HAEMATOLOGICAL STUDIES ON THE FRESHWATER MUSSEL *Lamellidens marginalis* (Lamark, 1819)

THESIS SUBMITTED TO

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

UNDER THE FACULTY OF ENVIRONMENTAL STUDIES

BY

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1993

CERTIFICATE

This is to certify that this thesis is a bonafide record of the research work carried out by Mr.K.Ramanathan, full-time research scholar, under my scientific supervision and guidance, in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the Cochin University of Science and Technology under the School of Environmental Studies, and no part thereof has been presented for the award of any other degree, diploma, or associateship in any University.

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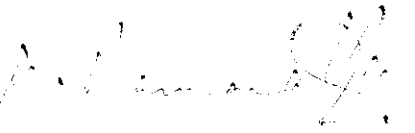
DECLARATION

I, K.Ramanathan, do here by declare that this thesis entitled " HAEMATOLOGICAL STUDIES ON FRESHWATER MUSSEL-*Lamellidens marginalis* (Lamarck,1819)" is a genuine record of the research work carried out by me under the guidance and supervision of Dr.A.Mohandas, Head, School of Environmental Studies, Cochin University of Science and Technology, and no part has been previously formed the basis for the award of any degree, diploma, or associateship of any university.



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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr.A.Mohandas, Head, School of Environmental Studies, Cochin University of Science and Technology, for giving me an opportunity to work under his supervision, encouragement, and for critical evaluation of the manuscript.

I also thank the authorities of the Cochin University of Science and Technology for providing me all necessary infra-structure facilities for the successful completion of my research work.

I express my sincere thanks to Dr. M.Chandrasekharan, Head, Center for Biotechnology, and Dr.K.Suresh for their invaluable help throughout the research work.

I also express my thanks to Dr.I.S. Bright Singh, Dr.M.Harindranathan Nair, and Dr.Ammini Joseph for their kind help during this thesis preparation.

I acknowledge with thanks Department of Science and Technology, Government of India, New Delhi and Cochin University of Science Technology for awarding me fellowships which helped me for the successful completion of my work.

Finally I express my sincere thanks to Mr.K.M.Mathew who was kind enough to type this thesis and Mr.V.Sathyamurthy, Mr.T.Karikalan, and Mr.K.Rajmohan for their invaluable help.

CONTENTS

CHAPTER - I

1.1.INTRODUCTION

Molluscs constitute the second largest phylum in the animal kingdom, next to arthropods in number of species and in diversity. They are of major interest to man from time immemorial. They occupy a significant position in the affairs of man as edible meat to aesthetic pearl, and in shell fisheries. They inhabit almost all niches, and some lead parasitic to paratenic mode of life. In India, molluscs constitute about half of the total shellfish resources. Mussels and oysters are the important molluscan fishery resources, and are foremost in total productivity (Nayar and Rao,1985). Some of the rural and coastal population's subsistence depend on clam and mussel meat because of their delicacy and dietary quality besides their abundant availability, and low cost. Apart from meat value, the shell is of commercial importance. Many of the cottage and small scale industries depend on the mineral content of the molluscan shell. Infact, many industries rely on shell lime for some of their industrial processes. In the export front not less than 25% of the marine products are clam meat, squids, and cuttle fish (MPEDA Newsletter,1990). But this comprises only meat products and not pearls and chemicals which form a part of the jewel and precious stone exports.

It is known that oysters, clams, mussels, and other molluscs suffer from diseases of both infectious and non-infectious nature. Infectious disease agents such as viruses, rickettsiae, chlamydae, fungi, bacteria, protozoa, and certain metazoa affect most molluscan forms, and their identification and pathological effects have been extensively reviewed (Kinne,1980, 1983; Andrews,1984).

The impact of infectious diseases on aquatic population can be immense. Mass mortalities and ecosystem disruptions have been reported (Andrews,1984; Jones et al.,1985; Sparks,1985). Damage to the gill due to iridovirus infection in Portuguese oyster *Crassostrea angulata* has been reported by His (1969). Further, the role of actinomycetes in death of Pacific oysters has been well documented (Elston et al; 1987). Also, many infectious diseases such as the one caused by the plasmodium, *Haplosporidium nelsoni*, in eastern oysters, and summer diseases in *Crassostrea gigas* due to bacterial infections (*Nocordia sp.*) which result in mass mortality in aqua cultural programmes were also recorded (Beattie et al.,1988). There are also reports of vibriosis infection in hatcheries of oysters in Japan (Beattie et al., 1988; Newell and Barber,1988). These studies have cautioned enough the threat posed by infectious diseases on economically important clam and oyster culture programmes.

Secondly, since the "Minamata" incident in the late 1950's, studies on the impact of heavy metals and other toxic pollutants on the aquatic system have received wide attention (Goldberg,1975; George et al., 1978; Patel,1983; Mix,1984; Ray,1984). Benthic invertebrates, especially molluscs, are found to concentrate a wide variety of heavy metals and other toxic matters from environment by several orders of magnitude above ambient levels (Phillips, 1976a,b; De Silva and Kureishy,1978; Leonzio et al., 1981; Roberts, 1976. Apart from accumulation, changes in the chemical configuration of the metals inside oysters further aggravate the toxicity. The accumulation of heavy metals and toxicants in Indian bivalves and their effect over biotic and metabolic activities also have been well documented (Lakshmanan,1982; Baby and Menon,1986; Shahulhameed and Mohanraj,1989; Rajalekshmi,1991). There have been some efforts to identify the different toxicity indices at the biochemical and physiological levels which may serve as warning system indicating the effects caused by environmental pollutants and susceptibility of the animals to diseases (Mix,1988). But our knowledge on the mode of infectious process in shellfish and effects caused by toxic chemical is fragmentary.

Finally, bivalves are reported to have the capacity to accumulate bacterial and viral pathogens from the environment where they live and transmit enteric diseases in man (Hart,1945; Kelly,1956; Dougherty and Altman,1962; Metcalf and Stiles,1965; Liu et al., 1966; Slanetz et al., 1968; Heffernan,1970; Surendran et al.,1986). Capacity to release microorganisms from body at favourable environmental conditions by bivalves, and the ambient factors that influence this release pattern have been well documented (Wells,1916; Dodgeson,1928; Arcisz and Kelly,1955; Erdman and Tennant, 1956; Wood,1961; Hedstrom and Lycke, 1964; Mitchel et al., 1966). The physiological processes that aid in the release of bacteria are the pumping rate of heart, the rate of transport, filtration efficiency, and elimination through epithelium by haemocytes *i.e.* diapedesis. Many molluscs act as intermediate host to protozoan and trematode parasites infecting man and animals.

Bivalves possess open circulatory system, and the internal organs are bathed in a pool of haemolymph. Haemocytes which are present in the haemolymph have access to all organs and are known to play a decisive role in internal defense through phagocytosis and encapsulation; in digestion by intercellular and intracellular processes; in transport, and in diapedesis. Thus, any change in the metabolism would reflect in the blood picture.

The effects of environmental factors on bivalves have been studied extensively on the whole animal, in tissues, and at cellular levels of the body (Johnson et al., 1985). The main orientation in those studies was the observation of responces to one or multiple variables like temperature, salinity, pollutants, etc. keeping other factors as constant as possible. These studies were aimed at organismal level and on short term effects. Particular attention was given to organs such as mantle, gills, gonads, and crystalline style, but seldom haemolymph and haemocytes were recognized or given importance as organ system, and fluctuation in value as possible index of stress (Suresh, 1988). Moreover, studies pertaining to haemolymph are direct, and haemolymph can be obtained from bivalves without unduly harming the animals severely (Feng et al., 1971; Ford, 1986). Hence, the study of haemolymph can be viewed as a method of nondestructive testing. Owing to the above mentioned advantages, attention was paid to the study of haemolymph and haemocytes.

Phagocytosis is the fundamental process in defense mechanism in bivalves, established as early as in 1891 (Metchnikoff, 1891) in invertebrates, and in bivalves by Stauber (1950). Later Cheng (1975, 1984) reported the role of haemocytes and haemolymph in internal defense process and their importance in bivalves.

The process of phagocytosis relies on a sequence of cellular events that include 1. nonself recognition, 2. cell spreading, 3. chemotaxis, 4. binding of foreign particle, 5. ingestion, and 6. intra cellular digestion (Fisher, 1986, 1988). During intra cellular digestion lysosomes fuse with phagosome to mix the foreign matter with degradation enzymes, eventually leading to either glycogen synthesis or making it nonviable (Cheng,1975; Auffret,1986; Fisher,1988). Further, it also results in increased synthesis of lytic enzymes, and the subsequent release of the same in to haemolymph (Cheng and Mohandas,1985).

It has been well documented that heavy metals do influence the rate of phagocytosis and release pattern of lysosomal enzymes in molluscs (Cheng and Sullivan, 1984; Cheng, 1988). Since elimination of both biotic and abiotic matters is mainly through the haemocyte and haemolymph, and since certain heavy metals have the ability in influencing the resistance potential of the animal, the combined effect (synergistic effect) on cellular defense mechanism was not fully elucidated so far.

With these background information, the present study was aimed at solving the following aspects in the freshwater mussel *Lamellidens marginalis* (Lamark,1819). Such a study, it is thought, would give some more information in the field of molluscan hematology, where contribution from India is limited.

Aim of the study:

1. To find out the activity pattern of haemocytes at different temperature conditions,
2. To examine the chemotactic activity and the preferential attachment of haemocytes towards different bacterial strains, both Gram positive, and Gram negative,
3. To find the role of serum in the activity of haemocytes,
4. To study in vivo phagocytic capability of haemocytes, and the role of ambient environmental parameters,
5. To estimate the activity of selected hydrolytic enzymes in the haemolymph, and
6. To examine the effect of heavy metals on the release pattern of selected haemolymph enzymes.

Finding answer to these questions, it is hoped, would help in understanding the problems more clearly thereby to formulate effective protective measures of mussels and oysters of economic importance against microbial and parasitic infections and diseases and pollutants that destroy or weaken the cultures and beds.

The thesis is divided into six chapters. The general introduction as given in this chapter is followed by chemotaxis of haemocytes in chapter two. In this chapter the spreading activity of haemocytes at different temperature for varying time-periods is reported, and the results properly analysed. The third chapter deals with studies carried out on in vivo

phagocytosis of haemocytes towards live and heat-killed *E.coli* at different temperatures. In the fourth chapter details of the study conducted on Acid Phosphatase (ACP), and Alkaline Phosphatase (ALP) in the haemolymph are reported. Both these enzymes were quantified at varying time-periods in the haemolymph of animals which were injected with saline, mild and heavy doses of live, and heat-killed *E.coli*. The fifth chapter includes the results of the studies on the effect of sublethal toxicity of two heavy metals Mercury (Hg), and Copper (Cu) on the activity pattern of ACP and ALP in animals challenged with *E.coli*. The salient features of the various experiments, and the conclusions drawn based on the results are included in the final chapter, followed by a list of references.

1.2.REVIEW OF LITERATURE

1.2.1.CELL TYPES

The unit of cellular defense in oysters and mussels is haemocytes. The study on the morphology of haemolymph cells dates back to 1891 when Cuenot first attempted to classify the haemocytes based on cytoplasm. Subsequent studies mainly dealt with the classifications based on the observations of cytochemical studies under light and electron microscopes and on the nature of cytoplasmic organelles their staining affinity and cytogenesis (Tanaka and Takatsuki, 1964; Feng et al,

1971,1977; Ruddel 1971 a,b,c; Narain, 1973; Cheng and Cali. 1974; Cheng et al., 1974; Cheng and Foley, 1975; Lowe, 1977; Rasmussen et al., 1985; Pal et al., 1986; Auffret,1988).

According to Cheng (1981) there are three basic types of haemocytes based on their morphology:

1. Hyalinocytes: cells with nearly clear cytoplasm containing few or no granules,
2. Granulocytes (Granular haemocytes): cells with well developed cytoplasm containing granules that range from scarce to numerous and more phagocytic in nature than hyalinocytes, and
3. Serous cells: known to be pigment bearing amoeboid cells whose function is mainly attributed to removal of degradation products of dead or moribund parasites and metabolic products of successful parasites (Takatsuki,1934b).

Auffret (1988) has expressed some reservation on the broad applicability of this classification to all bivalves. Based on comparative haemocyte study on many families of bivalves (Osteridae, Veneridae and Pectinidae) he observed that both hyaline and granular haemocytes are involved in phagocytosis while granular haemocytes were absent in the family Pectinidae.

1.3 . FUNCTIONS OF HAEMOCYTES

Bivalve haemocytes are known to be involved in diverse functions, such as 1. inflammation and wound repair, 2. shell repair, 3. digestion, 4. excretion and diapedesis, and 5. in internal defense (Bubel et al.,1977;Cheng,1981 1985; Feng,1988; Auffret,1988).

1.3.1. INFLAMMATION AND WOUND REPAIR

Inflammation is a process that involves both cellular and humoral elements of the host. This process begins following tissue injury alone or accompanied by invasion with a biological, chemical, or physical agent. Haemocytes are known to migrate in large numbers to the site of wound, form an amoebocyte plug which restrict haemolymph loss and contamination, and a wall of haemocytes surrounds the injury until they are replaced by epithelial cells growing over the plug (Fisher, 1986).

The major aspects of the process of wound healing are:

1. infiltration of the wounded area by large number of haemocytes,
2. aggregation of haemocytes to form a plug as well as delineating the area there by preventing loss of fluids and cells,

3. replacement of injured cells with elongated haemocytes resulting in healing which proceeds from interior of the lesion towards the surface (Cheng, 1981; Spark,1972;Feng, 1988),
4. deposition of collagen between cells comprising plug, and removal of collagens by the haemocytes in due course (Cheng, 1981; and Ruddel, 1971c),
5. infiltration of haemocytes (granulocytes) and removal of necrotic tissue debris by phagocytosis, and
6. restoration of normal tissue.

There are also reports of agranular amoebocytes and basophilic hyalinocytes involving in initial infiltration and phagocytosis (Ruddel,1971 a,b).

1.3.2. SHELL REPAIR

Bivalve leukocytes have been reported to play a limited role in shell repair, Dunachie (1963) and Beedam (1965), based on their studies in *Mytilus edulis* and *Anadara sp.* reported that transfer of calcium and organic matrix substances from the digestive gland to the site of repair is effected by haemocytes. But Wagge (1955), and Watabe (1983) observed that the migration of haemocytes to the mantle surface occurs only at earlier stages of repair, whereas according to Fisher (1988) the possible primary activity of the leukocytes on the mantle epithelium is to protect the damaged epithelial layer during repair process.

1.3.3. NUTRITION AND DIGESTION

Digestion in bivalves is both extra- and intra-cellular. Extra cellular digestion is effected in the alimentary canal by enzymes released from crystalline style. Intra cellular digestion occurs in two types of cells, in haemocytes and in digestive cells in the digestive diverticula (Yonge,1937; 1946). The role of haemocytes in digestion has been extensively reviewed (Takatsuki, 1934a,b; Wagge, 1955; Owen,1966; Purchon, 1968; Narain, 1973).Haemocytes endocytose both soluble and solid particles and once endocytosed digestion commences and they migrate deeper into tissues effecting transportation of nutrients (Yonge, 1926). Later studies confirmed the conversion of phagocytosed material into glycogen granules (Cheng and Cali, 1974; Cheng, 1975, 1981, 1983a; Mohandas, 1985).

The presence of carotinoid in haemocytes of *Mytilus corruseus* and *Crassostrea gigas* (Feng et al, 1977) indicates that molluscs have acquired them through pigment-bearing unicellular algae which comprise the diet in these animals. These observations imply that nutrient digestion and transport are clearly inter linked.

1.3.4. DIAPEDESIS AND EXCRETION

The role of haemocytes in elimination of unwanted materials has been studied (Stauber, 1950; Tripp, 1958,1960, Acton and Evans,1968; Cheng et al., 1969). The carmine laden phagocytes of the edible oyster *Ostrea edulis* exited through gonoduct and renal tubules (Takatsuki,1934a). Similarly erythrocytes laden haemocytes were observed in the gonoduct of *Crassostrea virginica*, and were seen to migrate through mantle epithelium into palial space (Feng and Feng, 1974).

In bivalves Keber's gland is known to be involved in excretion. This gland secretes serous cells, which play an important role in removal and degradation of moribund parasites and metabolic bye products of parasites (Mackin, 1951), are also available in the haemolymph (Cheng,1981). If serous cells are considered as third type of haemocytes (Cheng, 1981; Feng, 1988) then excretory function is also well convinced.

1.3.5. INTERNAL DEFENSE

Immunity or internal defense involves cellular and humoral factors. Studies on internal defense in bivalve commenced with the discovery of phagocytosis by Stauber (1950), who traced the ultimate deposition of India ink particles that were experimentally introduced into *Crassostrea virginica*.

The first prominent event observed in his study was occlusion of major blood vessels by ink particles followed by infiltration of haemocytes to the occluded lumen of the blood vessels and phagocytosis of the ink particles. Later,haemocytes emigrated with ink particles through arterial walls. Tripp (1958a, 1960) made similar observation in eastern oyster *Crassostrea virginica* injected with bacteria, yeasts and erythrocytes.

Phagocytosis involves five events: (Feng, 1988)

1. recognition of foreign particles,
2. adherence,
3. uptake,
4. destruction and
5. disposal .

