp, *Cyprinus carpio*, was studied by Siwicki et al. (1990). After intoxication, leukopenia as well as decreases in the phagocytic ability and phagocytic index of neutrophils were observed. The percentage of nitroblue tetrazolium (NBT)-positive neutrophils decreased while the ceruloplasmin activity in plasma increased in intoxicated fish.

Although there has been an increase in the use of methyl parathion worldwide, an understanding of its effects on the immune response of aquatic invertebrates is still very limited. Therefore, studies of such effects, especially in species with commercial value, should be encouraged.
III. CHEMOTAXIS ASSAYS

3.1 Introduction

Methyl parathion (o,o-dimethyl-o-4-nitrophenyl thiophosphate) is an organophosphorus insecticide which has been used extensively by food producers. At the current rate of usage, many aquatic systems are being contaminated by organophosphorus pesticides (Hassall, 1990). The mode of action of methyl parathion is based on its inhibition of the enzyme acetylcholinesterase (AChE) in the neural and neuromuscular junctions of the animal's nervous system (Corbett, 1974). Methyl parathion affects crustaceans primarily through changes in enzyme levels, especially an increase in adenosine monophosphate deaminase and adenosine deaminase which induces higher catabolism of amino acids. This results in higher ammoniagenesis and may cause ammonia toxicity to the cells (Reddy et al., 1988). The cellular immunological effects of methyl parathion include a decrease in acid phosphatase which is a major degradative enzyme in granular hemocytes (Reddy et al., 1986).

Chemotaxis is the first step in phagocytosis, which is the most important cellular defense mechanism in shrimp. Chemotactic migration resulting in the accumulation of phagocytes at sites of tissue injury is one of the recognized
components of the inflammatory response in mammals. The migratory leukocytes of fish are also believed to accumulate at inflammatory sites after migration from the vascular pool in response to the presence of local chemoattractants (Ellis, 1977). Similar migration of hemocytes in shrimp is thought to be the first step in eliminating invading foreign agents. Generally, less is known about the inflammatory response in invertebrates. The cells most actively involved in this response are the granular hemocytes (Paterson et al., 1976).

The factors influencing the migration of fish leukocytes suggest that the process is similar to that in mammals in many respects (Sharp et al., 1991; Weeks et al., 1986). There are a variety of host-derived factors including serum components (Griffin, 1984; MacArthur et al., 1985) and bacterial-derived products (Burrows and Fletcher, 1989) which are chemotactic. In invertebrates, the mechanisms underlying chemotaxis are not well understood, but chemotactic factors have been identified and placed into three categories:

1) bacterial factors which act directly
2) factors generated in serum by the action of bacterial substances or other stimuli such as beta-1,3-glucan, a component of the yeast cell wall (Issarasak et al., 1992)
3) factors generated by leukocytes or hemocytes themselves (de Chatlet, 1979)
In aquatic animals, many xenobiotics have been reported to alter the chemotactic ability of phagocytes (Weeks et al., 1986). The objective of this study was to determine if one of the most widely used organophosphorus pesticides, methyl parathion, has an effect on cellular immune responses of the giant black tiger shrimp, *Penaeus monodon* which is currently one of the most important species in the shrimp aquaculture industry. Chemotaxis, one activity of the cellular immune system, was examined in hemocytes from shrimp exposed to varying concentrations of methyl parathion. Evaluation of hemocyte chemotaxis was one of a number of studies designed to determine if shrimp health management can be accomplished through hemocyte monitoring programs.

3.2 Materials and methods

**Experimental Animals**

Giant black tiger shrimp, *Penaeus monodon*, weighing 30-40 grams, with an average length of 13 cm, were obtained from a shrimp farm in Samutsakorn, Thailand. Shrimp were maintained in 30 liter glass tanks with a static renewal system to maintain constant methyl parathion concentrations of 0, 1, 2, 3, 6, and 10 ppb. The water was aerated and maintained at 20 ppt salinity, 25°C, and pH 8.0. Each tank held 10 shrimp which were fed with commercial shrimp feed pellets daily. In a preliminary study, the LC$_{50}$ was determined to be 3 ppb.
PESTICIDE

Methyl parathion was initially solubilized in 100% methanol to yield a concentration of 10 ppm (Bodhipaksha, unpublished data). Desired concentrations were reached by diluting with water, and 3 ml of the desired concentration was added to each of the holding tanks. In the control group (0 ppb methyl parathion), 3 ml of methanol alone was added. After 96 hours, surviving shrimp were sacrificed by placing them on ice. Hemolymph samples and cardiac phagocytic cells were then immediately collected for the chemotaxis assays.

COLLECTION OF SHRIMP HEMOCYTES

Hemolymph Anticoagulant Media (HAM) (Bodhipaksha, 1994) was used to maintain the integrity and viability of collected hemocytes. The composition of HAM is:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Function</th>
<th>Amount (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>Balance salt solution</td>
<td>14.20</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Balance salt solution</td>
<td>13.60</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Ca⁺ chelator</td>
<td>2.92</td>
</tr>
<tr>
<td>Dextrose</td>
<td>Adjust osmolarity</td>
<td>varied with hemolymph</td>
</tr>
<tr>
<td>Sodium citrate 10%</td>
<td>Ca⁺ chelator</td>
<td>2.58</td>
</tr>
<tr>
<td>H₂O</td>
<td>Solvent</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

The solution was adjusted to a pH of 7.8 with 1 N NaOH (Bodhipaksha, 1994).
CIRCULATING HEMOCYTES

A hemocyte suspension was prepared by withdrawing hemolymph from the ventral sinus of each shrimp and placing it into a 5 ml syringe containing chilled (4°C) HAM (1 ml HAM: 1.5 ml hemolymph). Gentle mixing of the hemolymph suspension with HAM was periodically necessary to prevent hemolymph coagulation which would begin to form at the tip of the syringe. The hemolymph-HAM mixture was centrifuged at 500xg for 10 minutes to harvest the hemocytes. The supernatant was removed, and the hemocytes were resuspended in HAM to a final concentration of $2 \times 10^5$ hemocytes per ml. To avoid clotting of the hemocyte suspension, a small portion of HAM was added with gentle vortexing until a desired uniform cell concentration was obtained. The suspensions were used in the assay immediately following their preparation.

CARDIAC PHAGOCYTES

Shrimp were sacrificed by placing them in ice. The heart of the shrimp, which is located on the dorsal part of the posterior carapace, was removed under aseptic conditions and transferred to HAM supplemented with 500 units/ml penicillin, 0.3 mg/ml streptomycin and 10% fetal calf serum (Sharp et al., 1991). Cell suspensions were prepared by teasing the tissue with a glass rod over a stainless steel mesh screen in a petri dish (Weeks and Warinner, 1984; Weeks et al., 1988). Cell
suspensions were collected and centrifuged at 400xg for 5 minutes and then resuspended in supplemented HAM at a concentration of 1x10⁶ cells per ml. Cell suspensions of these non-circulating phagocytes were used in the assay immediately after collection.

PREPARATION OF CHEMOATTRACTANTS

Dry yeast (Saccharomyces cervisaeae, Baker's yeast) weighing 1.5 g was suspended in 7 ml of 0.1M phosphate buffered saline (PBS) and autoclaved at 120°C and 15 psi for 15 minutes. Cells were then washed twice in fresh PBS (Fryer and Bayne, 1990) and resuspended in supplemented HAM at a 1:10 dilution.