1.3.5.1 RECOGNITION AND ADHERENCE

The first two events could be brought about either passively by random collision, or actively by chemotaxis. Foreign particle recognition is probably determined by the surface properties of the particle and the receptor haemocytes, (Cheng and Howland, 1979, 1982; Howland and Cheng, 1982; Mohandas et al., 1985).

Chemotaxis and random collisions result in initial adherence and subsequent endocytosis of the particulate matter by haemocytes. Oyster haemocytes exhibit chemotactic responses to a variety of agents: metacercarial cysts of *Himasthala quissetensis* (Cheng et al.,1974); *Micrococcus variance, Bacillus megaterium* and *Escherichia coli* (Cheng and Rudo, 1976a; Cheng and Howland, 1979; Schmid, 1975). The findings of lesser activity of haemocytes towards heat-killed bacterial cells signify their preference and recognition sites (Cheng and Howland 1979). Howland and Cheng (1982) identified the agents from *Bacillus megaterium* and *Escherichia coli* that initiate chemotaxis in oyster haemocytes as proteins of about 10,000 dalton.

1.3.5.2. UPTAKE MECHANISM

Mode of endocytosis of foriegn substances by bivalves haemocytes was first reported by Bang (1961). The bacteria about to be phagocytosed by haemocytes of *Crassostrea virginica* and*Mercenaria mercenaria* initially adhered to the surface of the filopods and subsequently taken into the ectoplasm by gliding along filapodia and became enclosed in a phagosome(Bang,1961; Mohandas,1985). In the second uptake mechanism as a result of contact between bacteria and granulocytes invagination of the cell surface develops and the bacteria are taken into endocytic vacuoles without the

involvement of filapodia (Cheng,1975; Mohandas,1985). In the third type of mechanism, Renwrantz et al. (1979) observed that both granulocytes and hyalinocytes take in rat erythrocytes by producing a funnel shaped psuedopod through which the foreign cell glides into the phagosome in the cytoplasm.

1.3.5.3. INTRA CELLULAR DEGRADATION

Most experimentally introduced vegetative bacteria were eventually rendered nonviable by intra cellular digestion (Tripp,1960., Feng,1966a). The process is apparently the same for food particles, engulfed foreign molecules, organisms,or for toxic chemicals like heavy metals etc. (George et al.,1978; Cheng, 1981). Yoshino and Cheng (1976a) have demonstrated, by employing cytochemistry and electron microscopy, that these granules are true lysosomes. They serve as storage organelles for hydrolases and are therefore analogous to the granules in mammalian polymorphonuclear and monocytic leukocytes (Cheng,1981).

Thus, intra cellular degradation of phagocytosed bacteria and eukaryotic cells are accomplished by an array of enzymes; acid and alkaline phosphatases, nonspecific esterases, indoxyle esterase, glucuronidase, lipase, aminopeptidase, lysozyme and β hexaminidase (Mc Dade and Tripp, 1970; Eble and Tripp,1969; Feng et al., 1971, 1977; Rodrick and Cheng, 1974; Yoshino and

Cheng, 1976b; Moore and Lowe, 1977; Cheng, 1983a).

However, extra cellular digestion has also been reported. Elevated levels of haemolymph lysozyme in northern quahogs exposed to *Bacillus megaterium* were observed by Cheng et al. (1975), and Foley and Cheng (1975). The release of enzymes was effected by what has been considered as degranulation (Foley and Cheng,1977; Mohandas and Cheng, 1985b), a process involving migration of lysosomes to the surface of cell where the enclosed enzymes are discharged. The morphological basis for the release of haemocyte lysosomes into bivalve haemolymph has been well elucidated recently in a scanning electron microscope study by Mohandas et al.(1985) who showed that bacteria stimulated haemocytes of northern quahogs to extrude more and larger intact lysosomes into haemolymph than control group and noted that the contact between lysosomes and the bacteria caused the degradation of bacterial cell walls indicating extra cellular digestion.

1.3.5.4 ELIMINATION

Feng and Feng (1974) showed that erythrocyte laden haemocytes present in gonoduct actively migrate through mantle epithelium into palial space. Moreover, phagocytosis, intra cellular digestion, and migration of erythrocyte laden haemocyte were shown to be temperature-dependent processes (J.S. Feng,1966;

S.Y. Feng,1966b; Acton and Evans,1968; Feng and Feng,1974). Carmine laden phagocytes of edible oyster *Ostrea edulis* exited through gonoduct and renal tube as well (Takatsuki, 1934a). Besides, nondigestible *Bacillus mycoidus* spores were found in the faecal material for sixty days (Tripp,1960), and a *Psuedomonas*-like bacterium was isolated from morbid eastern oysters that exhibited relapsing course of infection at 22-27°C but not at 9°C (Feng 1966a). Thus,elimination of foreign substrate is not only determined by the digestibility of the matter but also on the ambient temperature at which the animals were maintained.

1.4. ENCAPSULATION

Encapsulation occurs when groups of haemocytes surround a particle, mostly parasites, too large for phagocytosis. Tripp (1961) found that particles too large to be ingested by single haemocyte often get enveloped in concentric layers of fibroblast like cells. Cheng and Rifkin (1970), and Harris (1975) have reviewed encapsulation and attempted to classify the different capsules formed in terms of both parasite and host material used in the capsule, and connective tissue fibres.

According to Fisher (1986), in many cases there may be no reaction to parasite due to the co-evolution of the host and parasite. Leukocytes infiltrate the area and are believed to

form fibroblasts-like cells which deposit fibres in the inter cellular matrix. The parasite is usually lined by a thin capsule of fibroblast-like cells which is then concentrically ringed by very thick fibrous and biochemically different capsules. Haemocyte cells stretch into fibroblast-like cells and form the inner layer of the capsule (Fisher, 1986). Postmortem studies have indicated that haemocytes that were joined together form multi nucleate giant cells (Sparks and Pauley, 1964).

1.4. NACREZATION

Nacrezation is a term coined by Cheng (1967) to describe the deposition of nacre around parasites of molluscs which irritate or invade the mantle region. This results in pearl formation. Although it has been recognized as a type of defense mechanism it does not usually involve haemocytes (Cheng, 1981). It is known that certain trematode metacercariae, when found between inner surface of the shell and mantle of marine pelecypodes, will stimulate the mantle to secrete nacre which becomes deposited around the parasites.

1.6. HAEMOCYTE ASSOCIATED HUMORAL FACTORS

The importance of understanding the link between phagocytic cells and humoral components of the blood or haemolymph was recognized in the early studies of endocytosis. Wright and Douglas (1903) discovered opsonin, a humoral component, that could render bacteria more susceptible to phagocytosis. In oyster the discovery of haemagglutinin and its functional role as an opsonin (Tripp, 1970), and characterization of lysosomes (Mc Dade and Tripp. 1967) are important in bivalve immunology.

These humoral principles were reported in blue and Californian mussels and Pacific oysters (Hardy et al., 1976, 1977b; Bayne et al.,1979b; Renwrantz and Stahmer, 1983). It is also reported that the haemocytes of eastern oyster internalize concavaline A (Yoshino et al., 1979). Vasta et al. (1982) identified two serologically distinct oyster haemolymph lectins and one associated with haemolymph membrane, and have identical specificity with one of the two haemolymph lectins. They suggested that the membrane associated lectins may serve as a membrane receptor in nonself recognition by oyster haemocytes. Renwrantz and Stahmer (1983) showed that the heightened phagocytosis of yeast is attributable to two haemolymph factors:

1. opsonising properties of the purified agglutinin, and 2. Ca^{++} ions. Since these recognition sites can be blocked by a purified antiagglutinin, immunologlobulin-G from the same haemolymph proteins, the haemolymph and membrane-associated lectins may share similar antigenic structures (Feng, 1988).

Lysozyme was detected in both haemolymph and haemocytes of eastern oyster (Mc. Dade and Tripp,1967; Feng et al., 1971; Rodrick and Cheng, 1974; Cheng and Rodrick, 1975). Wittke and Renwrantz (1984) reported that certain blue mussel haemocytes secrete cytotoxic substances against human group A erythrocytes. The presence of lysosomal enzyme in bivalve haemolymph led Cheng (1983a, 1985) to conclude that " hyper synthesized enzymes released into the serum play a protective role in destroying susceptible infectious, biotic agents, and the elevated level of serum lysosomal hydrolases may initiate autolysis in inflamed areas and is specific at the molecular level than what has been designated as nonspecific".

The morphological basis for the release of haemocyte lysosomes into bivalve haemolymph was illustrated by Mohandas et al. (1985) by scanning electron microscope study. They showed that bacteria stimulated haemocytes of northern quahogs to extrude more and larger intact lysosomes into haemolymph than those of normals ones. They reported that the contact between lysosomes and bacteria results in degradation of

bacterial cell walls, an indication of extracellular digestion. They suggested that the process of degranulation was due to the recognition sites on haemocyte membrane.

Although bivalve immunology has been categorized as being cellular and humoral, these are not to be confused or comparable with vertebrate immunology. It has been accepted that molluscs do not synthesize immunoglobulins nor have the complement system, and the acquired resistance is not based on antigen and antibody interaction (Cheng,1981).

Thus, it can be inferred that the internal defense in bivalves involve both Cell Mediated Immunity and Humoral Immunity. The cell mediated immunity is well pronounced in the form of phagocytosis and encapsulation. The humoral immunity is of haemocyte or cellular origin and is secondary. But its effects on the internal defense is well recognized (Auffret,1988).

CHAPTER-II

2.1.INTRODUCTION

Haemocytes are primarily responsible for the internal defense of bivalves, functioning in inflammation, wound repair, encapsulation and phagocytosis (Fisher,1988). Activities of haemocytes or haemolymph cells that accompany these known functions in the defense response are varied and complex. Phagocytosis depends upon nonself recognition, locomotion, binding and ingestion of foreign matter, and intra cellular digestion. These cells exist individually within the haemolymph and interstitial spaces of animals, where they encounter variations in temperature, salinity, nutrients and other substances. Hence, haematological manifestation, as response to internal and external stress caused by both biotic and abiotic factors, has been given importance.

2.2.1. CELL SPREADING

Haemocytes change their shape to be functional in defense roles (Ruddel, 1971a; Cheng,1981; Fisher,1988). Uninduced haemocytes in the haemolymph are spherical but they are found as flattened and elongated in wound plugs and capsules (Sparks Morado, 1988). In addition, an amoeboid shape is necessary for locomotion (Fisher and Newell,1986). The ability to change

shape is of primary importance and integrates complex changes in membrane fluidity and cytoskeleton. This process requires simultaneous participation of cytoplasm, cytoskeleton, membrane surface receptor, and also possibly nucleus (Fisher,1988).

Fisher and Newell (1986) have taken into consideration the spreading ability as response to environmental parameters. Since in vitro spreading is a prelude to amoeboid movement and phagocytosis, the study of spreading activity has become necessary. Also, in many bivalves the haemocytes tend to aggregate immediately after withdrawal from sinus, but tend to migrate from the aggregate upon a period of incubation. Further, spreading enhances the visibility of haemocytes morphology for detailed study.

The method adopted for assaying cell spreading in bivalves was to measure the time required for haemocytes to spread, *i.e.* the time taken by haemocytes to transform from a spherical shape to amoeboid shape in vitro. Fisher and Newell (1986) for the first time measured spreading activity with reference to time to demonstrate the influence of environmental parameters like salinity and temperature on eastern oyster haemocytes. It is a valuable measurement of haemocytes' activity since it represents the culmination of several cell processes like non-self recognition, locomotion and phagocytosis.

In the present study, before going into detailed examination of various functions of molluscan haemocytes that are to be undertaken, a preliminary assay was carried out to asses the effects of temperature on in vitro spreading activity of haemocytes of *Lamellidens marginalis*.

2.2.2. MATERIALS AND METHODS

2.2.2.1. COLLECTION OF SPECIMENS

1.Specimens of *Lamellidens marginalis* were collected from channels and marshes adjacent to Periyar River at Alwaye. 2. They were brought to laboratory immediately after collection in water taken from the collection site. 3. At laboratory, the specimens were maintained in pre-aerated (24 hours) tap water, and allowed to acclimate to room temperature for 48 hours before and until used for experiments. 4. They were reared in large fibre-glass tanks and care was taken to avoid crowding, and to ensure that oxygen did not act as a stress factor. 5. Throughout the experiment, and during the acclimation period the animals were fed with freshwater algae *Oocystis pusila* and 6. The tank water was changed regularly at every 24 hours, and approximately 25 ml. of algal broth per animal were added into the tank.

2.2.2.2.SIZE GROUP SELECTION

Only one size group, of 50-70 mm. in length was chosen owing to the inconsistent availability of number of animals in all size ranges throughout the year, especially during rainy season and summer, and also due to the diminishing population in the locality. Further, no significant variations in blood parameters was observed in the two size groups of 45-55 mm., and 65-75 mm. in during our prevoius studies (D.S.T. sponsored project SP/GO/CO1/ 1988).

2.2.2.3. COLLECTION OF HAEMOLYMPH

1. Haemolymph samples from the aniamls were collected after acclimation of the animals for 48 hours. 2. The specimens were taken out of water and dried with clean cloth. 3. With the help of a scalpel, the valves were forced open slightly taking care not to tear the adductor muscle. 4. By keeping the valves open with the help of thumb the inner mantle water was drained and blotted with blotting paper. 5.With the help of sterile needle (No.22) and tuberculin syringe from each specimen around 1 ml. of haemolymph was withdrawn from the posterior adductor muscle, slowly. 6. During the process care was taken to avoid air bubble getting into the syringe along with blood.

2.2.2.4. DETERMINATION OF SPREADING ACTIVITY

1.Immediately after withdrawal of haemolymph, 2 to 3 drops were placed slowly on a previously cleaned and dried glass slide. 2. A monolayer of haemolymph was prepared as outlined by Abdul Salam and Michelson (1980a), and atleast two mono layers were prepared of each haemolymph sample. 3. Adherence and spreading of haemocytes were facilitated by incubating the slides in a humidified chamber. 4. A large petridish with a fitting cover was used as the chamber. 5. At the bottom, a moistened filter paper was kept. 6. Provision was made to keep the slides above the damp base. 7. This whole set up was kept in a B O D incubator. 8. Spreading activity was determined at various temperatures (24^0C, 26^0C, 28^0C, 30^0C and 32^0C). 9.At the end of the incubation period (1 and 2 hours), haemolymph was drained from the glass slide leaving the monolayer of haemocytes firmly attached to the glass slide, and this layer was fixed immediately with 10% formalin. 10. The slides were washed with phosphate buffer (pH 6.8). 11. The total number of spread and nonspread haemocytes attached to glass slides was counted randomly from four corners of the smear and care was taken to avoid repetition. 12. The percentage of haemocytes exhibiting spreading was calculated as spreading index (I).

$$I = \frac{\text{Number of spread cells}}{\text{The total number of haemocytes counted}} \times 100$$

The results were statistically analysed . The two-tailed "t" test (Zar,1974) was employed to determine possible statistical differences in the values.

2.2.3. RESULT

The mean percentage value of spread and nonspread haemocytes after one and two hour of incubation at different temperatures is presented in Table 1, and Figure 1.

From the results it is inferred that maximum spreading occurred at 32^0C in both the incubation periods. In general, there was an increase in the percentage of spread haemocytes with increase in incubation temperature except at 26^0C at 1 hour incubation.

Statistically significant higher results have been found at 1 hr. at 28^0C over 26^0C,and at 30^0C over 26^0C ($p<0.005$) but was not in any other combination. At 32^0C there was a sharp rise in the percentage of spread haemocytes, and showed significantly higher result compared to the rest($p<0.05$).

In the case of two hour incubation, the observed results are as follows:- Statistically higher percentage of spread haemocytes was been noticed at 32^0C on comparing with percentage at other

TABLE-1

Perecentage of spread haemocytes of *Lamellidens marginalis* exposed to different temperatures for 1 and 2 hour periods

	1 hr time-period				2 hr time-period			
Temperature	N	Mean	SD±	Range	N	Mean	SD±	Range
24°C	4	22.5	5.772	16.0-30.30	4	11.5	2.179	8.0-14.0
26°C	4	14.8	2.586	11.0-18.0	4	22.6	4.648	16.3-28.0
28°C	4	27.9	4.193	22.1-32.3	4	28.4	3.399	24.8-33.1
30°C	4	36.0	2.663	33.0-40.1	4	38.0	5.813	31.5-46.6
32°C	4	79.96*	5.276	72.9-86.3	4	73.2*	5.423	68.3-81.6

* $p<0.001$ - Significantly higher than the rest.