Shrimp serum samples to be used for opsonization were collected by drawing hemolymph from the ventral sinus and allowing them to clot. The clot was then dislodged with a glass rod, and the hemolymph was incubated for 24 hours at 4°C. The serum was centrifuged at 500xg for 10 minutes to separate the clot from the plasma. After removing the clot, the serum was sterilized by filtering through a 0.45 μm pore size filter and stored at 4°C. Yeast cells were opsonized by suspending them in shrimp serum and incubating the suspension for one hour at 25°C. Two ml of opsonized yeast cell suspension was diluted with 15 ml supplemented HAM. The suspension was then centrifuged and washed twice. The final pellet was resuspended in 2 ml supplemented HAM to a
Concentration of $1 \times 10^6$ yeast cells/ml for use in the chemotaxis assay. The resuspended yeast cells were maintained at $4^\circ C$ until use. Figure 3.1 summarizes the steps in the preparation of the chemoattractant.

CHEMOTAXIS ASSAY

Employing Boyden chambers (Fisher) in duplicate (Boyden, 1962), 200 μl of an opsonized yeast cell suspension (1 mg/1 ml; $1 \times 10^6$ cells/ml) was added to the lower chamber. The chambers were equilibrated at room temperature to allow dissolved gas to dissipate. In each chamber, a 13 mm diameter, 5 μm pore size polycarbonate membrane filter (Nucleopore Corp.) was placed on top of the lower well. Two hundred μl of circulating or cardiac phagocyte suspensions ($5 \times 10^6$ hemocytes per ml) was placed in the upper chamber (Cheng et al., 1981). The chambers were incubated at room temperature in a humidified chamber for 90 minutes (Itami et al., 1989).

After each test, the membranes were air dried, fixed with 100% methanol for 5 minutes and then stained with Three Step Stain (Accra Lab, NJ.). The filters were mounted on microscope slides and examined using light microscopy. The number of phagocytes on the upper and the lower surfaces of the filter were counted. The cells which had migrated completely through the filter to its lower surface were classified as migrated hemocytes. The cells in 10 microscopic
fields (400x magnification) were used to derive the percent chemotaxis. The assay was repeated 4 times. The results were expressed as:

Percent chemotaxis = \frac{\text{Number of migrated hemocytes}}{\text{Total number of hemocytes counted on both surfaces of the filter}} (Maderazo and Waronick, 1978).

Analysis of variance was used to analyze the data. Comparisons of treatment to control groups were performed as described by Dowdy and Wearden (1991).

3.3 Results

The chemotactic responses were highest in the control groups for both circulating hemocytes and cardiac phagocytes. Migration was significantly reduced \((p<0.05)\) as the concentration of methyl parathion was increased \((1, 2, 3, \text{ and } 6 \text{ ppb})\). The shrimp exposed to methyl parathion at concentrations of 1, 2, 3, 6 and 10 ppb were not feeding, lethargic and very sensitive to external disturbances. Exposed shrimp also appeared to lose equilibrium and direction of movement.

A preliminary study to determine the LC\(_{50}\) of Penaeus monodon suggested that it was approximately 3 ppb at 96 hours. At the exposure dose of 3 ppb, the percent chemotaxis for circulating hemocytes was reduced by 37.9\% as compared to the
Figure 3.1 Preparation of chemoattractant. The yeast cell suspension was prepared by opsonizing yeast cells in shrimp serum for use in the chemotaxis assay. HAM, Hemolymph anticoagulation media; PBS, phosphate buffered saline.
Preparation of Chemoattractant

Hemolymph from ventral sinus
room temperature
Hemolymph clot
Break clot using glass rod
incubate 24 hrs. at 4° C
Centrifuge 500 g for 10 minutes
Serum
Filter through 0.45 micron pore size filter
Sterilized serum

Yeast 1.5 g.
Suspend in 7 ml PBS
Autoclave (120° C, 15 psi, 15 min.)
wash twice in PBS
Suspend in HAM (10:1)
Killed yeast cell suspension

incubate 25° C, 1 hr.
Opsonized yeast

Yeast suspension 2 ml. + 15 ml. HAM
Centrifuge + wash (twice)
Final pellet
Resuspend in 2 ml. HAM
(Store at 4° C)
47.0% when compared to the control group (Figure 3.2). The decrease in percent chemotaxis was significant (p<0.05) for both circulating hemocytes and cardiac phagocytes from all treatment groups as compared to the control group.

3.4 Discussion

Chemotaxis was reduced significantly after circulating hemocytes and cardiac phagocytes were exposed to methyl parathion at concentrations of 1, 2, 3, 6 and 10 ppb. The reduction in chemotaxis may be caused by interference with the mechanism of surface recognition. There are qualitative differences in the recognition sites on hemocyte surfaces which are capable of recognizing chemotactic signals (Cheng et al., 1981). The data suggest that recognition sites may have been altered by methyl parathion exposure and consequently were unavailable for subsequent recognition and chemotaxis (Cheng et al., 1980).

Chemotaxis is an important mechanism responsible for the aggregation of phagocytes to the site of foreign agents invading the body (Griffin, 1984). Therefore, the suppression of chemotaxis by methyl parathion may result in an increased susceptibility of shrimp to diseases such as *Vibrio vulnificus* which causes "black splinter" disease (Issarasak et al., 1991).

In vertebrates, the chemotactic mediators are generated and released by mast cells and basophils (Wasserman, 1991).
Figure 3.2 Chemotaxis of phagocytes. The percent chemotaxis of shrimp phagocytes was determined by measuring migration through membrane pores in a Boyden chamber. Cell counts were performed in 10 microscopic fields at 400x magnification. Four slides per sample were examined. Phagocytes studied include hemocytes and cardiac phagocytes.
Chemotaxis of Phagocytes
Shrimp exposed to methyl parathion

Methyl parathion concentration (ppb)

- Circulating hemocytes
- Cardiac phagocytes
In invertebrates, molecular species capable of modulating chemokinesis and chemotaxis of phagocytes are released during inflammation. Durliat and Vranckx (1989) indicated that a relationship between humoral factors and host defense mechanisms does exist in invertebrates, but the exact cell sources and their biological role remain to be further investigated (Wasserman, 1991).

Chemotaxis is considered to be a very important step in establishing contact between phagocytes and foreign invaders. When phagocytes encounter foreign substances, they respond by sending out finger-like pseudopods which enable them to migrate to the site of infection (Cooper, 1990).

Exposure to methyl parathion appears to affect the factors generated in the serum since the composition of serum enzymes changes following exposure to the pesticide (Chung and Secombes, 1987). Hemocytic factors are probably changed because alterations of the enzymes in mitochondria and other organelles have been noted in other invertebrate cells (Moorthy et al., 1985). In this study, the overall effect of methyl parathion on chemotactic migration of shrimp hemocytes was to decrease their activity. Therefore, lower cellular defense efficiency results, and the animal will be more susceptible to other opportunistic and pathogenic organisms (Issarasak et al., 1990).
IV. PHAGOCYTOSIS AND RESPIRATORY BURST ASSAYS

4.1 Introduction

Phagocytosis is the primary defense mechanism against invasion of pathogenic organisms in invertebrates (Olivier et al., 1988; Robohm, 1984). However, little is known about the intracellular mechanisms involved in the destruction of phagocytized microorganisms in decapods such as shrimp. Phagocytic hemocytes are believed to be the predominant cellular immune defense agents in crustaceans, where phagocytosis has been observed in vivo in circulating hemocytes and in phagocytes located within the heart muscle and liver tissue (Paterson and Stewart, 1974). The small granular hemocytes, some of which contain lysosomal enzymes, are the primary cells involved in the phagocytosis of foreign particles in shrimp. Various enzymes such as acid phosphatase, esterase and beta-glucuronidase have been found in the cytoplasmic vesicles within these cells. Large granular hemocytes are also capable of phagocytizing foreign material but do so with less frequency than the small granular hemocytes (Hose and Martin, 1989).