Percentage of spread haemocytes of *Lamellidens marginalis* at 1 and 2 hour periods at different temperatures

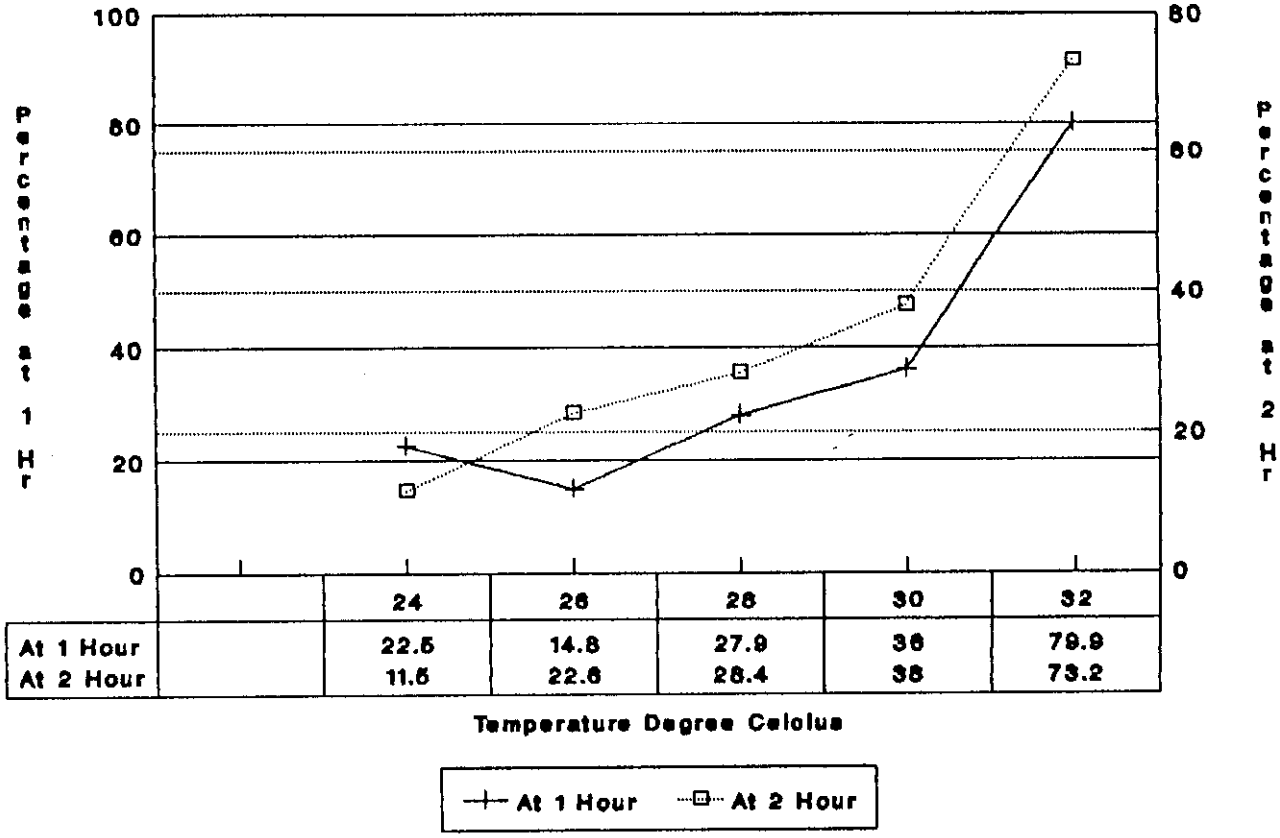


FIGURE-1

temperatures ($p<0.05$). At 24^0C the percentage of spread cells was significantly lower when compared to the percentage at 28^0C, and 30^0C ($p<0.05$,). But no significance was noticed at other temperatures.

2.2.4. DISCUSSION

Effect of temperature has been recognized as a method for microscopical evaluation of haemocyte activity in monolayers (Feng and Feng, 1974; Cheng, 1975; Abdul Salam and Michaelson, 1980a). In vitro studies by Foley and Cheng (1975) demonstrated higher phagocytic activity by cells from haemolymph of the pelecypod *Mercenariamercenaria* at higher ambient temperatures. Similarly, in the case of *Biamphalaria glabrata*, the haemocytes (in vitro) were more phagocytic at 27^0C than at 4^0C, 15^0C and 22^0C (Abdul Salam and Michelson, 1980). They also observed that there was decline in the activity of haemocytes at temperature above 27^0C .

Fisher and Tamplin (1988) reported higher and faster activity at 30^0C incubated sets than in 22^0C. They also observed that haemocytes of animals originated from environment where temperature was higher (estuarine) showed faster activity than those of animals collected from lower temperature (oceanic) area. They also mentioned that haemocytes which spread quickly

were also able to capture more foreign particles and took maximum of 20 minutes to spread.

Further, Fisher (1988) in his review reported that environmental stress reduces the spreading activity which may, in turn, have some effects on the phagocytosis. This was suggested based on the findings in the oysters obtained from higher temperature (25^{0}C) estuarine area and subjecting them to cooler temperature of 15^{0}C. Since recognition sites are found on the surface of the cell membrane any of the environmental factors that effect membrane could also affects the ability of haemocytes to recognize foreign material (Fisher, 1988). Temperature is known to have dramatic effects on biological membrane (Hochachka and Somero, 1973), and this influence might alter receptor site configurations (Fisher, 1988), which in turn affect the ability to distinguish between self and nonself material, the basic for internal defense (Cheng 1983b,c).

The results of the present study add evidence to the view that temperature influences the spreading activity of haemocytes. Thus, increase in temperature leads to increase in spreading activity and the maximum threshold was observed at 32^{0}C and at 34^{0}C there was destruction of haemocytes. Because of this reason 32^{0}C was taken for further in vitro studies of haemocytes.

2.3.1. CHEMOTAXIS OF HAEMOCYTES

Fundamental to defense response is the ability to distinguish between self and non-self materials. This is accompanied by the recognition sites or the receptor sites available on the external surface of haemocytes (Cheng, 1983b,c) which eventually lead to encapsulation or endocytosis of the foreign matter, or in some instances to release lytic enzymes by haemocytes (Cheng,1975, 1981; Mohandas et al., 1985). Foreign particles that bind are mostly reported to be endocytosed (Bang, 1961; Cheng, 1975; Renwrantz et al., 1979).

Adherence or binding, which is the result of foreign particle recognition by the receptor sites present on the haemocyte membrane,could be a valuable measurement of haemocyte defense activity, since it represents the culmination of the process of spreading, chemotaxis, and non-self recognition required in phagocytosis (Fisher and Tamplin, 1988).

Also, binding of foreign particle by haemocytes is reported to be used to asses the capacity of cellular defense pattern (Fisher et al., 1987), and their ability to distinguish various foreign subsatnces (Bang, 1961; Tripp and Kent,1967; Anderson and Good, 1976; Hardy et al., 1977a; Bayne et al., 1979; Cheng,1983a; Fisher and Tamplin,1988). Fisher (1988) attributed this differences in binding rate to the receptor

specificity of haemocyte membrane. The movement of haemocytes along a chemical gradient facilitates their contact with foreign particles for phagocytosis or encapsulation (Cheng and Howland,1979). The rate of chemotaxis by bivalve haemocytes has been demonstrated against a variety of bacterial strains (Cheng et al., 1974; Cheng and Rudo, 1976a; Cheng and Howland, 1979, 1982; Kumazawa et al.,1990, 1991; Kumazawa and Shimoji, 1991; and Kumazawa and Morimotto, 1992).

According to Cheng (1985) the surface receptors at work in the chemotaxis are the same as those that bind the foreign particles and hence binding could be taken as an indirect measure of chemotaxis and the variation in the foreign particle recognition ability of haemocytes in vitro. Moreover, this could be a rapid microscopical evaluation of cellular defense mechanisms against foreign substances in vitro and that might hitherto invade or enter into the body system of animal.

2.3.2. MATERIALS AND METHODS

Methods of collection of mussel, rearing,acclimation and collection of haemolymph were the same as described in the studies related to spreading activity (Section 2.2.2).
The number of animals employed for each set of experiment was 12. In vitro chemotaxis of haemocytes was assayed by incubating monolayers of blood cells with four different strains of

bacteria, both live and heat-killed. Heat-killing was done by autoclaving the bacterial suspension for 20 minutes.

2.3.2.1. PREPARATION OF MONOLAYERS FOR CHEMOTAXIS

1.The assay method described by Jeong and Heyneman (1976) was employed with some modifications. 2. A sample of 0.5 ml of haemolymph was withdrawn from each mussel from the posterior adductor muscle. 3. A thick smear was made on a clean cover glass slide. 4. The slides were incubated at 32^{0}C for 20 minutes to facilitate the attachment of haemocytes to and spread on slides in a humidified chamber. 5. A 0.2 ml of bacterial suspension was added to the smear and mixed very gently. 6. Slides were again incubated for 1 and 2 hour time-periods and excess haemolymph suspension was removed.
7. The cover glass sealed preparations were studied for the number of spread haemocytes with attached bacteria for the respective time-periods; *i.e.* 1 and 2 hours.

2.3.2.2.PREPARATION OF BATERIAL SUSPENSION

Four bacterial strains, namely two Gram negative: 1. *Escherichia coli*, and 2. *Vibrio alginolyticus*, and two Gram positive: 1. *Bacillus sp.* and 2. *Micrococcus sp.* were used to study the chemotaxis of haemocytes. The strains were cultured in nutrient agar plates for 24 hours, and harvested

TABLE-2 A

ercentage of *Lamellidens marginalis* haemocytes with attached
acteria when tested against four different live bacterial strains
t 1 and 2 hour periods.

	1 hr time-period				2 hr time-period		
	N	Mean	SD±	Range	N	Mean	SD±	Range
Escherichia-coli	12	62.45	5.27	54.00-71.88	12	63.12*	3.49	59.14-69.
Vibrio-alginolyticus	12	61.29	6.85	50.0-79.41	12	59.69	3.04	56.0-65.9
Bacillus sp.	12	57.89	4.89	48.9-67.81	12	60.47	3.37	53.85-67.
Micrococcus-sp.	12	61.36	3.23	54.93-66.67	12	62.99	4.32	56.67-70.

* $p<0.05$ - significantly higher than *V.alginolyticus*.

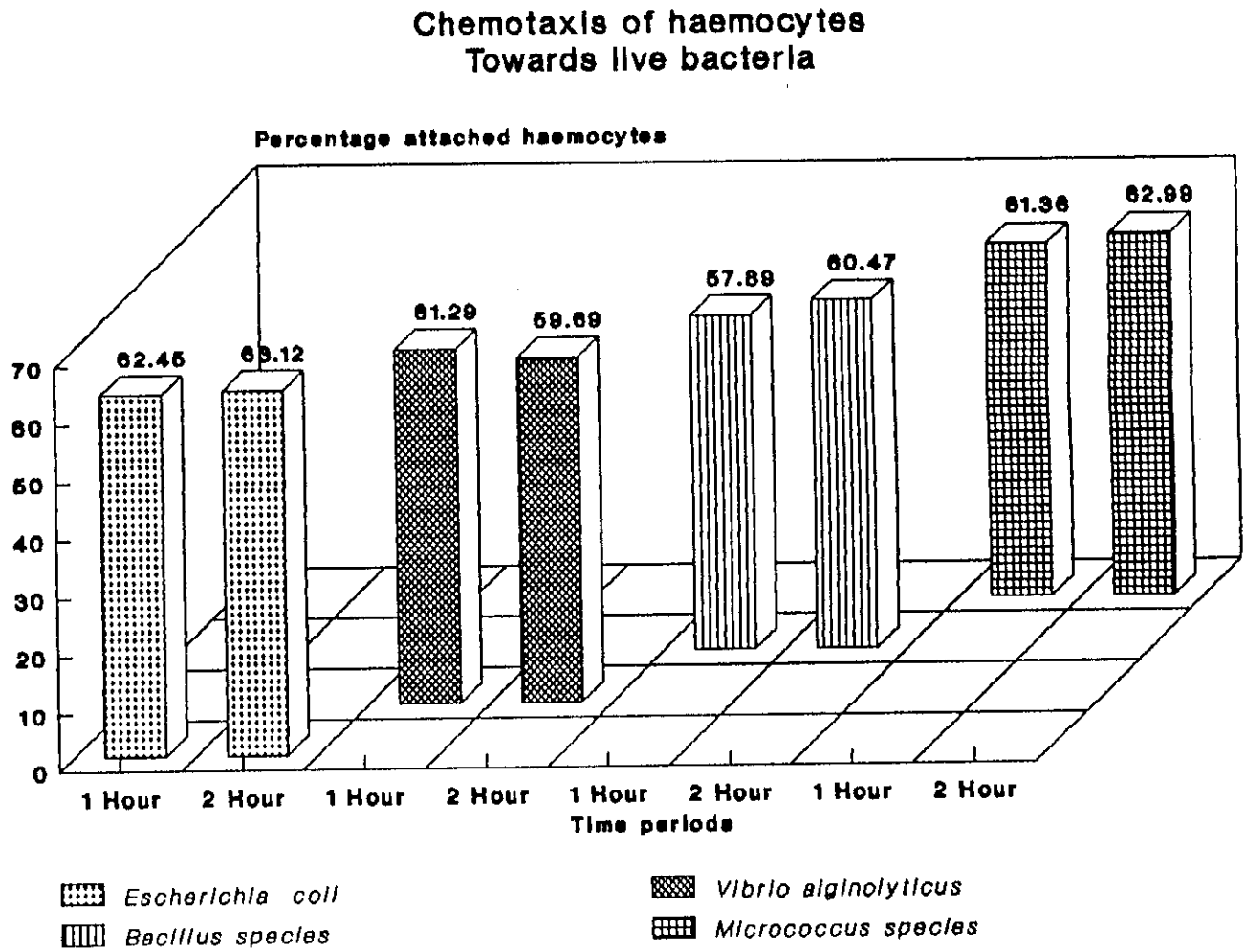

Chemotaxis of haemocytes
Towards live bacteria
Percentage attached haemocytes
62.45
61.29
59.69
57.89
60.47
61.36
62.99
70
60
50
40
30
20
10
0
1 Hour
2 Hour
1 Hour
2 Hour
1 Hour
2 Hour
1 Hour
2 Hour
Time periods
Escherichia coli
Bacillus species
Vibrio alginolyticus
Micrococcus species

FIGURE-2A

TABLE-2 B

Percentage of *Lamellidens marginalis* haemocytes with attached bacteria when tested against four different heat-killed bacterial strains at 1 and 2 hour periods.

	1 hr time-period				2 hr time-period			
	N	Mean	SD	Range	N	Mean	SD	Range
Escherichia-coli	12	28.91	3.36	23.53-36.67	12	41.75*	6.85	30.95-57.1
Vibrio-alginolyticus	12	30.27	5.38	18.84-36.14	12	38.78	6.95	27.16-51.4
Bacillus sp.	12	33.16	5.89	27.59-46.07	12	35.87	4.9	30.33-47.6
Micrococcus-sp.	12	28.81	4.61	20.62-36.21	12	28.12	3.73	21.16-33.0

* - $p<0.05$ Siginificantly higher than *Bacillus sp.* and *Micrococcus sp.*

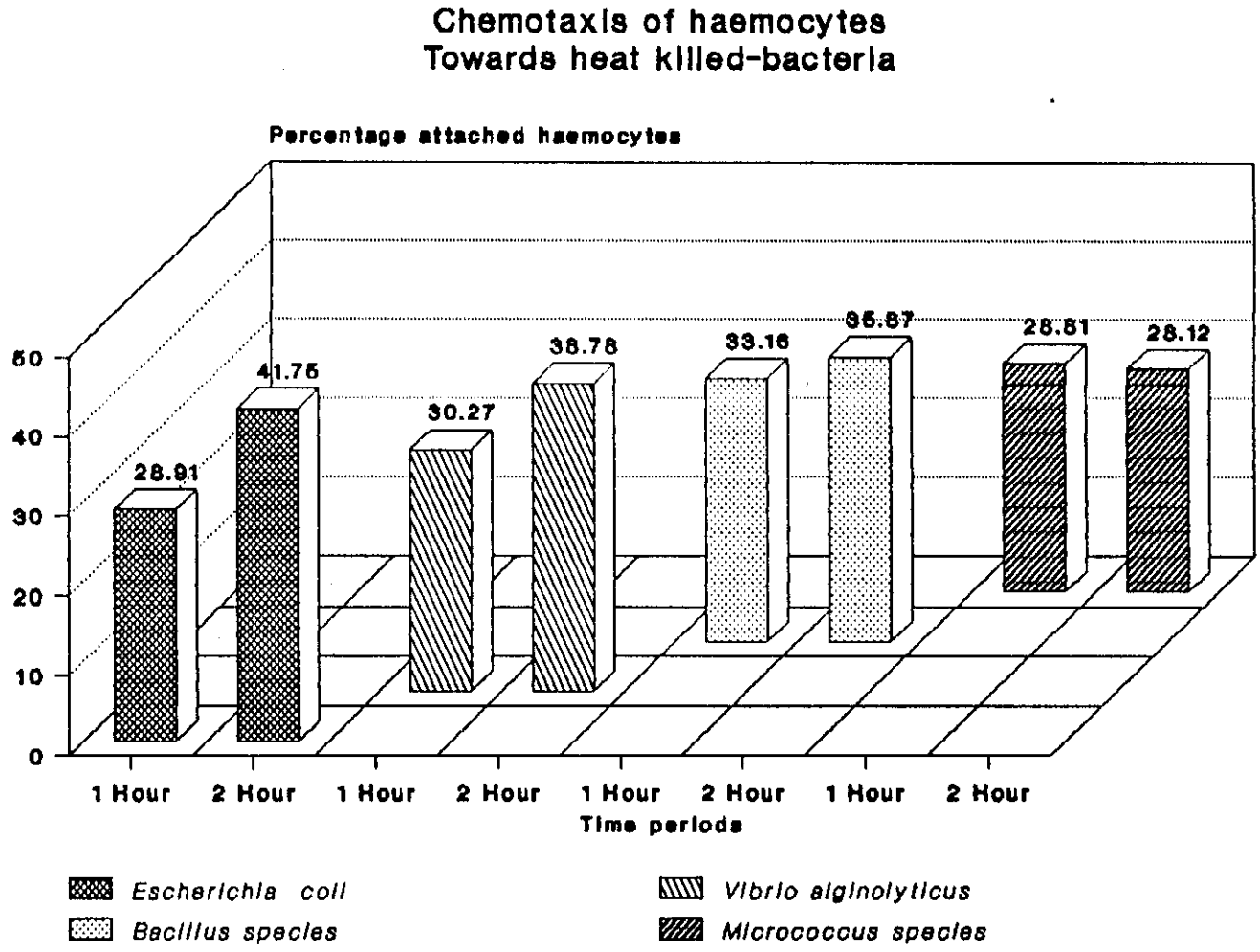

Chemotaxis of haemocytes
Towards heat killed-bacteria
Percentage attached haemocytes
50
40
30
20
10
0
28.81
41.75
30.27
38.78
33.16
36.87
28.81
28.12
1 Hour
2 Hour
1 Hour
2 Hour
1 Hour
2 Hour
1 Hour
2 Hour
Time periods
Escherichia coli
Bacillus species
Vibrio alginolyticus
Micrococcus species

FIGURE-2B

using 0.9% saline. Cell suspension in saline was adjusted to a concentration of 1.2×10^6 cells per ml by dilution technique.