In decapods, some cardiac cells also have phagocytic capabilities (Johnson, 1987). Their ability to clear toxic
substances through pinocytosis has been demonstrated (Ghiretti-Magaldi et al., 1977), but little is known about the effects of pesticide exposure on the phagocytic ability of circulating and cardiac cells.

Methyl parathion is an organophosphorus pesticide that functions by inhibiting acetylcholinesterase and pseudocholinesterase in insects. Methyl parathion is metabolized into methyl paraoxon which reacts with acetylcholinesterase at esteric sites and causes a phosphorylated enzyme complex to form. This complex acts as an irreversible cholinesterase inhibitor. As a result, the hydrolysis of acetylcholine is inhibited and organic processes, including immunological responses, may be impaired. Phase one of this study was to determine if methyl parathion is capable of adversely affecting the phagocytic ability (percent phagocytosis and phagocytic index) of shrimp phagocytes. The second phase was designed to measure the effect of methyl parathion on the production of superoxide anions ($O_2^-$). Because stimulation of the hemocyte cell membrane during phagocytosis triggers the production of microbicidal oxygen free radicals or reactive oxygen species ($O_2^-, H_2O_2, OH^-, 'O$), the quantification of the $O_2^-$ produced by phagocytes can serve as a measure of the respiratory burst (Secombes, 1990).

The respiratory burst has been studied extensively in fish macrophages (Chung and Secombes, 1988; Secombes et al.,
1988; Warinner et al., 1988; Anderson et al., 1990; Mathews et al., 1990; Kelly-Reay and Weeks-Perkins, in press). The stimulation produced by the phagocytic process in fish macrophages involves the hexose monophosphate shunt (HMP) and nicotinamide adenine dinucleotide-2'-phosphate (NADPH) oxidase which triggers the production of reactive oxygen species. In the membrane, intracellular superoxide anion ($O_2^-$) radicals, one component of the respiratory burst, are produced (Secombes, 1990) as demonstrated in Figure 4.1.

Stimulation of phagocytes at the membrane level can be achieved by using a soluble activating agent such as phorbol myristate acetate (PMA). Hydrogen peroxide, hydroxyl ions and singlet oxygen are produced from the superoxide which is generated upon stimulation. The production of hydrogen peroxide is catalyzed by the enzyme, superoxide dismutase (SOD) (Findovich, 1978). The production of these antimicrobial agents within phagocytic cells prevents invading foreign agents from damaging body systems.

Intracellular superoxide ($O_2^-$) can be measured in vitro by quantifying the reduction of the redox dye, nitroblue tetrazolium (NBT) into an insoluble formazan using a spectrophotometer. Superoxide production can be verified by preventing its reduction with exogenous SOD which dismutates $O_2^-$ and generates $H_2O_2$ (Secombes et al., 1988). The optimum PMA concentration and incubation time are unique for each species and must therefore be determined individually.
Figure 4.1 The production of reactive oxygen species during the respiratory burst.

Reaction 1. During phagocytosis, reactive oxygen species or superoxide anions are produced. Nicotinamide adenine dinucleotide-2-phosphate (NADPH) oxidase converts NADPH and O\textsubscript{2} to NADP and superoxide ions. These reactions occur at the membrane level of the cells.

Reaction 2. Production of hydrogen peroxide is catalyzed by the enzyme superoxide dismutase (SOD). Hydrogen peroxide is produced from the intracellular superoxide radical which leads to the antimicrobial process within phagocytic cells.
1) \[ 2O_2 + \text{NADPH} \rightarrow 2O_2^- + \text{NADP}^- + H^+ \]

NADPH Oxidase

2) \[ 2H^- + 2O_2^- \rightarrow O_2 + H_2O_2 \]

SOD

(Chung and Secombes, 1988)
The optimum range of PMA for the production of intracellular O$_2^-$ was 1 $\mu$g/ml for shrimp phagocytes which corresponds to the optimum range of 0.2-2 $\mu$g/ml for superoxide production in fish macrophages.

Methyl parathion is an organophosphorus pesticide whose widespread use increases the opportunity for water supply contamination. Thus far, studies conducted on the effects of this pesticide on aquatic fauna have focused on enzyme systems, mainly involving the inhibition of acetylcholinesterase (Corbett, 1974). However, since fish macrophages have been shown to have reduced phagocytosis and reactive oxygen species production when exposed to pollutants such as polynuclear aromatic hydrocarbons (PAH) (Weeks and Warinner, 1984; Warinner et al., 1988; Weeks-Perkins and Wong-Verelle, in preparation), this study focuses on the effects of methyl parathion on phagocytosis and respiratory burst activity in giant black tiger shrimp, Penaeus monodon.

4.2 Materials and methods

ANIMALS

Giant black tiger shrimp (Penaeus monodon), approximately four months old, were collected from a commercial earth pond in Samutsakorn, Thailand. The average weight and length of the shrimp were 35 grams and 13 cm, respectively. The shrimp were maintained in aerated static systems containing 30 liters of water at 20 ppt salinity, 25°C and pH 8.0. Exposure to
methyl parathion was carried out by adding methyl parathion in 3 ml of methanol (1, 2, 3, 6, and 10 ppb) to the holding tanks. In the control group, 3 ml of methanol alone was added. At 96 hours, surviving shrimp were sacrificed by placing them in ice.

SURVIVAL RATE DETERMINATIONS

After exposure to pesticide for 96 hours at different concentrations of methyl parathion (0, 1, 2, 3, 6 and 10 ppb), surviving shrimp were counted, and the LC₅₀ was calculated. Four replications were performed.

PHAGOCYTE ISOLATION

Circulating Phagocytes

Hemolymph was drawn from the ventral sinus of shrimp using a 5 ml syringe with a 20 gauge needle. Cooled hemolymph anticoagulation media (HAM) supplemented with 2% fetal calf serum (FCS) and L-15 medium (Bodhipaksha, 1994) was first placed in the syringe at a ratio of 1 ml/1.5 ml hemolymph. The syringe containing hemocytes and HAM was gently mixed to prevent clotting. The hemolymph-HAM mixture was centrifuged at 500xg for 10 minutes to harvest the hemocytes. The supernatant was removed, and the hemocytes were resuspended in HAM to a final concentration of 1x10⁶ cells/ml. Viability of the cells was determined by trypan blue exclusion. The nuclei
of the non-viable cells appeared pycnotic and took up the blue stain while the viable cells remained unstained.

Cardiac Phagocytes

Hearts were aseptically removed and transferred to HAM supplemented with 500 IU/ml penicillin, 0.3 mg/ml streptomycin and 10% FCS (Sharp et al., 1991). Cell suspensions were prepared by teasing the tissue with a glass rod over a stainless steel mesh screen in a petri dish. Cell suspensions were collected, centrifuged at 400xg for 5 minutes, and resuspended in supplemented HAM at a concentration of 1 x 10^6 cells/ml. Phagocytes were used in the phagocytosis and NBT assays immediately after collection.