2.3.2.3. ASSAY OF CHEMOTAXIS

At the end of 1 and 2 hour incubation periods the extent of chemotaxis was determined by random counting of atleast 100 spread haemocytes from each slide. The percentage of haemocytes exhibiting chemotaxis (chemotaxis index) was calculated as percentage of haemocytes with attached bacteria.

$$\text{Chemotaxis Index I} = \frac{\text{Number of haemocytes with attached bacteria}}{\text{Total Number of spread haemocytes counted}} \times 100$$

2.3.3.RESULTS

Table 2a,b and Figure 2a,b represents the average percentage of haemocytes in association with bacteria, live and heat killed, at 1 and 2 hour time-periods.

In the case of live strains sets, at 1 hour time-period, there was no significant difference amoung all the four strains. But at 2 hour time-period, *E.coli* showed significantly higher result than that of *V.alginolyticus* ($P<0.05$) but not with other strains.

In heat-killed bacteria-haemocyte association at 1 hour period there was no significant difference in the chemotaxis index amoung all the four bacterial strains. But at 2 hour time-period *E.coli* showed significantly higher chemotaxis index than those of *Bacillus sp.* and *Micrococcus sp.* ($P < 0.05$).

2.3.4. DISCUSSION

Chemotaxis of molluscan haemocytes towards different foreign substances is well known (Cheng et al.,1974; Schmid,1975; Cheng and Howland,1979; Kumazawa et al.,1992). Chemotaxis denotes the directional movement of cells in a chemical gradient (Cheng and Howland, 1979). Further there are reports of differential chemotaxis to different substances (Cheng and Howland,1979; Kumazawa et al.,1992). Attempts were made to relate the differences to the ability of haemocytes to distinguish between foreign matters, and host-parasite relationship, pathogenicity, receptor sites and impaired recognition (Sinderman,1970; Cheng et al.,1974; Cheng and Howland,1979; Mohandas et al.,1985; Kumazawa et al.,1990). Cheng et al. (1974) have reprted that there is chemotatic attraction between haemocytes of *Crassostrea virginica* and the parasitic metacercarial cyst of the trematode *Himasthala quissetensis* .

Schmid (1975) observed that haemocytes of *Viviparous malleatus* are attracted to heat killed *Staphylococcus aureus* (Gram positive), and N-acetyl-D-glucosamine a cell wall sugar compound (peptydoglycon) which forms part of the cell wall of *S.aureus*. Cheng and Rudo (1976a) have reported that *Crassostrea virginica* haemocytes are chemotactically attracted to *Micrococcus varience*. According to them, molluscan cells were attracted to live *Bacillus thurungiensis* and *Shigella soni* while the haemocytes were not attracted to *Micrococcus roseus*, *Staphylococcus aureus*, *Salmonella typhii*, *Pseudomonas aeruginosa and Vibrio parahaemolyticus* .

Cheng and Howland (1979) observed during chemotactic assay on *Crassostrea virginica* haemocytes with four bacterial strains (Gram positive) *Bacillus megaterium*, *Bacillus varience*, and (Gram negative) *Escherechia coli* and *Vibrio parahaemolyticus* that chemotactic attraction was higher to *E.coli*, *B.megaterium*,and to *B.varience* but was less to *V.parahaemolyticus*. Further, they also observed a lesser attraction towards heat-killed bacteria by all strains. From this observation they suggested that higher attraction towards live bacteria is due to some molecules emitted by living cells irrespective of Gram negative or positive nature of the strains. The lesser attraction to *Vibrio parahaemolyticus* was attributed to its pathogenicity to marine palecypods (Sinderman,1970). He also reasoned this with similar findings,

such as inability to phagocytose *Bacillus thurungiensis* by lepedopteran insects and the haplosporidan *Minchina nelsoni* by oysters. Abdul Salam and Michelson (1980a) found that the in vitro phagocytosis was directly proportional to the time of the incubation in the case of *Biamphalaria glabrata* haemocytes with formalized sheep erythrocytes.

Cheng and Howland (1982) found that inhibition by colchicine and cytochalasin B significantly reduced chemotaxis of *C.virginica* haemocytes towards live *B.megaterium*. They emphasized the need of intact cytoskeleton system for chemotactic response since cytochalasin B and colchicine are known to decrease the micro filament bundles and retraction of pseudopods, and inhibit microtubules assembly respectively. This, in turn, affects chemotaxis because microtubules stabilize the cell during chemotaxis in mammalian leukocytes (Cheng, 1981).

Further, Cheng (1985) has suggested that surface receptors at work in chemotaxis and those that bind the foreign particles are the same. Noda and Loker (1989) described in vitro phagocytosis activity of haemocytes of the snail *Biomphalaria glabrata* infected with *Echinostoma paraensei* towards fixed rabbit RBC, and sheep RBC and yeast (*Saccharomyces cerevisiae*) and found that infected snails showed lesser in vitro phagocytic activity than uninfected snails. According to them

thisimpaired recognition of haemocytes and the inhibitory effect of phagocytic activity to viable parasites. Further, they proposed that the parasite produced molecules which selectively interfere with recognition of parasite antigens or prevent the destruction of parasite by haemocytes.

Kumazawa et al. (1990) found that chemotactic activity of haemocytes of the estuarine gastropod *Clithon retropictus* towards live and heat killed *Vibrio parahaemolyticus* and *E.coli* strains was defective, where as marine gastropod *Nerita albicilla* showed greater chemotactic activity. They attributed this to direct correlation in survival and clearance rate of *V.parahaemolyticus* and *E.coli* in these estuarine and marine gastropods. However, they did not observe any pathological change or excessive proliferation of *V.parahaemolyticus* in *Clithon retropictus*. Kumazawa Shimiji (1991) compared the chemotactic activity of haemocytes from two neritid gastropods *Nerita albicilla and Heminerita japonica* to *V.parahaemolyticus* and *E.coli*. They found that both gastropods are chemotactic to the two strains in live condition. But the level of chemotaxis by *Heminerita japonica* to *E.coli* was lower than that of *V.parahaemolyticus*. This they attributed again to the clearance rate of *V.parahaemolyticus* within three days, and preservation of *E.coli* for atleast 14 days before elimination in these animals. Kumazawa and Morimotto (1992) found that chemotaxis of the haemocytes of clam *Corbicula japonica* to

V.parahaemolyticus was higher than that of *E.coli* in the presence of plasma and in C B S S (Chernin's Balanced Salt Solution). They suggested that the possibility of chemoattractant generated from *E.coli* might be lesser than that from *V.parahaemolyticus*.

Results of the present study and the time course adopted clearly indicate that the rate of chemotactic activity is proportional to time of incubation, and in turn showed the degree of recognition ability of haemocytes towards live bacterial strains. The sigificant variation in the degree of attachment of haemocytes with live and heat-killed *E.coli* can be attributed to the presence of specific molecules on live *E.coli* that induced the enhancement of chemotactic activity of haemocytes towards live and heat-killed *E.coli*.

Further, greater attraction towards both live and heat-killed *E.coli* compared to the two Gram positive strains at 2 hour post-challenge suggests the possibility of activation of specific recognition sites on the haemocytes membrane for greater attachment with *E.coli* strain. This in turn indicates the distinguishing ability of haemocytes to different foreign biotic substances. Hence, *E.coli* was selected for further studies. Further, element of ambiguity in the random collision of bacteria and haemocytes has been nullified since the assay was taken as an average of 12 animals for each set, and the

comparison was drawn based on these results analysed statistically. Secondly, all bacteria taken for assay are not motile (*Micrococcus sp.*).

2.4.1 ROLE OF SERUM/HUMORAL FACTORS IN CHEMOTAXIS

In marine bivalves humoral factors, agglutinins, lectins, and enzymes of lysosomal origin appear to be innate and nonspecific (Chu, 1988) though certain amount of ambiguity exists in the distinction between agglutinins and lectins in molluscan context.Many have mentioned the immunological role of lectins and agglutinins in molluscs (Hardy et al., 1977b; Lackie,1980; Warr,1981; Vasta et al., 1982; Cheng et al.,1984; Coombe et al.,1984). Chu (1988) generalized the roles of agglutins and lectins in defense mechanism as follows.

1. agglutinins and lectins inactivate bacteria or parasites by agglutination and may lead to lysis or endocytosis,
2. serum agglutinins and lectins serve as opsonins to link receptors with similar glycosyle moieties on the surface of nonself particles and haemocytes (Hardy et al.,1977b, Arimoto and Tripp,1977; Sminia et al., 1979; Van der Knaap et al., 1982).
3. agglutinins and lectins attach to haemocytes and function as cell surface recognition factors to bind nonself particle bearing appropriate glycosyle moieties (Yoshino et al.,1979; Cheng et al., 1980; Vasta et al., 1982)

2.4.2.MATERIALS AND METHODS

Method adopted was similar to the one described in 2.3.2.with minor changes. 1. The haemolymph samples from posterior adductor muscle were taken from each mussel. 2. A 0.2 ml. sample was expelled on each clean cover glass slides to which live *E coli* suspension was added immediately, and incubated for 1 hour at 32^{0}c. 3.To incubate the haemocyte and bacteria in the absence of serum the following method was adopted:
The haemolymph samples of 0.2 ml on glass slides were incubated for 10 minutes in a humidified chamber to allow the haemocytes to spread and adhere to glass. After 10 minutes the serum was drained and the slides were rinsed with iso-osmotic saline several times. It was then drained and iso- osmotic saline equal to the volume of serum was overlaid to the attached haemocytes. To this 0.1 ml of bacterial suspension was added as in the control (With-serum), and incubated for 1 hour at 32^{0}C.

2.4.3. RESULT

Table 3 shows the percentage of haemocytes with attached bacteria in presence of serum and in its absence. The result indicates that there is significantly higher haemocyte-bacteria association in the presence of serum than in its absence ($p < 0.001$).

TABLE-3

Percentage of haemocytes of *Lamellidens marginalis* with attached bacteria (*E.coli*)

	With serum	With saline
N	10	10
Mean	8.5049 *	3.095
SD	3.491	1.304
Range	3.00 - 14.00	1.00 - 5.00

* $p<0.001$ - significantly higher.

2.4.4.DISCUSSION

Two serum and one cell bound lectins have been discovered in the eastern oyster *Crassostrea virginica* (Vasta et al., 1982; Cheng et al., 1984). Each of these serum lectins was shown to have a distinct serological agglutination specificity. Cheng et al. (1984) postulated that during phagocytosis and encapsulation serum lectins facilitate attachment of haemocyte and nonself material through binding of appropriate sugar moieties.

Kumazawa and Shimoji (1991) reported plasma dependent chemotaxis of haemocytes of the gastropod *Clithon retropictus*. They found that haemocytes from adult gastropod were chemotactically attracted to *Vibrio parahaemolyticus* and *E. coli* strains. Chemotaxis was stimulated by plasma, in adult *C. retropictus* whereas juvenile specimen were attracted to *V. parahaemolyticus* and *E. coli* in the presence of plasma and only with *E. coli* and not with *V. parahaemolyticus* in the absence of plasma. Hence, they suggested the need of plasma factors to exhibit the chemotactic activity. Kumazawa and Shimoji (1991), based on their studies on two marine gastropods, *Nerita albicilla* and *Heminerita japonica*, reported the following findings. In *Nerita albicilla* both juvenile and adult showed plasma dependent chemotaxis towards *V. parahaemolyticus* and *E.coli* (live) but was reduced in C B S S.

In the case of *Heminerita japonica* though there was reduced chemotaxis towards *E. coli* compared to *V. parahaemolyticus* there appeared to be no enhancement in the rate of chemotaxis in the presence of serum.

In the brackish water clam *Corbicula japonica* Kumazawa and Morimotto (1992) found that haemocytes were attracted to *V parahaemolyticus* and *E coli* in CBSS but enhanced attraction was noticed in the presence of *Corbicula japonica* plasma. Further, they also found that the rate of activity was faster in the presence of plasma (90 minutes) whereas for CBSS incubated it took more time (180 minutes).

In the present the study enhanced association of haemocytes with *E.coli* in the presence of serum over saline-incubated ones shows the role of serum in recognition and chemotaxis of haemocytes towards live bacteria, *E coli*. Besides the active role of serum in chemotaxis, probably the serum factor is involved in mediation of receptor sites.

CHAPTER III

3.1. PHAGOCYTOSIS OF HAEMOCYTES (IN VIVO)

3.1.1.1 INTRODUCTION

Phagocytosis is a well known type of internal defense mechanism in molluscs (Stauber, 1950; Tripp, 1960; Feng, 1965b; Cheng, 1967, 1975, 1981; Bernard, 1989). It involves the uptake of foreign material by blood cells of the host, and protects the body tissues from the effects of reactions of such materials of both biotic and abiotic nature. To provide insight into the defense mechanism, investigators have utilised several basic experimental approaches-injection of particulate materials and soluble substances (antibodies) into the circulatory system, and monitoring their reaction and distribution with respect to time (Feng, 1967; Cheng, 1967; Foley and Cheng, 1975).

Most of the experimentally introduced substances are endocytosed over a period of time. But the fate of these phagocytosed substances vary. While degradation of digestible particles (certain bacteria) and micromolecules takes place within the haemocytes (Tripp, 1958 a, b, 1960; Feng, 1959, 1965b; Cheng, 1975; Cheng and Mohandas, 1985), indigestible particles and macromolecules are voided via migration of

foreign material-laden phagocytes across the mantle and epithelial borders (Stauber, 1950; Tripp, 1958a,1960; Feng,1965b; Feng and Feng, 1974).

It is further noted that differences prevail in the rate of phagocytosis and elemination of various foreign substances like bacteria and parasites (*Hexamita nelsoni*, and bacteriophage in *Crassostrea virginica*, and in echinostome infected *Biamphalaria glabrata*) (Feng 1966 a, 1967; Feng and Stauber, 1968; Noda and Loker,1989). It is also known that in vivo recognition capability of the host blood cells and the interference in the recognition process before endocytosis depend upon the pathogenicity of the invading organisms (Prytherch, 1940; Mackin, 1951; Michelson, 1961; Noda and Loker, 1989; Kumazawa et al.,1990).

There are reports of environmental factors such as temperature and salinity influencing haemocyte phagocytic activity (Feng, 1966a; Feng and Feng, 1974; Foley and Cheng, 1975; Fisher and Newell, 1986; Fisher and Tamplin, 1988). The direct influence of temperature on phagoctic activity was noticed by Fisher (1986). Hence it was tought to study the effect of temperature on the in vivo phagocytosis of live and heat-killed *E.coli* by haemocytes of *L.marginalis*, so as to throw some light towards better understanding of the environmental influence on internal defense.

3.1.2 MATERIALS AND METHODS

1. Specimens of *Lamellidens marginalis* were collected and maintained as described in chapter II. 2. One group of 30 specimens of *Lamellidens marginalis* was reared in each of the three tanks with water temperature of $22^{0}C$, $27^{0}C$ and $32^{0}C$ respectively for 24 hours and were fed with alga *Ocyctis pussila*. 3. One more group was set exactly in the same way, and in identical manner. 4. After 24 hours of maintenance the mussels of first group were injected with 0.02 ml. of live *E.coli* and the second group was injected with heat-killed *E.coli* suspension at the posteriar adductor muscle sinus. 5. The number of bacteria in both the suspensions was 1.03×10^{9} cells per ml (in 0.9% saline). 6. For injection of bacterial suspension, and for withdrawal of haemolymph, tuberculine syringe with No. 23 gauge needle was used. 6. After injection of bacterial suspension the animals were again maitained in the respective temperature for specified time-periods, in tanks. 7. At the end of 2, 4 and 8 hour period haemolymph samples were withdrawn and the number of phagocytosed haemocytes were counted.

3.1.2.2 PREPARATION OF HAEMOLYMPH SMEAR

1. At 2, 4, and 8 hour post-injection, a 0.3 ml. sample of haemolymph was withdrawn from posteriar adductor muscle of each

animal. 2. To previously cleaned, clear glass slides haemolymph sample from individual mussel was discharged and a thick smears made. 3. The slides were kept in a humidified chamber (as described in Chapter II), and incubated at $32^{0}C$ in a B.O.D. incubator for 30 minutes.

3.1.2.3. STAINING OF SMEARS

1. At the end the of incubation period the serum was drained. 2. The adhered haemocytes were fixed in 10% formalin for 2 minutes. 3. The fixative was drained and the slides were flooded with phosphate buffer ($Na\ H_2\ PO_4\ H_2O$, pH 7.0 ±0.2) 4. The slides were then stained with a few drops of crystal violet solution in the presence of buffer for 10 minutes. 5. The slides were again flooded with phosphate buffer washed,and air-dried. 6. The slides were prepared in the same way for both live, and heat-killed *E.coli* injected groups. 7. The haemocytes were counted randomly for the number of individual phagocytosed spread cells, under a powerful microscope (Nikon, Japan).

3.1.2.4. ASSAY OF PHAGOCYTOSIS

1. A minimum of 100 spread cells were counted from each slide. 2. The percentage of haemocytes with phagocytosed bacteria was calculated for each slide as and expressed as Phagocutic Index.

$$\text{Phagocytic Index} = \frac{\text{Number of phagocytosed haemocytes}}{\text{Total Number of spread haemocytes counted}} \times 100$$

3. The mean percentage of phagocytosed haemocytes was calculated for each test group at 2, 4 and 8 hour periods. 4. The data obtained were statistically analysed for possible significant differences .

3.1.3.0 RESULTS

Table 4 and Figure 3 represent the percentage of phagocytosed haemocytes in *L.marginalis* injected with live, and heat-killed *E.coli* at 2, 4 and 8 hour post-injection in temperatures 22^0C, 27^0C, and 32^0 C.

3.1.3.1 COMPARISON BETWEEN TEMPERATURE GROUPS

In experiments involving live *E.coli*, at 27^0 C, there was significantly higher percentage of haemocytes with phagocytosed bacteria at all time-periods (2, 4 and 8 hour) when compared to values at 22^0C ($p< 0.005$, $p<0.005$, $p<0.05$ respectively), and at 2 and 4 hour periods when compared with values at 32^0C ($p<0.05$, $p<0.005$ respectively).

TABLE-4

Percentage of invivo phgocytosis at different temperatures and time-periods in *Lamellidens marginalis* when injected with live and heat-killed *E.c* suspension.

		Live *E.coli*			Heat-killed *E.coli*		
		22°C	27°C	32°C	22°C	27°C	32°C
2 Hr	N	10	10	10	10	10	10
	Mean	3.533	9.930 ab	5.361	3.662	5.462	4.246
	SD	1.857	3.307	2.163	1.232	2.211	0.856
	Range	1.26-7.41	4.56-14.1	2.9-10.68	1.97-5.21	2.88-10.48	2.16-5.
4 Hr	N	10	10	10	10	10	10
	Mean	2.898	6.23 ab	3.095	3.392 x	4.934	2.793
	SD	1.394	1.682	1.483	0.974	1.359	1.466
	Range	1.42-4.71	3.97-8.8	0.94-5.66	2.28-5.02	2.73-7.13	0.99-6.
8 Hr	N	10	10	10	10	10	10
	Mean	2.606	3.711 a	2.927	3.079	3.972	3.973
	SD	0.901	1.199	0.848	1.161	1.466	1.007
	Range	1.48-4.02	2.3-6.25	1.28-4.19	1.36-5.44	1.72-7.2	2.5-5.8

a Significantly higher than the value at 22°C ($p<0.05$)

b Significantly higher than the value at 32°C ($p<0.05$)

x Significantly higher than the value at 22°C and 32°C ($p<0.05$)

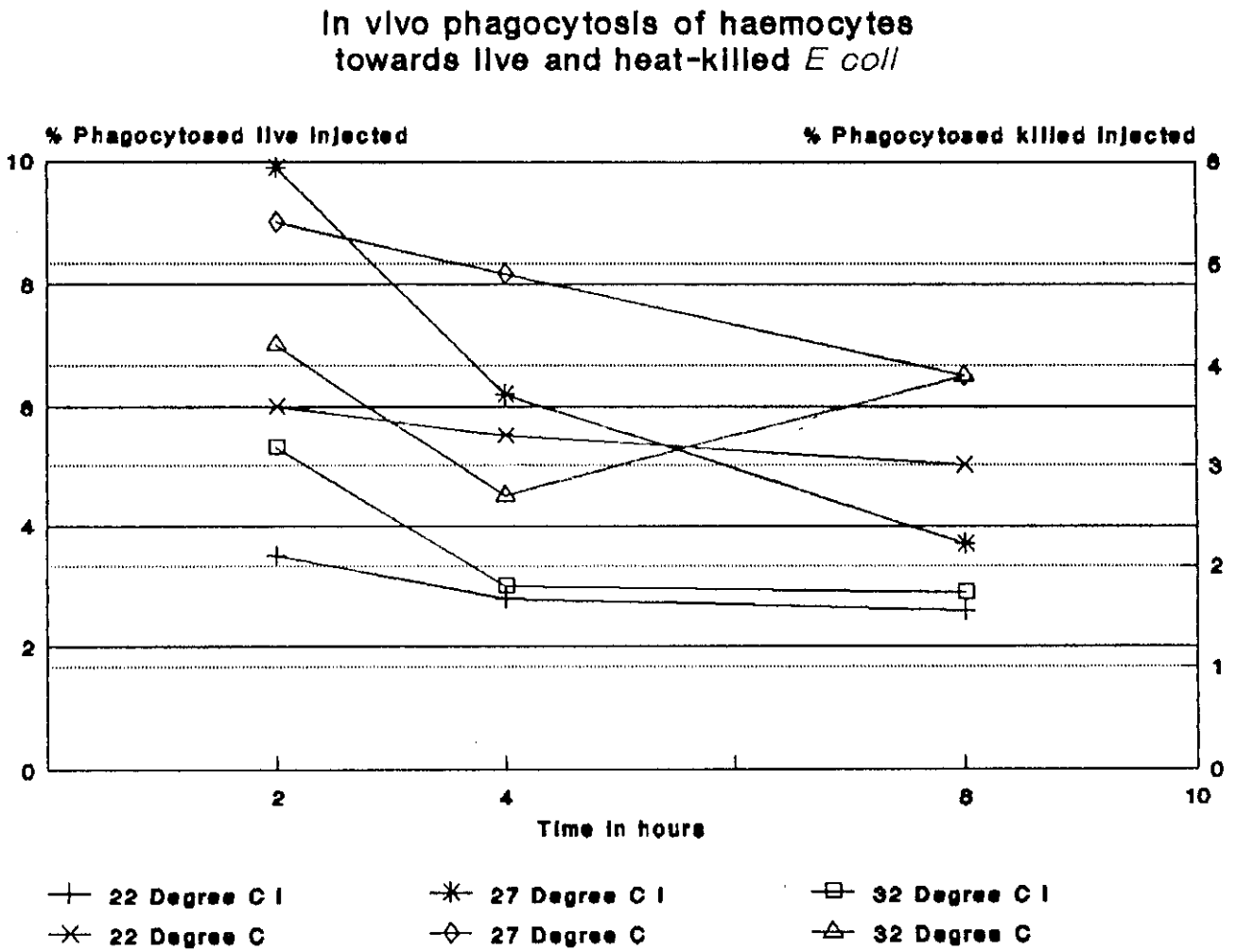

In vivo phagocytosis of haemocytes towards live and heat-killed E coli
% Phagocytosed live injected
% Phagocytosed killed injected
10
8
6
4
2
0
6
5
4
3
2
1
0
2
4
8
10
Time in hours
22 Degree C I
27 Degree C I
32 Degree C I
22 Degree C
27 Degree C
32 Degree C

FIGURE-3

In the case of heat-killed *E.coli* injected groups, at 27^0C there was significantly higher percentage of phagocytosed haemocytes at 4 hours when compared with values at 22^0C and 32^0C ($p<0.05$,) but not at other time-periods.

3.1.3.2 COMPARISON BETWEEN TIME-PERIODS WITHIN THE SAME TEMPERATURE REGIME

In the case of live *E.coli* injected mussels there was a general reduction in the percentage of phagocytosed haemocytosis with respect to time. At 27^0C, mussels at 2 hour post-injection showed significanly higher percentage of haemocytes than at 4 and 8 hour period ($p<0.05$, $p<0.005$ respectively) and the value at 4 hour post-injection was higher than the value at 8 hour post-injection ($p<0.005$). In the case of mussels at 32^0C, significantly higher percentage of phagocytosed haemocytes was observed at 2 hour time-period than at 4 and 8 hour time-period ($p<0.05$, $p<0.05$ respectively) but no significant difference in values between 4 and 8 hour periods. At 22^0C, there was no significant change in values in the three time-periods.

In studies involving heat-killed *E.coli* injected mussels also there was a general reduction in values with respect to time at all temperature regimes, except at 4 hour at 32^0C. Statistically, however, these changes in values were not significant.

3.1.3.3 COMPARISON BETWEEN LIVE AND HEAT-KILLED ONES AT ALL TEMPERATURES

At 22^0C there was no statistically significant difference in the percentage of phagocytosed haemocytes at all time-periods between live and heat-killed injected groups. At 27^0C,live injected mussels at 2 hour post-injection showed significantly higher percentage of phagocytosed haemocyts compared to heat-killed injected ones ($p<0.005$), but there was no significant change in the percentage at 4 and 8 hour post-injection. At 32^0C there was no significant change in values between live and heat-killed injected mussels at 2 and 4 hour periods, but at 8 hour heat-killed *E.coli* injected mussels showed significantly higher phagocytosed haemocytes than in live-injected mussels ($p<0.05$).

3.1.4 DISCUSSION

In vivo phagocytosis of foreign particles was first delineated by Stauber (1950) in *Crassostrea virginica*. He observed that by eighth day of post-injection emigration of phagocytosed heamocytes commenced through arterial walls and epethelia. Similar results were also observed in *C.virginica* injected with bacteria, yeast, and erythrocytes (Tripp,1958a, 1960; Feng and Feng, 1974). There are also similar reports in gastropods *Littorina scabra* (Cheng et al., 1969; Chorney and Cheng,1980),

in *Helix pomatia* (Bayne and Kinne, 1970), and in the bivalve *Tridacna maxima* with carbon particles (Reade and Reade, 1972)

Further it was also found that the rate of cardiac turbulance influences the number of circulating haemocytes during feeding and excretion cycles, and temperature on the rate of clearance of foreign particles by haemocytes with respect to time, in *C.virginica* (Feng,1965a;J.S.Feng, 1966; Feng and Feng, 1974). In *Biamphalaria glabrata*, it was reported that the maximum phagocytic activity of haemocytes occured at 30^{0}C, decreased at elevated temperature of 37^{0}C and at lower temperature of 22^{0} C, but was inhibited below 15^{0}C (Abdul Salam and Michelson, 1980a).

Thus, it is clear that temperature influences in vivo phagocytic activity. This influence of temperature on phagocytic activity was attributed to, atleast, in part to its effect on haemocyte locomotion which has a Q 10 of about 2 (Fisher, 1986). Further, it was also reported that lower particle clearance, in vivo, at lower temperatures in bivalves was due to decreased ability of haemocytes to locomote and phagocytose heterolougous materials (Bang, 1967), and continuous loss of phagocytic cells loaded with bacteria to the exterior through mantle epithelia (Houser, 1964; Cheng et al., 1969; Malek and Cheng, 1974). Fisher and Tampline (1988) have reported that in vitro activities of haemocytes

(locomotion and foreign particle binding) were faster in oysters that were collected from warmer eustuarine areas (25^{0}C and salinity 13 ppm) than from oceanic lower temperature habitate (24^{0}C and 33 ppm). Thus, besides temperature salinity also influences haemocyte activities.

Uptake and elimination of coliform bacteria, *E.coli*, by four marine bivalves have been studied by Bernard (1989). He reported that in all the four bivalves, *Mytilus edulis*, *Crassostrea gigas*, *Protothaca staminea*,and *Mya arenarea* there was a positive correlation between coliform accumulation and temperature rise. Further, he observed that bacterial accumulation by bivalves was rapid initially and declined after 3 to 4 hour. He suggested that a plateau phase may be reached, where uptake is equivalent to digestion and pseudo-feacal elimination.

In the present study also the initial rise (at 2 hour post-injection) in the percentage of phogocytosed haemocytes denotes their response to foreign substances and the primary role of internal defense. The higher percentage at 27^{0}C for both live and heat-killed bacteria injected mussels indicates the optimum temperature for maximum (threshold) activity,and the reduction at the later time-period suggests the scarcity of bacteria since much of them were phagocytosed immediately after injection. The lesser percentage of phagocytosed haemocytes

at 32^{0}C compared to that at 27^{0}C may be attributed to faster haemolymph circulation at elevated temperature in these animals.

Since bivalves are poikilotherms the elevated temperature might have increased the heart pumping rate (Q 10) which inturn reflects the faster blood circulation. This could result in either the bacteria being carried away in the blood flow as well as bacteria-laden haemocytes from the injection site.

Further, the discripancy in the percentage of chemotaxis in vitro with the *E.coli*, and the percentage of phagocytosis in vivo with the *E.coli* may be due to the following factors.
1. In the in vitro studies the bacteria-haemocyte ratio is known but in in vivo study the ratio is unknown and the volume of haemolymph and number of haemocytes are larger. In other words, in in vivo study for limited quantity of bacteria there are unlimited number of haemocytes, and obviously the number of bacteria availble for each haemocytes is very limited. Moreover, chemotactic attraction need not necessarily end up in phagocytosis . 2. There are also reports of failure to spread and attach to glass by haemocytes that have already phagocytosed (Noda and Loker, 1989). This, though not estasblished in *L.marginalis*, could also amount to significant loss of haemocytes in the counting procedure.

3.2.0 HEAMOLYMPH GLYCOGEN

3.2.1 INTRODUCTION

Bivalve heamocytes are involved in a wide range of physiological functions such as digestion, excretion, and transport besides defense (Narain, 1973; Bayne et al., 1980; Cheng, 1981; Feng, 1988). In many cases all these process are closely interconnected (Cheng, 1981). It is established cytochemically that digestible particles are degraded intracellularly within oyster heamocytes, and converted into glucose and glycogen granules through unknown pathway (Cheng, 1975, 1981, 1983a) and are subsequently released into serum. Further, there is also quantitative evidence that the amount of glycogen in the heamolymph of bacteria phagocytosed oyster *C.virginca* (Cheng, 1975) is higher than that in the normal untampered animals.

Hence an assay of haemolymph glycogen in bacteria-challenged mussels would indirectly indicate the fate of phagocytosed substances, and the digestibility of the same by haemocytes.

3..2.2.1 MATERIALS AND METHODS

1. Specimens of *L.marginalis* were collected as described in Chapter II, and acclimated. 2. Fifty specimens were injected

with live suspension of *E.coli* and another fifty with suspension of heat-killed *E.coli*. 3. Identical number of saline-injected, and untampered controls were also maintained.

3.2.2.2 CHALLENGING ANIMALS WITH BACTERIAL SUSPENSION

1. A 0.02 ml. of bacterial suspension of live, and heat-killed *E.coli* and 0.9% saline were injected into the posterior adductor muscle sinus of each mussel of the respective groups. 2. The bacteria in the suspension was 1.03×10^9 per ml. for both live and heat-killed *E.coli*. 3. Heamolymph samples were collected at 2, 4, 8, 12 and 24 hour post-injection with a tuberculine synringe fitted with No.23 needle, for glycogen estimation.

3.2.2.3 ESTIMATION OF GLYCOGEN

1. Glycogen in the heamolymph was estimated following the method of Montgomery (1957) after deproteinising the sample with 10% TCA. 2. For this, to 1 ml. of 10% TCA, 0.2 ml. of haemolymph from each mussel was pippeted and mixed. 3. It was then centrifuged at 2500 r.p.m. for 15 minutes. 4. The supernatant was gently decanted into another centrifuge tube for assay. 5. For determination of glycogen, 1.2 ml. of 95% ethyl alcohol added and mixed. 6. It was then kept over night in a refrigerator and then centrifuged at 2500 r.p.m. for 15

TABLE-5

Glycogen (µg/ml) in the haemolymph of *Lamellidens marginalis* at different time-periods when challenged with live and heat-killed suspension of *E. co*

		2 hr	4 hr	8 hr	12 hr	24 hr
Un-tampered	N	10	10	10	10	10
	Mean	16.0	12.0	20.4	24.0	22.8
	SD	7.469	7.469	10.37	14.879	11.556
	Range	6-27	3-24	12-42	9-60	12-45
Saline injected	N	10	10	10	10	10
	Mean	6.0	14.7	21.3	15.6	26.7
	SD	4.242	7.28	7.40	9.656	15.72
	Range	3-18	3-24	12-33	6-39	6-51
Live *E.coli* injected	N	10	10	10	10	10
	Mean	12.0	18.8 x	26.3	15.9	12.9
	SD	7.937	5.440	18.480	8.800	9.585
	Range	3-27	9-27	9-66	6-36	3-39
Heat-killed *E.coli* injected	N	10	10	10	10	10
	Mean	12.3	18.7 x	18.6	18.3	20.7
	SD	7.523	5.529	12.059	8.637	8.100
	Range	3-27	3-27	6-42	9-30	6-33

x Significantly higher than control ($p<0.05$).