OPSONIZATION OF YEAST CELLS

Commercial Baker’s yeast (Saccharomyces cervisae) was prepared for phagocytosis assays by suspending 1.5 g in 7 ml phosphate buffered saline (PBS) and autoclaving the suspension at 120°C and 15 psi for 15 minutes. The yeast cells were rendered non-viable so that a uniform preparation of phagocytized yeast cells were available in all experiments (Bodhipaksha et al., 1992). After two washes in PBS, the yeast cells were resuspended in HAM at a 1% packed suspension (Fryer and Bayne, 1990). Just prior to use, cells were resuspended at 8.0 X 10^7/ml in HAM.
Opsonization of antigen in plasma has been shown to increase phagocytosis (Goldenberg and Greenberg, 1983). Yeast cells were opsonized by incubating for one hour in shrimp serum which was obtained from the hemolymph drawn from the ventral sinus and centrifuged at 1500 rpm for 5 minutes to separate the hemocytes from the serum. The yeast cells were then washed twice with PBS and centrifuged at 2000 rpm for 5 minutes before being resuspended in HAM at a concentration of 1% packed suspension. The opsonized killed yeast cell suspension was freshly made before each experiment to avoid any alteration in the opsonic property during storage (Fryer and Bayne, 1989).

Prior to conducting phagocytic assays, yeast cells were stained with the fluorescent dye, phloxine B (Sigma) solution (10% in PBS). This was used to facilitate microscopic examination of phagocytized yeast cells using light and fluorescent microscopy.

PHAGOCYTOSIS ASSAYS

The phagocyte suspensions were placed onto glass slides (100 ul/slide), and the cells were allowed to adhere for 30 min. The slides with attached phagocyte monolayers were rinsed gently with HAM to remove unattached phagocytes. Opsonized yeast cell suspensions (50 µl) were overlain on each phagocyte monolayer (providing a yeast cell to phagocyte ratio of 50 to 1). After gently mixing with a pipette, the slides
were incubated at room temperature in a humidity chamber for 90 minutes. Non-phagocytized yeast cells were removed by washing each slide twice with 100 µl of HAM using a pipette. To facilitate quantification of phagocytized yeast cells, 100 µl of trypsin solution (0.2-0.5% trypsin dissolved in HAM and adjusted to pH 7.6) was dropped on each slide for 1 minute to loosen adsorbed but non-phagocytized yeast cells, before rinsing with HAM. This step in the process improved accuracy in counting since only phagocytized yeast cells remained on the slides. Slide preparations were then fixed with Davidson’s fixative (Shaw and Battle, 1957) for 10 minutes, rinsed with HAM, air dried and stained with Hematology Three Step Stain (Accra, N.J.) and phloxine. The phagocytized yeast cells were easily observed using fluorescent microscopy and distinguished from other organelles or foreign particles. Phagocytized yeast cells appeared to be at a focal level similar to the phagocytes. Two hundred phagocytes were counted and characterized as being active (containing phagocytized yeast cells) or non-active (not containing yeast cells). The individual yeast cells within active phagocytes were also counted. The percent phagocytosis (number of active phagocytes/100 phagocytes) and the phagocytic index (number of yeast cells phagocytized/phagocyte) were determined for both circulating and cardiac phagocytes. Four replicates were performed.
SUPEROXIDE ANION DETECTION

The detection of intracellular superoxide anion ($O_2^-$) which has been accepted as a direct and accurate method of quantifying the intensity of the respiratory burst was detected by reduction of the redox dye, nitroblue tetrazolium (NBT) (Secombes, 1990).

Phagocyte monolayers were prepared in microtitre plates (NUNC) by adding 100 microliters of hemolymph or cardiac cell suspension to each well. The plates were incubated for two hours at room temperature and unattached cells were then removed by washing with L-15 medium.

One hundred microliters of a solution containing 1 mg/ml NBT dissolved in L-15 medium and 1 $\mu$g/ml PMA were added to each of the wells. The plates were incubated for 30 minutes at 25°C. Superoxide dismutase (SOD) was added to some of the NBT-PMA solution and placed in the wells as a control. During this incubation, NBT was reduced by $O_2^-$ into an insoluble blue formazan. After removal of the excess medium, 70% methanol was used to fix the cells for ten minutes. The wells were then washed with methanol 3 times and allowed to air-dry. To dissolve the formazan, 120 $\mu$l of 2 M KOH and 140 $\mu$l of dimethyl sulfoxide (DMSO) were added to each well and mixed with a pipette. The concentration of the turquoise blue colored solution was measured in a multiscan spectrophotometer (Dynatech MR5000) at 630 nm using KOH/DMSO as a blank.
STATISTICAL ANALYSIS

The statistical analysis of data was performed using analysis of variance for determination of significant differences in values for control and exposed groups of shrimp. The least significant difference (LSD) method of multiple comparisons was used to identify significantly different responses among treatment groups (Dowdy and Wearden, 1991).

4.3 Results

SURVIVAL RATE

The survival rate of shrimp exposed to methyl parathion decreased with increasing concentrations of pesticide as shown in Figure 4.2. The LC₅₀ was calculated to be approximately 3 ppb. Surviving shrimp showed decoloration of the body and were very weak, especially at concentrations higher than 2 ppb.

VIABILITY

The results showed that the viability of both cardiac and circulating phagocytes was approximately 80% or more after 3 hours at room temperature using HAM as the hemocyte media. Therefore, phagocytic assays were performed at room temperature.

After 96 hours of exposure, the average viability of circulating hemocytes was reduced with increasing
Figure 4.2 Survival rates of shrimp exposed to methyl parathion. Survival rates after 96 hours of exposure showed a decrease in % survival as the concentration of pesticide was increased. The LC$_{50}$ was approximately 3 ppb.
Survival rates of shrimp exposed to methyl parathion

Survival rate (%) vs. Methyl parathion concentration (ppb)

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concentrations of methyl parathion. At 10 ppb, the percent viability was reduced to 76% as compared to 96% for the control group. Viability of cardiac phagocytes was reduced to 82% at 10 ppb as compared to 98% viability for the control group as shown in Figure 4.3.

MORPHOLOGICAL STUDIES

The phagocytes formed numerous filopodia and showed exocytosis of granules. The circulating hemocytes, when they were freshly removed from the animal, were observed to have a round shape. However, when attached to the surface of glass slides, the cells spread out, became flattened, and extended pseudopodia. Spreading cells usually had a higher cytoplasmic to nuclear ratio than non-spreading ones. The round hemocytes exhibited less phagocytic ability than the spreading ones. Phagocytosis of yeast cells occurred following opsonization by incubating the cells with hemolymph (Soderhall and Smith, 1986). Phagocytosis of yeast cells by shrimp hemocytes was observed using fluorescent microscopy.

PHAGOCYTOSIS ASSAYS

Results of phagocytosis (percent phagocytosis) assays for circulating and cardiac phagocytes are shown in Figure 4.4. Percent phagocytosis for cardiac phagocytes decreased significantly ($p < 0.05$) in all treatment groups (1, 2, 3, 6 and 10 ppb) as compared to the control. For circulating
Figure 4.3 Phagocyte viability from shrimp exposed to methyl parathion. The average viability of hemocytes was measured using the trypan blue exclusion method. After 96 hours of exposure, the decreases in viability of circulating hemocytes and cardiac phagocytes were significant (p<0.05) at 10 ppb as compared to the control group.
Hemocyte viability
Shrimp exposed to methyl parathion

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Figure 4.4 Phagocytic activity of phagocytes from shrimp exposed to methyl parathion. Phagocyte counts were determined using light microscopy (400x magnification). Non-ingested but adsorbed yeast cells were minimized by using trypsin prior to hemocyte staining.
PHAGOCYTIC ACTIVITY OF SHRIMP PHAGOCYTES

PHAGOCYTIC ACTIVITY (% PHAGOCYTOSIS)

METHYL PARATHION CONCENTRATION (ppb)

CIRCULATING
CARDIAC

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phagocytes there was a significant (p < 0.05) decrease in phagocytosis at 2, 3, 6, and 10 ppb as compared to the control group. In each treatment group, there were no significant differences between circulating and cardiac phagocytic activity.

The results of the phagocytic index assays for both circulating and cardiac phagocytes (Figure 4.5) indicated the presence of a decreasing trend as the methyl parathion concentration was increased from 0 to 10 ppb. The decreases were significant (p < 0.05) for groups exposed to 3, 6 and 10 ppb as compared to the control group.

RESPIRATORY BURST ASSAYS

Superoxide anions were released during the respiratory burst by circulating and cardiac phagocytes after the shrimp were exposed to methyl parathion for 96 hours (Figure 4.6). For circulating phagocytes, no significant differences (p < 0.05) were found between the control groups and the groups exposed to 1, 2 and 3 ppb methyl parathion. For cardiac phagocytes, the experimental group exposed to 3 ppb, but not to 1 and 2 ppb, showed a significant (p < 0.05) increase in superoxide production. In shrimp exposed to 6 and 10 ppb, significant (p < 0.05) increases in superoxide anion production were found in both circulating and cardiac phagocytes as compared to the controls. Cardiac and circulating phagocytes showed similar increasing trends in
Figure 4.5 The phagocytic index of phagocytes from shrimp exposed to methyl parathion. Phagocytic indices were expressed as the number of phagocytized yeast cells per phagocyte. Yeast cells were counted using light microscopy and confirmed with fluorescent microscopy after staining with the fluorescent dye, phloxine.
AVERAGE NUMBER YEAST CELLS/PHAGOCYTE

PHAGOCYTIC INDEX OF SHRIMP PHAGOCYTES

CIRCULATING
CARDIAC

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Figure 4.6 Superoxide production by phagocytes from shrimp exposed to methyl parathion. Superoxide anion production during the respiratory burst was determined in circulating hemocytes and cardiac phagocytes after shrimp were exposed to methyl parathion for 96 hours. The turquoise blue color resulting from the reduction of NBT was read using a multiscan spectrophotometer at 630 nm with KOH/DMSO as the blank.
SUPEROXIDE PRODUCTION BY PHAGOCYTES FROM SHRIMP EXPOSED TO METHYL PARATHION

METHYL PARATHION CONCENTRATION (ppb)

OPTICAL DENSITY (630 nm)
respiratory burst activity when shrimp were exposed to methyl parathion. However, for all groups, superoxide production was greater ($p < 0.05$) for circulating hemocytes than for cardiac hemocytes.

4.4 Discussion

The phagocytic ability of phagocytes from shrimp exposed to methyl parathion was studied using immunological assays for phagocytosis. Phagocytosis is an important part of the cellular immune system and functions to protect the host by destroying infectious agents. The results of this study demonstrated that methyl parathion exposure caused a significant decrease in the percent phagocytosis for circulating and cardiac phagocytes especially above the LC$_{50}$ which was determined to be 3 ppb. In these experiments, the average percent phagocytosis in the control group was 8.75 for circulating hemocytes and 10.88 for cardiac phagocytes.

In shrimp, opsonic factors exist in the form of proenzymes in the small granular and large granular phagocytic cells. Phagocytosis is initiated when the yeast cells come in contact with phagocytes. Autoclaving yeast cells at 120°C and 15 psi for 15 minutes killed the cells but their non-self recognition was maintained (Bodhipaksha et al., 1992). Opsonization of yeast cells with cell free hemolymph as demonstrated by Hose and Martin (1989) was shown to significantly increase maximal phagocytic rates in small
granular cells. Opsonic factors in crustaceans are thought to be either products of an opsonization activating system (Smith and Soderhall, 1983; Soderhall et al., 1986) or agglutinins, specifically lectins. Some of the carbohydrate specificities of crustacean opsonins have been defined by Goldenberg and Greenberg (1983) who showed that D(+) glucose and D(+) mannose caused a significant inhibition of the opsonins and decreased the percent phagocytosis by hemocytes. Methyl parathion exposure at sublethal concentrations may cause changes in the glucose metabolism of invertebrate cells (Moorthy et al., 1985).

After freshwater mussels were exposed to methyl parathion, glucose-6-phosphate dehydrogenase activity increased, suggesting enhanced oxidation of glucose and reduction in the aerobic oxidation of glucose (Moorthy et al., 1985). In the same manner, this exposure may cause an increase in cell metabolites (Hoar, 1983) and a decrease in the non-self recognition necessary for phagocytosis. In this study, the percent phagocytosis by hemocytes decreased with an increase in exposure to methyl parathion. Also, since methyl parathion has been shown to cause both abnormalities in mitochondrial oxidative metabolism and a decrease in ATPases (Moorthy et al., 1985), the energy produced for phagocytic activity was probably decreased; and therefore, there was a decline in the number of cells capable of phagocytosis. Another possible factor that could lower the amount of energy
that hemocytes can use for phagocytosis is the elevation in serum glucose induced by stress of pesticide exposure for 96 hours. Lynch (1973) and Lynch and Webb (1973) indicated that the elevation of serum glucose is an indicator of stress in the crab, *Callinectes sapidus*.

The attempt to quantify phagocytosis accurately depends on the removal of all loose and adsorbed particles (Prowse and Tait, 1969). Adsorption of untreated or opsonized particles to hemocytes is sufficiently strong so that washing alone does not remove adsorbed particles. In describing the effect of trypsin on the adsorption of particles by invertebrate hemocytes, Scott (1971) showed that adsorption depends on a protein receptor site. Therefore, trypsin was used, with success, in these experiments to eliminate adsorption of yeast cells to shrimp hemocyte.

The phagocytic index (average no. of yeast cells/phagocyte) also showed significant decreases with increasing concentrations of methyl parathion for both circulating and cardiac phagocytes. Further work will be required to determine the mechanism of impaired phagocytosis caused by methyl parathion.

This study confirmed that circulating and cardiac phagocytes collected from shrimp responded to the membrane activating agent PMA and produced superoxide anions ($O_2^-$) during the respiratory burst. After exposing shrimp to methyl parathion for 96 hours, both circulating and cardiac
phagocytes exhibited significant increases in superoxide production at 6 and 10 ppb, as well as at 3 ppb in the case of cardiac phagocytes. In freshwater mussels exposed to methyl parathion, Moorthy et al. (1985) found an increase in glucose-6-phosphate dehydrogenase activity suggesting enhanced oxidation of glucose via the hexose monophosphate (HMP) shunt. The HMP shunt generates NADPH which provides the one electron necessary for the reduction of oxygen into superoxide. Therefore, the increases in superoxide may be attributed to an elevation in glucose-6-phosphate dehydrogenase activity. This suggests that phagocytes in shrimp exposed to methyl parathion may have undergone increased glucose oxidation via the HMP shunt, and the decreased aerobic oxidation of glucose induced higher $O_2^-$ production by phagocytes following stimulation with PMA.