Haemolymph glycogen in
Lamellidens marginalis **exposed to**
live and heat-killed *E coli*

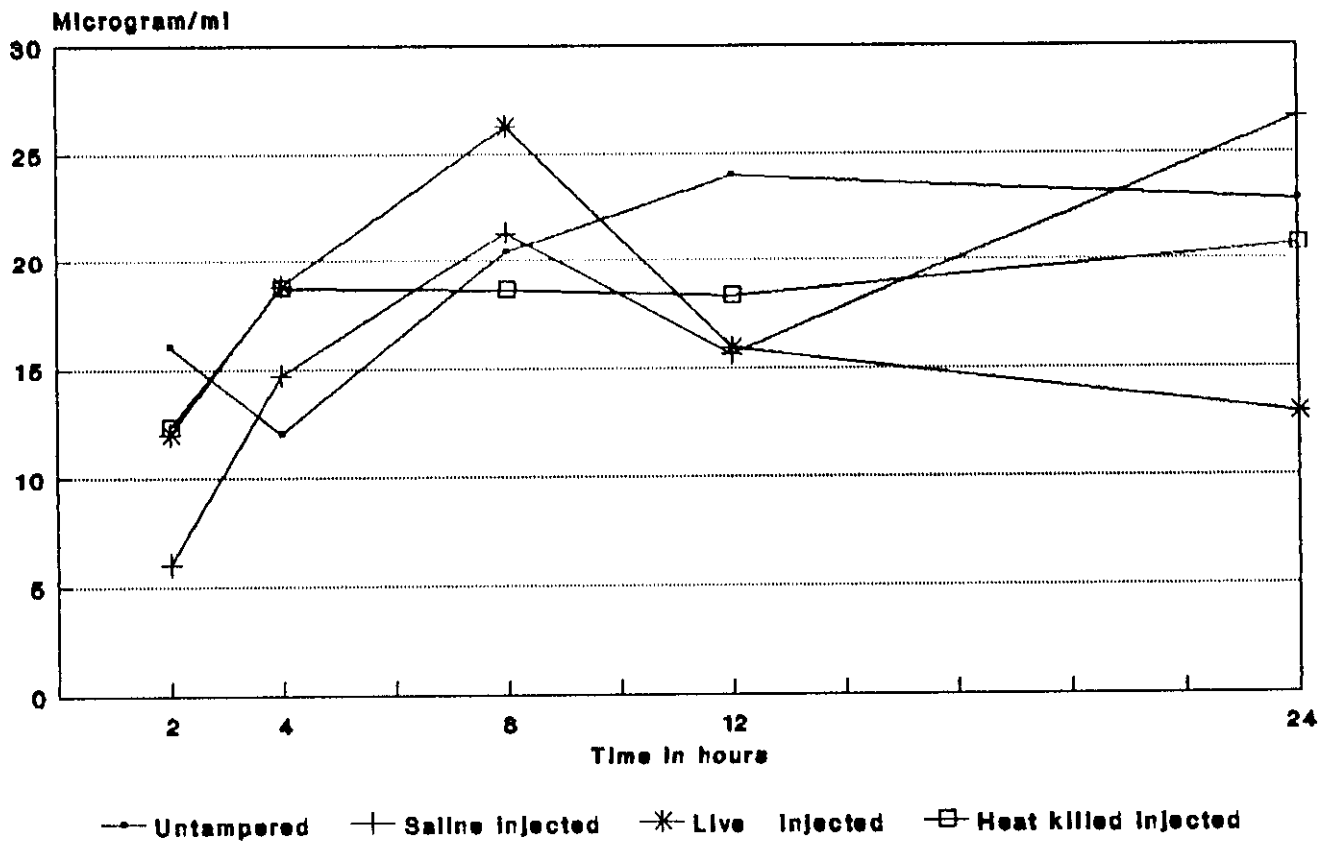


FIGURE-4

minutes. 7. The supernatant was carefully decanted. 7. To the precipitate 2 ml. of distilled water and 0.1 ml. of 80% phenol were added and mixed well. 8. To this 5 ml. of concentrated sulphuric acid was added forcefully to aid mixing. 9. It was then left at room temperature for 30 minutes. 10. After cooling the optical density was read at 490 nm. 11. The concentration of glycogen in the samples was found out from standard graph prepared by employing oyster glycogen (Sigma Company USA) as standard.

3.2.3 RESULTS

Table 5, Figure 4 gives the mean value, standard deviation, and range of heamolymph glycogen in the four groups of animals at 2, 4, 8, 12 and 24 hours post injection. On comparing haemolymph glycogen levels between groups the following results were observed.

At four hour period live and heat-killed injected animals showed significantly higher haemolymph glycogen compared to untampered ones ($p<0.05$,), but not with saline injected animals. At all other time-periods there was no significant change between test groups and control.

On comparing time-periods within each group, the following observations were made. In the case of untampered specimen

there was no significant difference in value among the time-periods. But in saline-injected animals value at 2 hour time-period was significantly lower than those of all other time-period ($p<0.05$). In the case of live-injected animals value at 2 hour time-period was significantly lower than the value at 8 hour-period ($p<0.05$), and in the case of heat-killed injected animals, value at 2 hour time-period was significantly lower than the value at 24 hour ($p<0.05$).

3.2.4.DISCUSSION

Degradation of foreign particles by haemocytes is well documented and also the rate of degradation (Cheng, 1975; Cheng and Rudo,1976b; Rodrick and Ulrich, 1984). Cheng (1975) reported significant rise in heamolymph glycogen in *C.virginica* at 24 hour post-challenge with *E.coli* (in vivo), and in the case of *Mytilus campechiensis*, *C.virginica*, and *Anadora ovalis* at 1 hour post-injection with *E.coli* and *Vibrio anguillarum* (Rodrick and Ulrich,1984). It was also demonstrated in *C.virginica* that degradation of ^{14}C labelled *Bacillus megaterium* by phagocytic haemocytes leads to synthesis of glycogen from sugar of bacterial origin, and its release from phagocytes (Cheng and Rudo, 1976b; Cheng,1977b). This was based on the detection of ^{14}C activity in the glycogen extracted from sera at 24 hours post-injection. Electron microscopic study on the heaemocytes of *C.viginica* (Cheng and

Cali, 1974) and in *Mercenaria mercenaria* (Mohandas, 1985) have revealed that subsequent to bacterial challenge they are degraded in the phagosomes by the lysosomal enzymes, and glycogen thus formed is released into serum.

Suresh (1988) also established cytochemically the presence of glycogen granules in bacteria (*Vibrio sp.*) phagocytosed heamocytes, and proved through biochemical studies of the increased haemolymph glycogen levels in bacteria injected bivalves. In the present study the higher glycogen level in both live and heat-killed *E.coli* injected animals can also be attributed to the synthesis of glycogen from the degradation of challenged bacteria.

3.3.0. HAEMOLYMPH PROTEIN

3.3.1 INTRODUCTION

In molluscs, serum protein plays a major role in immunity, homeostasis, osmolality, transport, and detoxication (Simkiss and Mason, 1983; Cheng, 1986). It has been reported that the circulatory fluid of bivalves contains specific proteins which are responsible for lysis of bacteria, and intracellular degradation of foreign substances (Tripp,1960;Cheng et al., 1966). These specific proteins or lysosomal enzymes available in the haemolymph are of cellular origin and inducible (Cheng, 1983 a, b, c; Mohandas, 1985). Since, there is no report of existence of antigen-antibody system in bivalves, the primary reactions to non-self material is in the form of encapsulation and phagocytosis by haemocytes, resulting in the release of lytic enzymes into serum.

In the present study haemolymph protein was assayed in mussels challenged with bacteria and the results are presented.

3.3.2 1 MATERIALS AND METHODS

Methods of collection of mussels, rearing,acclimation, and the method of challenging the mussels with bacteria both live and heat-killed were the same as described in part 3.2.2.

Haemolymph was collected from the mussels of the untampered, saline, live and heat-killed injected ones at 2, 4, 8, 12, and 24 hour post-challenge.

3.3.2.2. ESTIMATION OF PROTEIN

1. Estimation of protein was done following the method of Lowry et al.(1951). 2. One ml. of haemolymph was withdrawn from posterior adductor muscle sinus by a tuberculine syringe fitted with No.22 gauge needle of each mussel, and expelled into separate test tubes. 3. A 0.2 ml. of sample of the expelled haemolymph was then pippeted into a centrifuge tube containing 1 ml of 10% TCA. 4. It was shaken well and centrifuged at 2500 r.p.m. for 15 minutes. 5. The supernatant was carefully decanted out. 6. The precipitate was dissolved in 1 ml. of 0.1 N NaOH. 7. To this, 5 ml of alkaline copper reagent was added and shaken well. 8. After 15 minutes, 0.5 ml. of Folin's phenol reagent was added and shaken well. 9. After 45 minutes, the optical density and the corresponding concentrations were found out from standard graph employing bovine serum albumin as standard.

3.3.3. RESULTS

Table 6, Figure 5 represent the mean value, standard deviation, and range of haemolymph protein in the four groups of animals

TABLE-6

Protien (μg/ml) in the haemolymph of *Lamellidens marginalis* at different time-periods when challenged with live and heat-killed suspension of *E. coli*

		2 hr	4 hr	8 hr	12 hr	24 hr
Un-tampered	N	10	10	10	10	10
	Mean	381	413	455	494	561
	SD	83.12	114.19	126.35	80.27	114.14
	Range	260-560	270-730	260-680	370-620	400-780
Saline injected	N	10	10	10	10	10
	Mean	468	495	458	499	659
	SD	128.90	213.41	69.25	137.29	164.28
	Range	320-790	260-1030	330-560	330-710	470-1070
Live *E.coli* injected	N	10	10	10	10	10
	Mean	420	524	455	523	582
	SD	185.09	121.34	124.11	91.43	109.61
	Range	130-840	370-760	130-580	370-730	360-720
Heat-killed *E.coli* injected	N	10	10	10	10	10
	Mean	487	466	477	495	664
	SD	130.69	88.11	140.36	159.51	142.28
	Range	180-610	310-560	270-730	110-710	450-930

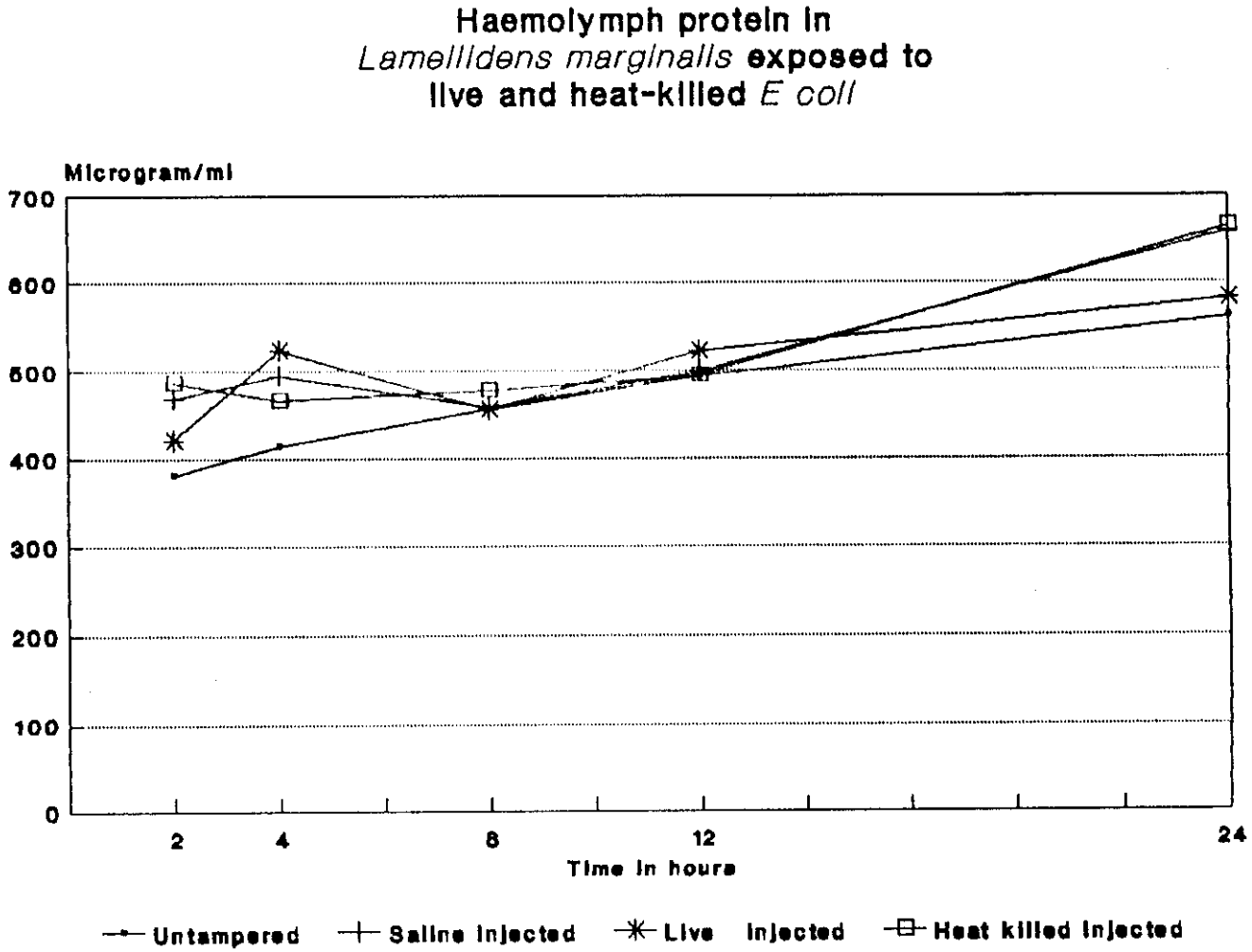

Haemolymph protein in
Lamellidens marginalis exposed to
live and heat-killed E coli
Microgram/ml
700
600
500
400
300
200
100
0
2
4
8
12
24
Time in hours
Untampered
Saline injected
Live injected
Heat killed injected

FIGURE-5

at 2, 4, 8, 12, and 24 hour post-challenge.

From the data it is found that statistically there was no significant variation in the protein content between the experimentals and the control at all time-periods.

3.3.4 DISCUSSION

In bivalves it is found that specific proteins that are responsible for the lysis of foreign susbstances such as bacteria, protein and other degradable macromolecules are lysosomal enzymes (Cheng,1983a; Cheng and Mohandas,1985; Chu,1988). Availability of such enzymes both in haemolymph and haemocytes is reported but seldom expressed as total proteins.

In vertebarates, it is well established that the antigen-antibody system is well developed. The antibodies sysnthesised are specific and precepitable from serum. These antibodies are proteins and quantitative procedures for measuring proteins could be applied to the estimation of precipitated antibodies also. They increase with increase in antigen titre (Davis et al.,1980). But it is widely accepted that invertebrates, including molluscs, do neither synthesis immunoglobulins nor have the complement system, and hence internal resistance is not based on antigen-antibody interaction. Suresh (1988) observed no change in the haemolymph protein of clams

Sunetta scripta, and *Villorita cyprinoides var cochinensis* injected with *Vibrio sp.*

In the present study also, no change in protein level in the blood can be attributed to the absence of antigen-antibody system in *L.marginalis*. Also, the changes in the lytic enzyme constituent as a measure of protein in the blood would be clear only if one examined the blood protein level in both the serum and the haemocytes separately. Further, the non-significant increase in the protein level in live *E.coli* injected animals at four hour may be due to the migration of more haemocytes to the injected sites for internal defense. If this could be, then, such an increase in live *E.coli* injected mussels only substantiates the activity of heamocytes in internal defense.

CHAPTER IV

HAEMOLYMPH ENZYME ACTIVITY.

4.1.0 INTRODUCTION

The role of haemolymph lysosomal hydrolases in bivalves is generally presumed to be involved in host defense and digestion (Cheng,1983a;Cheng and Mohandas,1985; Chu, 1988). Their activity (along with cell mediated immunity) to invading parasites and microorganisms is of considerable interest. Hence, estimating haemolymph enzyme activity levels in bivalves may allow us to infer the resistance potential or tolerance to infections and diseases by these animals.

The presence of many hydrolytic enzymes such as acid and alkaline phosphatases (ACP and ALP, respectively), amino-peptidase, amylase, lipase, lysozyme, and β-glucuronidase in the haemolymph has been established by both histochemical and biochemical studies in many molluscs (Cheng et al.,1975, 1978;Cheng,1976b; Cheng and Rodrick, 1974, 1975; Cheng, 1977a; Yoshino and Cheng, 1976a,b; Foley and Cheng 1977; Cheng and Garrabrant, 1977; Cheng, 1980; Cheng and Mohandas, 1985). Jeong and Heyneman (1976), and Yoshino and Cheng (1976a) have demonstrated that the hydrolysing enzymes are restricted to lysosomal granules of molluscan haemocytes.

These lysosomal enzymes are known to be active both inside the haemocytes and in the serum (Rodrick and Cheng,1974b; Cheng and Rodrick, 1974; Cheng et al., 1975). Inside the haemocytes they become active after fusing with phagosomes and cause the lysis or digestion of the phagocytosed substance (Cheng, 1975). Further, it was also established that haemocytes represent a major site of lysosomal enzymes synthesis (Foley and Cheng,1977; Cheng, 1983 c; Mohandas,1985). Under normal conditions these hydrolases are restricted within the lipoprotein lysosomal membrane in the latent phase in the form of granules (Yoshino and Cheng, 1976a). If challenged by biotic or abiotic substances, the lysosomes are destabilised and enzymes are either released into the cytoplasm of cell resulting in intra-cellular lysis (Moore et al., 1977; Cheng, 1980), or released from cells into serum through the process of degranulation (Cheng et al.,1975; Cheng and Yoshino, 1976a,b; Cheng, 1983a; Cheng and Mohandas, 1985; Yoshino and Cheng, 1976b; Foley and Cheng, 1977; Mohandas,1985) resulting in extra-cellular digestion or lysis. Thus lysosomal enzymes availability in the serum is effected.

Further, variation in the synthesis and release pattern of these lysosomal enzymes also have been noted when animals are challenged with foreign substances (Cheng,1983a; Cheng et al., 1977; and Cheng and Mohandas, 1985). It has been found that

the activity of enzymes also varies (incited or suppressed) (Rodrick and Cheng 1974b;Lie,1977) depending on the bacteria or parasites challenged, and their pathogenicity and chemical configuration. This variation in enzyme activity or in synthesis was attributed to the qualitative and quantitative differences in the recognition sites available on the haemocytes membrane (Cheng, 1983a; Cheng and Mohandas, 1985; Mohandas and Cheng, 1985a,b).

Of the reported lysosomal hydrolytic enzymes, acid phosphatase (ACP) is one of the widely acknowledged marker enzymes (Yoshino and Cheng,1976a; Cheng and Mohandas, 1985), besides its hydrolysing character. Since elevated lysosomal enzyme levels in haemolymph are believed to be a response to many changes induced by parasites and microbes, and known to play a prominent role in internal defense, in the present study the activity pattern of haemolymph ACP was chosen as an indicator of lysosomal disruption caused by of different doses bacterial challenge (*E.coli*) in *L. marginalis* and measured at different time-periods.

Secondly, the lysosomal system, in general, has been shown to be very sensitive to changes in the intra-and-extra-cellular environment, and their involvement in regulating the changes amounts to release of the enzymes either into cytoplasm or into serum. Plasma membrane, which forms the bounding membrane, is

likely to be the first target of such changes. Hence, alkaline phosphatase, which is considered as a marker enzyme for the plasma membrane destabilisation (Bogitsh, 1974), and also known to be involved as catalyst in the hydrolytic cleavage of phosphoric esters with the pH optimum in the alkaline range, has also been chosen for the present study. Further, it is also treated as one of the lysosomal enzymes (Cheng and Rodrick, 1975; Huffman and Tripp, 1982).

4.2.0 MATERIALS AND METHODS

Methods of collection of mussels, rearing of mussels, acclamation, size group selection, and statistical analysis of data were the same as described in Chapter II.

4.2.1.

1. A group of forty animals for each experiment was challenged with mild, and heavy doses of live and heat-killed *E.coli*, separately. 2. The assay was done at 2, 4, 8, and 24 hour post-challenge. 3. Saline injected and untampered controls were also maintained and assayed for all time-periods.

4.2.2. CHALLENGING BACTERIA (*E.coli*)

1. Two different concentrations, mild and heavy,of live and heat-killed *E.coli* suspensions were taken for the assay of ACP

and ALP.2. Mild dose suspension contained about 1.06 x 10^9 cells per ml.,while heavy dose suspension contained about 2.06 x 10^9 cells per ml. 3. The animals of the respective groups were injected with 0.02 ml of the respective suspensions at the posterior adductor muscle sinus.4. After 2,4,8, and 24 hour post-injection, the haemolymph was withdrawn and immediately taken for assay of ACP and ALP.

4.2.3. ESTIMATION OF ACID PHOSPHATASE ACTIVITY (ALP)

1. Acid phosphatase activity was determined following the methodology using Sigma Technical Bulletin No: 104 with some modifications. 2. To study the enzyme activity, 0.1 M citrate buffer of pH 4.0 was used. 3. The incubation temperature was $37^0 \pm 0.5^0$C. 4. To one ml of frozen buffer containing 100 µMole of NaCl, 0.1ml of whole haemolymph was added using 0.1 ml pipette, and immediately frozen till analysis. 5. At the time of analysis, the buffer-enzyme (haemolymph) mixture was kept in a water bath at 37^0C. 6. When the temperature of the buffer-enzyme mixture reached 37^0 C, 0.1ml of the substrate (2 mg of para-nitrophenyl phosphate Sodium salt (Merk) in 0.1 ml. distilled water) was added using a 0.1 ml. pipette to start the reaction. 7. After incubating for 1 hour at 37^0C the reaction was stopped by adding 2 ml. of 0.25 NaOH. 8. The yellow colour of para-nitrophenol in the alkaline medium was read at 410nm in Hitachi-U 2000 UV-Vis Spectrophotometer. 9. The concentration

of para-nitrophenol formed was found from the standard graph prepared for para-nitrophenol. 10. From this, μMoles of para-nitrophenol liberated per ml. of haemolymph per minute was calculated.

4.2.4. ESTIMATION OF ALKALINE PHOPHATASE ACTIVITY (ALP)

1. Alkaline phosphatase activity was determined following the methodology given in Sigma Technical Bulletin No: 104 with some modifications. 2. To study the enzyme activity, 0.05 M glycine-NaOH buffer of pH 9.6was used. 3. The incubation temperature was $37^0 \pm 0.5\ ^0C$. 4. To one ml of frozen buffer containing 100 μMole of NaCl and 0.1 mg of $MgCl_2$, 0.1ml of whole haemolymph was added using 0.1 ml pipette, and immediately frozen till analysis. 5. At the time of analysis the buffer-enzyme (haemolyhmph) mixture was kept in a water bath at 37^0 C. 6. When the temperature of the buffer-enzyme mixture reached 37^0C, 0.1ml of substrate (2 mg of para-nitrophenyl phosphate Sodium salt (Merk) in 0.1 ml. of ditilled water) was added using a 0.1 ml. pipette to start the reaction. 7. After incubating for 1 hour at 37^0 C the reaction was stopped by adding 2 ml. of 0.25 NaOH. 8. The yellow colour of para-nitrophenol in the alkaline medium was read at 410 nm in Hitachi-U 2000 UV-Vis Spectrophotometer. 9. The concentration of para-nitrophenol formed was found from the

standard graph prepared for para-nitrophenol. 10. From this, μMoles of para-nitrophenol liberated per ml. of haemolymph per minute was calculated.

4.3.0 RESULTS

4.3.1 Acid phosphatase activity in the haemolymph of *L.marginalis* challenged with *E.coli* at different time-periods is given in Table 7, Figure 6.

Table gives the n, mean, standard deviation, and range of haemolymph acid phosphatase activity in animals exposed to mild and heavy doses of live, and heat-killed *E.coli* at 2, 4, 8, and 24 hour post-injection, and also in saline injected and untampered control specimens.

The Figure-6 outlines the general trend in the ACP activity pattern at all time-periods for all test groups.

The results of the experimental group were compared with those of saline and untampered controls for all time-periods, and also between time-periods in each test group. The result indicated that there was no statistically significant difference in the enzyme activity between untampered, saline, and both mild and heavy dose of heat-killed injected groups at all time- periods. In the case of both live mild and live heavy dose injected specimens significantly lower activity was

TABLE-7

Acid phosphatase activity (μ moles/ml haemolymph/minute) pattern in the haemolymph of *Lamellidens marginalis* challenged with mild and heavy dose of live and heat-killed *E. coli* suspension at different time-periods.

		2 hr	4 hr	8 hr	24 hr
Un-	N	10	10	10	10
tampered	Mean	1.976	1.966	1.6609	1.4415
	SD	0.5669	1.1619	1.381	0.654
	Range	1.266-2.888	0.833-4.222	0.333-5.110	0.721-2.722
Saline	N	10	10	10	10
injected	Mean	2.042	1.894	1.702	1.855
	SD	2.0327	0.855	0.9699	0.988
	Range	0.600-7.666	0.777-3.722	0.443-3.333	0.666-3.888
Live	N	10	10	10	10
E.coli	Mean	2.472	1.019 [a]	1.185	2.360 [b]
injected	SD	0.7896	0.674	1.0168	0.432
(mild dose)	Range	0.944-3.721	0.1667-2.277	0.2217-2.722	0.610-2.000
Live	N	10	10	10	10
E.coli	Mean	1.379 [a]	1.111 [a]	1.707	2.255 [b]
injected	SD	0.440	0.360	0.914	0.654
(heavy dose)	Range	0.722-2.111	0.7217-2.055	0.333-3.443	1.111-2.943
Heat-killed	N	10	10	10	10
E.coli	Mean	2.982	1.468	1.557	1.909
injected	SD	1.356	1.199	1.263	1.602
(mild dose)	Range	1.50-4.943	0.277-3.777	0.388-4.166	0.333-5.444
Heat-killed	N	10	10	10	10
E.coli	Mean	1.836	1.875	1.899	2.299
injected	SD	0.772	0.529	1.240	1.438
(heavy dose)	Range	1.00-3.110	0.333-5.222	0.444-4.388	0.388-5.944

[a] Significantly lower than untampered ($p<0.05$).

[b] Significantly higher than untampered ($p<0.05$)

Haemolymph ACP
Lamellidens marginalis **injected with**
live and heat-killed *E coli*

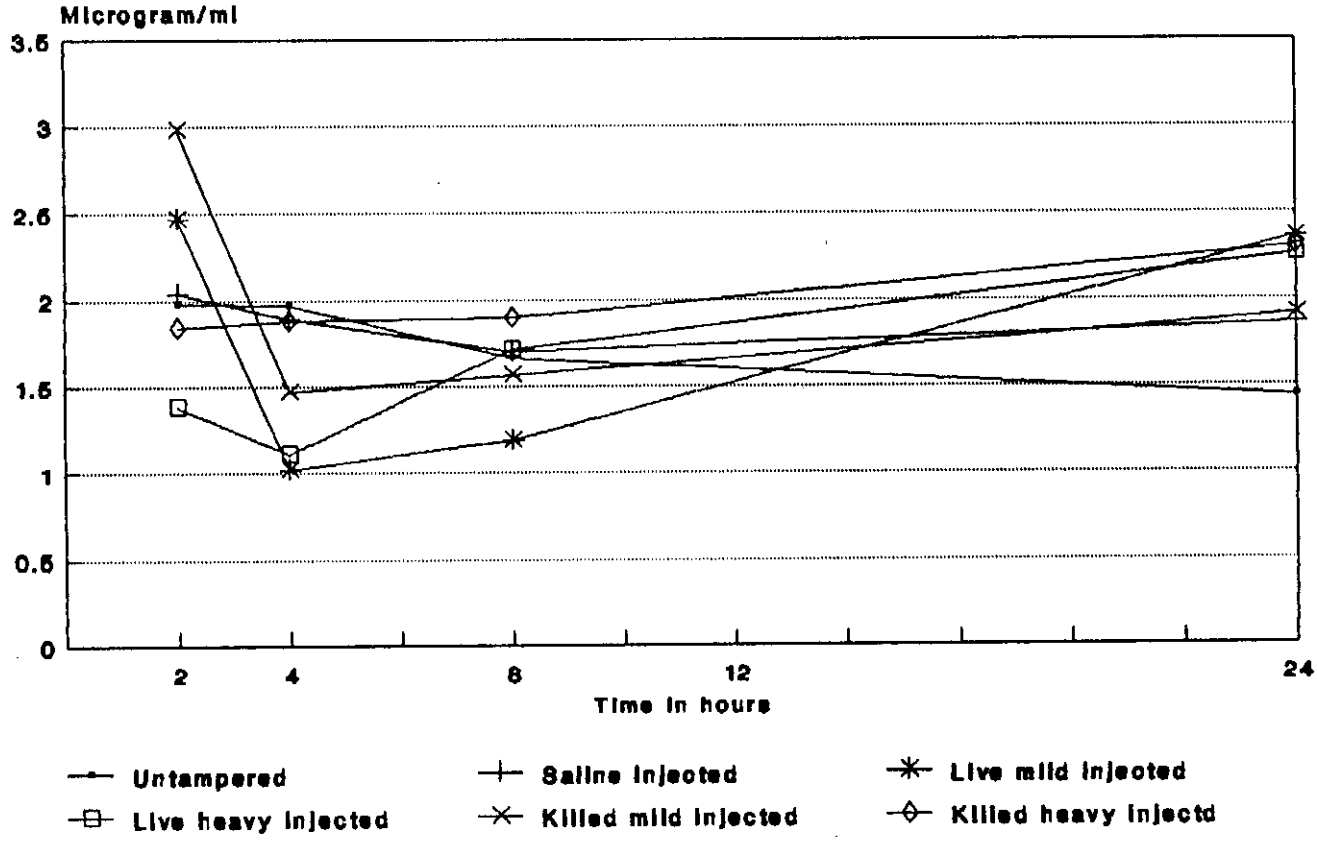


FIGURE-6

noticed at 4 hour post-challenge ($p<0.05$) compared to the activities in untampered and saline injected specimens, and at 2 hour post-challenge in the case of live heavy injected animals ($p<0.05$) compared to the activities in untampered, and live mild injected specimens.

At 24 hour post-challenge, live injected specimens (mild and heavy dose) showed significantly higher enzyme activity compared to that in untampered ($p<0.05$ and $p<0.005$, respectively) controls.

Within each test group there was no significant changes in activity between time-periods in the case of untampered, the saline, and heat-killed mild and heavy injected groups. In the case of live mild injected group, activities at 4 and 8 hour period were signficantly lower compared to those at 2 and 24 hour period ($p<0.05$ and $p<0.005$, respectively), whereas in live heavy injected group, 2 and 4 hours showed significantly lower activity compared to the activity at 24 hour ($p<0.05$ and $p<0.005$, respectively).

4.3.2. Alkaline phosphatase activity in the haemolymph of *L.marginalis* challenged with *E.coli* at different time-periods.

Table 8 gives n, mean value, standard deviation, and range of haemolymph alkaline phosphatase activity in animals challenged

TABLE-8

Alkaline phosphatase activity (μ moles/ml haemolymph/minute) pattern in th haemolymph of *Lamellidens marginalis* challenged with mild and heavy dose of live and heat-killed *E. coli* suspension at different time-periods.