Enzymatic activities, especially microsomal enzymes (Corbett, 1974), are particularly important in determining toxicity tolerance to organophosphorus pesticides in invertebrates. These enzymes can metabolize pesticides into non-toxic substances, especially those which are lipid soluble. Further investigation as to how cellular enzymes affect phagocytosis and superoxide production is needed.

Phagocytosis and respiratory burst assays have the potential to be used in shrimp as a biomarker to assess organophosphorous pesticide contamination in the environment.
V. HISTOPATHOLOGY AND ELECTRON MICROSCOPIC STUDIES

5.1 Introduction

Since methyl parathion was found to affect the defense mechanisms of giant black tiger shrimp, Penaeus monodon (Chapters 3 and 4), studies were conducted to determine what fine structural changes occurred in hemocytes after exposure to the pesticide. In addition, tissue changes were evaluated utilizing histological techniques.

A major concern about organophosphorus pesticides such as methyl parathion involves the presence of tissue residues resulting from enzymatic alterations of methyl paraoxon. Even in very low concentrations, this is a concern. It was postulated that the primary pesticide and/or its metabolized form (methyl paraoxon) can cause morphologically visible damage to various organs. These include the hepatopancreas, an important detoxifying organ; gill, a respiratory organ which is exposed directly to the environment; heart, a part of the circulatory system which can be exposed to toxicants by hemolymph flow; nerve, the target organ of action for the pesticide; and muscle, an effector organ of the peripheral nervous system (Barrington, 1967). It was also assumed that these changes could be observed using histopathological procedures.
Circulating hemocytes and cardiac phagocytes play an important role in elimination of foreign particles but have not been studied extensively. Effects of methyl parathion on both types of phagocytes were considered as part of the immunological assessment of shrimp health.

This investigation attempts to demonstrate the structural changes which occur in shrimp phagocytes and selected tissues following exposure to methyl parathion. The knowledge concerning these morphological alterations may be useful in assessing the effects of various concentrations of a variety of aquatic pollutants on shrimp health. A determination of the effects of differing levels of aquatic toxicants can assist in the establishment of reasonable water quality standards.

5.2 Materials and methods

EXPERIMENTAL ORGANISM

Giant black tiger shrimp (Penaeus monodon) approximately 4 months old with an average weight of 35 grams and an average length of 13 cm were obtained from a local shrimp farm in Samutsakorn, Thailand. They were maintained in 30 liter glass tanks containing 10 shrimp per tank. Water quality was monitored and maintained at a constant salinity of 20 ppt, a temperature of 25°C and a pH of 8.0 with continuous aeration.
PESTICIDE EXPOSURE

Using a static system, methyl parathion was added to each tank at concentrations of 0, 1, 2, 3, 6 and 10 ppb. The experiments were completed after 96 hours of exposure in replicates of four. Water was not changed during the 96 hours.

HISTOPATHOLOGICAL STUDIES

The shrimp from each tank were removed and sacrificed by placing them in crushed ice. Tissues from the shrimp were removed and fixed in Davidson’s fixative (Shaw and Battle, 1957). Tissues and organs studied were the hepatopancreas, gill, heart, skeletal muscle and ventral nerve fibers. After fixing for 24 hours at room temperature, the samples were dehydrated in a graded ethanol series, embedded in paraffin, sectioned at six µm and stained with hematoxylin and eosin (Krol et al., 1989). Histological sections of gill, hepatopancreas, skeletal muscle, ventral nerve and heart were observed using bright field light microscopy.

ELECTRON MICROSCOPY

Preparation of samples for electron microscopy was performed using a modification of procedures described by Perkins et al. (1981) and Krol et al. (1989). Circulating hemocyte samples were collected by drawing hemolymph from the...
ventral sinus without using an anticoagulant. The cell suspension was centrifuged in a 2 ml centrifuge tube at 2000 rpm for 10 minutes, during which time the clot formed and separated from the plasma. The clot containing hemocytes was cut into pieces approximately 1 mm³ and fixed in 2% glutaraldehyde at 4°C in 0.1 M Millonig’s phosphate buffer (pH 7.2-7.4).

To collect cardiac phagocytes for fixation, the heart was removed from the shrimp and dissected to gain access to the endocardium where cardiac phagocytes are found between heart muscle fibers. The fixation was as performed as described above.

After fixing the samples of circulating hemocytes and cardiac phagocytes in glutaraldehyde for 2-3 hours, the samples were transferred to 0.025 M sodium cacodylate-buffered natural seawater at pH 8.0. The samples were then postfixed in buffered 1.0% osmium tetroxide in the same buffer at pH 8 for 1 hour at 4°C, dehydrated in a graded ethanol series, and infiltrated and embedded in Spurr’s resin (Spurr, 1969). Thin sections were placed on copper grids for staining with lead citrate and uranyl acetate. The sections were then examined with a Hitachi HU 11B transmission electron microscope at 75 or 80 kV.
5.3 Results

HISTOPATHOLOGICAL FINDINGS

Tissues of untreated shrimp which are considered normal, are represented in Figure 5.1A, 5.2 A, 5.3A, and 5.4A. The normal structures for gill, hepatopancreas, ventral nerve, skeletal muscle and heart were comparable to those described by Bell and Lightner (1988). No changes in skeletal muscle were observed in shrimp exposed to various pesticide concentrations as compared to the control group; therefore they are not illustrated. Morphological appearances of the shrimp tissues after exposure to methyl parathion were as follows:

CONCENTRATION: 1 ppb

Gill: Cells were similar to the control cells with gill lamellae being slightly congested (Figure 5.1 B).

Hepatopancreas: Normal structure of tubules with slight separation of tubules from the surrounding connective tissue (arrow, Figure 5.2 B).

Nerve: Normal appearance (Figure 5.3 B).

Heart: Normal appearance (Figure 5.4 B).

CONCENTRATION: 2 ppb

Gill: Nuclei became more basophilic with some marginated nuclei. Gill lamellae was slightly swollen (Figure 5.1 C).

Hepatopancreas: Some sloughing off of tubular epithelium. Hemocytic infiltration in the hemocoel increased (arrow, Figure 5.2 C).
Nerve: Normal appearance (Figure 5.3 C).
Heart: Normal appearance (Figure 5.4 C).

CONCENTRATION: 3 ppb

Gill: Marked lamellar swelling and slight melanization in some areas (arrow) (Figure 5.1 D).
Hepatopancreas: Necrosis with marked separation of tubules from surrounding connective tissues. Most of the F-cells (basophilic cells) were still intact and appeared normal (Figure 5.2 D).
Nerve: Some of the nuclei of the peripheral nerve cord were pycnotic (arrow) and marginated (Figure 5.3 D).
Heart: Normal appearance (Figure 5.4 D).

CONCENTRATION: 6 ppb

Gill: Marked melanization of gill lamellae with cell aggregations in some areas (arrow). Both primary and secondary lamellae were swollen with congestion in hemolymph sinuses (Figure 5.1 E).
Hepatopancreas: Severe degeneration of hepatopancreatic tubules and separation from surrounding connective tissue. Cell debris present in the lumen of the tubules (Figure 5.2 E).
Nerve: Pycnosis of nerve cell nuclei (arrow, Figure 5.3 E).
Heart: Normal appearance (Figure 5.4 E).

CONCENTRATION: 10 ppb

Gill: Extensive necrotic areas in the gill lamellae. Nuclei of gill cells were marginated and pycnotic (arrows) (Figure 5.1 F).
Hepatopancreas: Large necrotic areas containing only cell debris and connective tissue structural elements. A few other areas still contained distinguishable hepatopancreatic tubules and cells which had sloughed off the tubules. Cell debris filled the tubule lumens. Most of the nuclei of the distinguishable cells were either pycnotic or margined. Most of the phagocytes were enlarged and contained cell debris (Figure 5.2 F).

Nerve: Most of the cell nuclei were pycnotic with some separation of nerve fibers (separation is not visible in micrograph) (Figure 5.3 F).

Heart: Cardiac phagocyte numbers were slightly elevated as compared to lower concentrations of pesticide and controls. Most of the phagocytes were enlarged. Muscle fibers appeared normal (Figure 5.4 F).

ELECTRON MICROSCOPY

Electron microscopy results were as follows:

CIRCULATING GRANULAR HEMOCYTES

1 ppb There was no noticeable difference in hemocytes as compared to the control group (Figure 5.5 A,B).

2 ppb The granules appeared normal, but the cell membranes of some hemocytes were extended to form surface projections or "spines" (not visible in Figure 5.5 C).

3 ppb There was a slight alteration of some granules in that electron-lucent areas appeared in the matrix and profile of some of the granules were altered (Figure 5.5 D).

6 ppb Many of the granules appeared to lose their rounded profiles and coalesced with adjacent granules to form a reticulum of electron dense material in the cytoplasm (Figure 5.5 E).
Figure 5.1 Histopathological changes in gill of shrimp exposed to methyl parathion. Gills of the control group are shown in Fig 5.1A. The normal cells can be compared with the slightly congested gill lamellae at 1 ppb (B) and the margined nuclei at 2 ppb (C). At 3 and 6 ppb (D and E) melanization areas with cell aggregations were seen (arrows). At 10 ppb (F) cell necrosis in the form of margined and pycnotic nuclei was observed (arrows). (400X magnification)
Figure 5.2 Histopathological changes in the hepatopancreas of shrimp exposed to methyl parathion. Tubule structures of the control group are shown in Fig. 5.6A. Separation of tubules from surrounding connective tissue started at 1 ppb exposure (B, arrow). There was an increase in hemocyte infiltration in the tissue at 2 ppb (C, arrow). The separation of tubules and degeneration of tubule cells increased at 3, 6, and 10 ppb (D, E, F) (400X magnification).
Figure 5.3 Histopathological changes of ventral nerves of shrimp exposed to methyl parathion. Nerve structure appeared normal at 0, 1 and 2 ppb (A, B, C). Some of the nuclei were pycnotic (D, arrow) or marginated at 3 ppb; and more pycnotic cells were observed at 6 (E, arrow) and 10 ppb (F) (400X magnification).
Figure 5.4 Histopathological changes in the heart muscle of shrimp exposed to methyl parathion. No changes were observed in heart muscle structure at the various treatments of 0, 1, 2, 3, 6, and 10 ppb exposures (A, B, C, D, E, and F). A slight increase in cardiac phagocyte number was noted at 10 ppb (F) (100X magnification).
Figure 5.5 Electron microscopic studies of circulating granular hemocytes of shrimp exposed to methyl parathion. Normal structure of hemocytes was observed at 0, 1 and 2 ppb (A, B and C). Granule degeneration was noticed and increased with exposures of 3, 6 and 10 ppb (D, E, and F). N, nuclei; G, granules; M, mitochondria. (7,560X magnification). 

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10 ppb Electron-dense material in the cells was more evident and was found both as a reticulum and as granules with smooth profiles. There was clumping of intranuclear material (not shown in Figure 5.5 F).

CARDIAC PHAGOCYTES

There appeared to be no ultrastructural changes in cardiac phagocytes. At exposures of 1, 2, 3, 6 and 10 ppb methyl parathion, all cells were similar to those seen in the control group.

5.4 Discussion

HISTOPATHOLOGY

The effects of methyl parathion on the organs of the shrimp were observed to be a function of the concentration of pesticide exposure. The most significant effects were seen in the hepatopancreatic cells which comprise many cell types (Doughtie and Rao, 1983). After exposure to methyl parathion at 1, 2, 3, 6 and 10 ppb, gradual and increasingly severe changes in the hepatopancreas were seen as the concentration of methyl parathion was increased.

Massive necrosis was clearly evident on gross observation. At lower exposure concentrations, the affected hepatopancreas was abnormally orange in color, appeared slightly swollen, and lesions were found at the central and
portal lobular areas. At higher concentrations, the total structure of the hepatopancreas collapsed and appeared liquified when the carapace was cut open.

Histopathological studies showed progressive extension of lesions across the lobules. Interestingly, shrimp survived with only a very small area of intact cells remaining in the hepatopancreas. At lower concentrations, the damage to hepatocytes was multifocal, and no fibrosis was observed. These necrotic foci may be repaired by removal of dead hepatocytes and replacement by adjacent hepatocytes (Popp and Cattley, 1991). In the present study, at 10 ppb massive necrosis was observed. Destruction of a considerable portion of the hepatopancreas was still compatible with life as evidenced by the fact that shrimp can remain alive even though this hepatopancreas was mostly liquidfied. It is believed that the hepatocytes, remaining as small islands of unaffected or reversibly affected cells, effectively repair the damage by their regeneration. However, complete destruction of hepatocytes in a lobule renders that lobule incapable of participating in the regenerative process, and therefore the lobule is permanently lost from the hepatopancreas (Popp and Cattley, 1991).

The effects of methyl parathion on acetylcholinesterase function may also affect the function of the heart in both the movements of cardiac muscle and functioning of the pacemaker. After shrimp were exposed to methyl parathion, the hemolymph
provided the vehicle by which exposure of heart cells occurred. Methyl parathion and its metabolites in the hemolymph provide maximum exposure concentrations to the heart due to its unique function as the hemolymph pump. As a consequence, the risk of myocardial damage increased with the duration of exposure (Van Fleet et al., 1991).

After 96 hours at 10 ppb exposure, the number of cardiac phagocytes seemed to increase. Cardiac phagocytes, which have been called "phagocytic reserve cells" (Johnson 1987), lie on the basal laminas of tissues facing the hemal space. Fontaine and Lightner (1974) suggested that the function of these cells are in the clearance of toxic substances by releasing the contents of the reserve inclusions in the neighborhood of tissues adversely affected by a toxic compound. In so doing, they may also provide additional sustenance during stressful times. The need for a greater number of cells increases as the concentration of a toxic compound increases. Therefore, this could explain the increase in number of these cells evidenced in the histopathological study at 10 ppb exposure.

In this study, the effects of methyl parathion on muscle were observed by behavioral changes more readily than by histological changes. Since arthropod skeletal muscles are innervated by a small number of axons (usually 2 or 5), they supply a significant percentage of all the cells that comprise the muscle. Motor units are absent and each of the axonal branches to a muscle fiber is subdivided into numerous fine
branches that contact the muscle cells at many points (Hoar, 1983). Since methyl parathion can induce a clinical response that is not associated with demonstrable microscopic alterations in skeletal muscle, the observation of behavioral changes may result from extreme motor nerve excitation rather than from direct toxicity (Kakulas, 1982).

In rats, injection of paraoxon results in a progressive myopathy which ultimately leads to necrosis and phagocytosis of muscle fibers. The myopathy is attributed to an excessive concentration of acetylcholine (Van Fleet et al., 1991). In vertebrates and higher invertebrates, the acetylcholine action sites are the endings of motor nerves as well as the endings of autonomic preganglionic and parasympathetic postganglionic nerves. In arthropods however, acetylcholine appears to be the excitatory transmitter at some central synapses (Pichon, 1974) and serves as a transmitter at sensory nerves (Florey, 1967). Therefore, the effect of methyl parathion on shrimp skeletal muscle would be an indirect effect from the over stimulation of sensory nerves rather than an over stimulation of motor nerves as in vertebrates. The direct mechanism of the effects of methyl parathion on neuromuscular transmission in shrimp includes the interference of acetylcholine release and the impairment of muscle membrane conductance (Van Fleet et al., 1991). The reduced inhibition was observed in the tissues within 7 days after transferring shrimp to toxicant-free water (Reddy et al., 1987). There is also the
possibility that the toxicant may provoke immunologic reactions which lead to generalized muscle weakness (Harvey, 1980).

In the present histopathological studies of the nerve, nerve cell damage was found to increase with increasing methyl parathion concentrations. In most animal species, the effects of organophosphorus compounds can be divided into acute and delayed effects. The acute effects are based on the inhibition of acetylcholinesterase, leading to immediate clinical signs (involuntary excretion, muscle twitching, weakness, convulsion and death). The delayed effects result in a neuropathy and have no direct relationship to the acute effects. The delayed action results in a distal axonopathy of large axons and peripheral nerves occurring several days to weeks after exposure (Asbury and Brown, 1980). In this experiment, the acute effects which resulted in death were not seen since all animals used in these studies were those that survived 96 hours of exposure. Therefore, the delayed effects may be an important consideration for the effects on tissues. A loss of microtubules and neurofilaments in affected axonal regions was seen in animals with delayed neuropathy (Davis and Richardson, 1980). Light lesions are often subtle and difficult to detect. They are, however, present concurrently with the first clinical signs such as axonal degeneration, and then the myelin secondarily disintegrates (Davis and Richardson, 1980). Since the effects of exposure to 1 and 2
ppb methyl parathion on the nerve were not detectable, the mechanism of delayed toxicity is not clear (Koestner and Norton, 1991); therefore, further studies of the physiological changes in the nerves following toxicant exposure must be carried out.

The present histopathological studies showed that there was damage to gills in shrimp exposed to 2, 3, 6 and 10 ppb methyl parathion. The extent of damage increased with increasing pesticide concentrations. One of the cell types which was probably involved was the brachial podocyte which is found in gills of many different decapods. The gill is a complex organ that serves in osmotic control, respiration, excretion and probably in the storage of waste materials (Johnson, 1980b). Under normal conditions, podocytes are larger than other gill cells and contain voluminous cytoplasmic vacuoles, smaller satellite vacuoles and a peripheral nucleus. The function of brachial podocytes in detoxification and elimination of hemolymph components by phagocytosis and pinocytosis has been described in grass shrimp, Palaemonetes pugio (Doughtie and Rao 1981). After the shrimp were exposed to methyl parathion, gill cellular damage, edema and epithelial sloughing increased with increases in pesticide concentration. Similar results have been obtained with the freshwater teleost, Cyprinus carpio, exposed to lethal and sublethal concentrations of methyl parathion (Ramamurthy et al., 1987). The detachment of gill epithelium,
gill edema and cell damage were the characteristic lesions observed. This results in respiratory distress which is also seen in malathion poisoning in the gills of bluegill, *Lepomis macrochirus* (Richmonds and Dutta, 1989), rainbow trout, *Oncorhyncus mykiss* (Walsh and Ribelin, 1975), snake head minnow, *Channa punctatus* (Dubale and Shah, 1979), walking catfish, *Clarius batrachus* (Mandel and Kalshrestha, 1983), tilapia, *Saratherodon mossambi* (Shukla et al., 1984) and channel catfish, *Ictalurus punctatus* (Areechon and Plumb, 1990).

In the present study, the most severe damage resulting from pesticide exposure was observed in the hepatopancreas which is the site of metabolism of methyl parathion into the more toxic form, methyl paraoxon (Hassal, 1990). Damage to gill cells also occurred probably because the gill, as an external organ, provides entry and elimination of toxicants to and from the water (Doughtie and Rao, 1981). Ventral nerve and heart showed morphological changes at higher concentrations of methyl parathion. In shrimp exposed to low concentrations, other workers have observed some external morphological changes after a longer period of exposure (Asbury and Brown, 1980).

In conclusion, with increasing concentrations of methyl parathion, the overall histopathological effects in shrimp were progressive increases in cell and tissue damage to the tissues of the hepatopancreas, gill, and ventral nerve.
ELECTRON MICROSCOPY

The majority of damage in the circulating granular hemocytes of shrimp exposed to methyl parathion was in the cytoplasmic granules. The level of destruction increased with increasing methyl parathion exposure. However, changes in the nuclei were not observed. Ham et al. (1979) showed that changes in arthropods exposed to the pesticide occur in the nucleolus, the first organelle to develop morphological changes when the rate of RNA synthesis is altered. Popp et al. (1985) observed that the nucleoli were fragmented and the subcomponents segregated. Segregated nucleoli had the granular and fibrillar components separated into distinct homogenous zones. However, such changes were not in this study, a difference which may be related to sample fixation.

One possible reason that there were no significant differences in the ultrastructure of cardiac phagocytes between the control and exposed groups is because there may have been a renewal of the cell population from other sites. Since mitotic figures were never seen in phagocytic cells of the heart, many of the fixed phagocytes may have been destroyed during the course of methyl parathion exposure and may have been replaced by extrinsic cells (Johnson, 1980a). This would explain why the newly recruited cardiac phagocytes exposed to the pesticide were ultrastructurally similar to the control phagocytes. Replacement by cardiac phagocytic cells attached at the basal lamina may assist in faster elimination.
of methyl parathion from the hemolymph circulation. It has been suggested that after cardiac phagocytes take up the toxicant by mechanisms similar to those described by Fontaine and Lightner (1974), the cells are damaged, dropped from the basal lamina and eliminated from the hemolymph circulation by other organs. At the same time, there is an activation at the basal lamina of the heart to induce replacement of cells (Johnson et al., 1981).
GENERAL DISCUSSION AND CONCLUSION

Because it provides a high safety margin for humans, methyl parathion has been used extensively worldwide. Many aspects of the effects of this organophosphorus pesticide on vertebrates and invertebrates have been studied previously. The present study was intended to emphasize its effects on cellular immune functions of giant black tiger shrimp (Penaeus monodon) which is one of the most important species in crustacean aquaculture.

This study indicates that methyl parathion exposure, even at a very low concentration, reduces cellular immune responses in giant black tiger shrimp. Exposure at concentrations of the LC_{50} and above results in alterations in the overall cellular (phagocytic) immune response. Therefore, one can assume that shrimp are more susceptible to invading organisms and other stressors. This reduced immunocompetence could lead to higher morbidity and mortality during shrimp production. Therefore, it is important for users of the pesticide, aquaculture farmers, environmental scientists and consumers to be concerned about the effects of the pesticide. Since tissue residues can create problems in shrimp immune systems, and residues in those which survive, if high enough, can affect humans who consume the animals, there is reason for concern.
More studies of the effects of methyl parathion on other aspects of shrimp biology should be encouraged in order to find ways to provide higher yields in shrimp production. More discriminating use of the pesticide will undoubtedly result in better health for mankind.
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