		2 hr	4 hr	8 hr	24 hr
Un-	N	10	10	10	10
tampered	Mean	1.9416	1.8166	0.8416	1.3416
	SD	1.5995	1.4298	0.3023	1.2622
	Range	0.555-5.444	0.722-4.833	0.388-1.333	0.444-5.000
Saline	N	10	10	10	10
injected	Mean	1.0527	1.5194	1.0527	1.9582
	SD	0.7062	1.3946	0.4705	2.1263
	Range	O.611-3.110	0.555-5.555	0.444-2.000	0.388-7.944
Live	N	10	10	10	10
E.coli	Mean	1.911	0.991	1.0638	1.0361
injected	SD	2.1879	0.6194	0.5306	0.8386
(mild dose)	Range	O.444-8.222	0.444-2.333	0.444-2.388	0.500-3.110
Live	N	10	10	10	10
E.coli	Mean	1.3277	1.8107	1.9333	0.8861
injected	SD	1.0992	2.2819	1.5732	0.2352
(heavy dose)	Range	O.500-4.00	0.722-8.609	0.500-4.666	0.555-1.2776
Heat-killed	N	10	10	10	10
E.coli	Mean	1.8527	1.5416	1.599	0.8499
injected	SD	1.3889	0.9289	1.0118	0.6315
(mild dose)	Range	O.722-4.666	0.888-3.888	0.500-3.6109	0.388-2.666
Heat-killed	N	10	10	10	10
E.coli	Mean	1.811	1.3277	1.1611	1.5694
injected	SD	1.2129	0.8152	1.3112	1.6638
(heavy dose)	Range	O.500-4.888	0.444-3.222	0.333-4.999	0.361-5.500

Haemolymph ALP in *Lamellidens marginalis* injected with live and heat-killed *E coli*

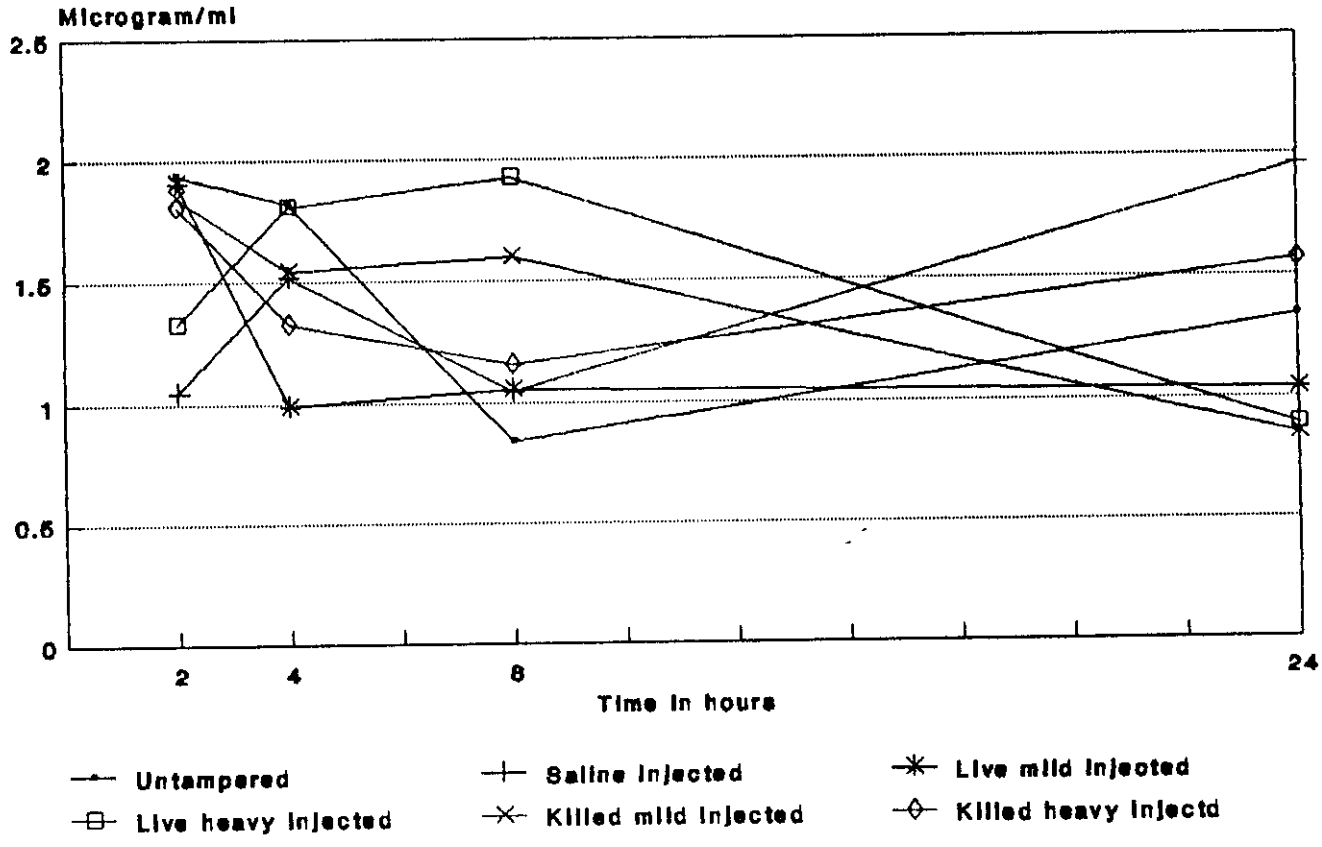


FIGURE-7

with mild and heavy dose of live and heat-killed *E.coli* at 2, 4, 8, and 24 hour time-periods, and also in saline and untampered control specimens.

The Figure 7 outlines the general average trend in the ALP activity pattern at all hours.

The results of the experimental groups were compared with those of saline and untampered control for all time-periods, and also between time-periods within each test groups.

The result indicated that there was no significant change in the activity pattern at all time-periods in all test groups compared to saline injected and untampered controls. Within each group also there was no stastically significant changes between different time-periods.

4.4.0 DISCUSSION

The occurrence of lysosomal hydrolases in the haemolymph has beeen reported date back to 1970 by Mc Dade and Tripp in *Crassostrea virginica* . The presence of the hydrolytic enzymes ACP and ALP in the haemocytes of *Crassostrea virginica* challenged to bacteria has been established (Feng et al., 1971; Foley and Cheng, 1972). They found that the hydrolases are present in the form of acidophilic and basophilic lysosomal

granules in the cytoplasm of haemocytes of phagocytic nature, and hence treated ACP as marker for lysosome presence.

The release of these lysosomal hydrolases through degranulation to the exterior induced by bacterial challenge has been reported in *Crassostrea virginica* (Foley, 1974). Further,it was also reported that haemolymph lysosomal enzymes in *Crassostrea virginica*, *Mya arenaria*, and *Mercenaria mercenaria* were found to be active but vary against many bacterial strains such as *Bacillus megaterium*, *Micrococcus lysodiekticus*, *Bacillus subtilis*, *Gaffkya tetragena*, *Salmonella pullorum*, and *Shigella sonei* but not active against *Staphylococcus aureus* (Cheng and Rodrick, 1974; Rodrick and Cheng, 1974a; Cheng, 1983a). Since the activity pattern of the hydrolases changes with each strain they attributed this to the possible role of cell wall of these bacterial strains. Cheng (1975), discovered higher amount of these enzymes in the haemocytes challenged with heat-killed *Bacillus megaterium*, and during phagocytosis in the haemolymph and reported increased activity during phagocytosis.Cheng and Garrabrant (1977) found accumulation of ACP-rich haemocytes around *Schistosoma mansoni* sporocysts challenged in the snail *Biamphalaria glabrata*.

Further, there are also reports of elevated lysosomal enzymes in haemolymph during phagocytosis in the bivalves *Mercenaria mercenaria*, (Cheng et al., 1975), *Crassostrea*

virginica (Yoshino and Cheng,1976b), *Mya arenaria* (Cheng and Yoshino, 1976a), and in the gastropod *Biomphalaria glabrata* (Cheng and Yoshino, 1976b; Cheng et al.,1977). It is also noted that lysosomes even in single cell types are variable in their enzymatic constitution (Yoshino and Cheng, 1976a; Dean, 1977) and divergent in functional activities (Schellens et al., 1977). Moreover, the cytoplasmic granules in haemocytes of several molluscs were considered to be true lysosomes (Mohandas et al., 1985). Cheng et al.,(1975) reported elevated levels of haemolymph hydrolytic enzymes in *Mercenaria mercenaria* injected with *B.megaterium*. They are of the opinion that lysosomal enzymes are released by haemocytes in response to bacterial challenge and the release occurs concurrently with phagocytosis of bacteria and not as delayed response. Further, they also found that release of lysozyme into serum is not the result of destruction of plasma membrane of the haemolymph cells, since no change was found in the LDH activity in the haemocytes and in serum. Yoshino and Cheng (1976b), based on their in vitro studies in *Crassostrea virginica*, reported elevated levels of lysosomal enzyme aminopepdidase to bacterial challenge and suggested the increased synthesis of lysosomal enzymes by haemocytes.

Cheng et al.,(1977) reported that elevated lysosomal activity was observed in *B.glabrata* injected with heat-killed *B.megaterium* at one hour in haemocytes, and at 2 and 4 hours in

the haemolymph, and attributed this increased activity to either increased synthesis of lysozyme, or to the increase in number of haemocytes to the bacterial challenge. They also suggested that the increased lysosomal activity was due to non-specific inducible humoral defence system.

It has been demonstrated that serum acid phosphatase level in *B.glabrata* challenged with heat-killed *B.megaterium* was significantly elevated at 1, 2, and 4 hour post-injection (Cheng and Butler, 1979). Further, it was also found that there was no increased ACP activity in serum of *B.glabrata* infected with *S.mansoni* at 1, 12 and 24 hours, but slight increase was noticed after 2 weeks (Granath and Yoshino, 1983), and they attributed this to failure in specific response to parasite infection. Thus, it is clear that enhancement of host reactions does not appear to be the case always. It is influenced by many factors such as recognition sites, rate of synthesis of enzymes, and chemical configuration of the invading bacteria or parasites.

Besides, it was also found that (Moat,1979) majority of Gram negative bacteria possess multilayered cell wall, an outer membrane composed of lipopolysaccharide and lipoprotein complexes external to the peptodoglycan layer particularly in *E.coli*, *Salmonella sps.* and *Schigella sp.* This lipopolysaccharide layer is known to act as dominant antigenic

determinant ('O' antigens), and entotoxin of Gram negative bacteria. They also function as barrier to action of many lytic enzymes. Treatment with these enzymes results in formation of spheroplast which provides a protective mechanism against phagocytosis.

In the present study also in the case of ACP, the lower activity in live heavy injected animals at 2 hour, and at 4 hour in the case of live mild and live heavy injected animals may probably be due to the spheroplast formation with cell wall of *E.coli* there by bring down the lytic action of hydrolases and protecting the bacteria. But the elevated level of ACP at 24 hour in both dosages of live injected animals clearly signifies the capacity of animals to synthesis more lytic enzyme and in turn recognise the non-self biotic substance and their protective role.

The reduction in the activity pattern at both 2 and 4 hour period in live heavy injected animals compared to only at 4 hour period in live mild injected one also shows that in heavy injected animals since the number of bacteria was higher than in mild injected group (almost twice the number), the prolonged inhibition of activity has also been noticed.

The insignificant change in the release pattern of ALP further corroborates the study reported by Cheng et al., (1975), and Mohandas et al.,(1985) that the release of hydrolytic enzymes by haemocytes is not the result of destruction of plasma membrane of haemocytes but through the budding of lysosomal vacuoles or granules to the exterior through degranulation.

CHAPTER V

EFFECT OF HEAVY METALS ON THE ACTIVITY PATTERN OF HAEMOLYMPH ENZYMES

5.1.0. INTRODUCTION

The ability of an organism to resist an invading foreign agent is normally determined, in part, by the functional capacity of its internal defense mechanisms. If these defense mechanism are compromised due to pollution or stress/ unfavourable conditions the outcome of the infection would be unfavourable to the host, whereas if the normal defense mechanism is functional or in cases enhanced then the infection would be sometimes resolved (Anderson,1988).

There are many reports available regarding the effect of toxic pollutants on various physiological aspects of bivalves. This includes measuring the changes in structure and function of tissue, and cellular organelles such as lysosomes, endoplasmic reticulum, mitochondria, and plasma membrane which are known to be disturbed by xenobiotics (Fowler et al., 1975; Sternlieb and Goldfischer, 1976; Moore and Stebbing, 1976; Moore, 1977, 1980; Zaba and Haris, 1978; George, 1983 a, b, Pickwell and Steinert, 1984; Moore et al., 1984; Akbarali et al., 1984; Suresh, 1988).

But relatively very few information is available on the immunological competency during sublethal exposure to toxic heavy metals. The changes in the immunological competency could adversely affect the health and survival of these animals.

The important findings in that line of research are the reports of high incidence of infestation by unicellular organisms in animals from polluted area (Jeffries,1972), abnormal cellular conditions like proliferative neoplastic cells due to polynuclear aromatic hydrocarbons (Lowe and Moore, 1978; Mix et al., 1979), the abnormal occlusion of haemolymph sinuses by large number of haemocytes termed granulocytomas (Lowe and Moore, 1979) in oysters from polluted environmental localities, and depressed phagocytic activity in response to phenol (Fries and Tripp,1980).

Further, there are also reports of lysosomal disruptions in mussels in response to heavy metals and chemicals (Pickwell and Steinert, 1984; Moore et al., 1984), and extra cellular release of lysosomal enzymes (Harrison and Berger,1982), and inhibition of cellular immunity in molluscs (Cheng and Sullivan, 1984). Mussels and periwinkles proved sensitive to sublethal chemical perterbations that are expressed at cellular and subcellular levels of organisations (Bayne et al., 1981, 1982; Dixon, 1982).

It is well known that lysosomes act as detoxication system by compartmentalisation and accumulation of many organic chemicals and metals (Allison, 1969; Moore, 1980; Viarengo, et al., 1981,1984; George, 1983a,b), and these xenobiotics in excess induce alteration in the bounding membrane of lysosomes leading to destabilisation (Viarengo, 1981a,Moore and Lowe, 1985), and lead to release of degradative lysososmal enzymes into cytoplasm (Moore, 1976; Baccino, 1978). The quantitative relationship between the magnitude of stress and destabilisation of membrane resulting in the release of hydrolytic enzymes into cytoplasm and extracellular environment has been reported (Bayne et al., 1976, 1979 a, 1982; Moore and Stebbing, 1976; Pickwell and Steinert, 1984). But seldom it is taken as an inference when animals challenged with biotic subtances while under sublethal exposure to toxicity and as a measure of synergistic stress, and as a possible indicator of susceptibilty to infection during environmental stress.

Hence, measuring the changes in the heamatological parameters would provide valuable diagnostic and predictive information towards better understanding of the defense system. In the present study the activity patterns of the lysosomal hydrolytic enzymes ACP and ALP have been investigated as possible indicators of the immunological compatibility or otherwise of the animals under sublethal exposure to heavy

metals and challenged with bacteria. Futher, this would also provide some insights towards better understanding of their defense system under biotic and abiotic stress and may be useful in predictions for the control of natural populations.

5.2. MATERIALS AND METHODS

5.2.1. TOXICITY, AND $L.C._{50}$ STUDIES

Methods of collection of specimens, rearing, and acclimation were the same as described in Chapter- II.

As a preliminary study to find the tolerance of mussels to sublethal concentration of heavy metals, mercury ($HgCl_2$), and copper ($CuSO_4\ 5H_2O$), 96 hour LC 50 values were worked out.

1. Three sets of ten animals each were used for each heavy metal at 3 different concentrations (Hg 1.0 ppm, 1.5 ppm and 2.0 ppm, and copper 2.0 ppm, 3.0 ppm and 4.0 ppm respectively).
2. The water in the tanks was changed at every 24 hours, and the animals were fed with the algae *Oocystis pussila*.
3. After 96 hour, the LC 50 value were calculated.

The 96 hr LC 50 values for *L.marginalis* are as follows.

Copper as $CuSO_4\ 5H_2O$ = 3.11 ppm and

Mercury as $HgCl_2$ = 1.18 ppm.

5.2.2. SUBLETHAL EXPOSURE OF THE ANIMALS TO HEAVY METALS Hg AND Cu

1. After acclimation, the mussels were dosed with heavy metals, Hg and Cu separately at sublethal concentration of 0.9 ppm and 2.3 ppm, respectively for 96 hours. 2. The water was replaced at every 24 hours, and the animals were fed with algal broth.

5.2.3. CHALLENGING WITH *E.coli*

1. At the end of 96 hour exposure to heavy metals the animals were injected with mild, and heavy dose of live and heat-killed bacteria 2. Untampered and saline injected controls were also maintained. 3.All the animals continued to remain exposed to heavy metals after challenge. 3. The haemolymph samples were collected for the estimation of ACP and ALP activities at 2, 4, 8, and 24 hour post-challenge. 4. The number of animals for each time-period was 10 in the case of mercury exposed group, and five in the case copper exposed group. 5.The bacterial concentration in suspension and procedure for estimation of ACP and ALP were the same as described in Chapter IV.

5.3.RESULTS

5.3.1. ACP activity in Hg exposed mussels

Table 9 gives the n, mean value, standard deviation, and range of haemolymph ACP activity at 2, 4, 8 and 24 hours post-challenge.
The figure 8 outlines the general trend in ACP activity pattern at all time-periods for all the test groups.

5.3.1.1 Comparison between experimental groups.

From the data obtained it is infered that at 2 hour post-challenge live mild and live heavy injected groups showed significantly lower activity compared to those with saline injected sets ($P<0.005$). But no change has been observed in values between untampered, saline, and both doses of heat-killed injected sets at any of the time-period.

5.3.1.2. Comparison between time-periods

In the case of untampered, saline, heat-killed mild, and heavy dose injected animals there was no statistically siginificant changes in values between time-periods. But in live mild injected set, value at 2 hour post-challenge was significantly

lower when compared to the value at 4, 8, and 24 hour time-periods ($P<0.05$).

5.3.2 ALP activity in Hg exposed groups.

Table 10 gives the n, mean value, standard deviation, and range of haemolymph ALP activity at 2, 4, 8 and 24 hours post-challenge.

The Figure 9 outlines the general trend in ALP activity pattern at all time-periods for all test groups.

5.3.2.1. Comparison between experimental groups

At 2 hour post-exposure, live mild, live heavy, and saline injected animals showed signficantly lower activity when compared to the activity in the untampered ($P<0.05$). In the case of 4 hour post-challenge, live heavy showed significantly higher activity compared to activities in saline injected and heat-killed heavy dose injected sets ($P<0.05$). At 8 hour post-injection, both live mild and live heavy injected animals showed significantly lower activity compared to those in saline injected, and both heat-killed mild and heavy injected animals ($P<0.05$).

TABLE-9

Acid phosphatase activity (μ moles/ml haemolymph/minute) pattern in the haemolymph of *Lamellidens marginalis* challenged with mild and heavy dose of live and heat-killed *E. coli* under sublethal exposure to $HgCl_2$.

		2 hr	4 hr	8 hr	24 hr
Un-tampered	N	10	10	10	10
	Mean	1.204	1.185	1.440	1.467
	SD	0.783	0.720	0.752	0.559
	Range	0.166-2.830	0.541-2.833	0.500-3.040	0.750-2.166
Saline injected	N	10	10	10	10
	Mean	1.3958	0.854	1.3667	1.4540
	SD	0.4997	0.561	0.579	0.500
	Range	O.5416-2.416	0.166-1.875	0.75-3.00	0.916-2.50
Live *E.coli* injected (mild dose)	N	10	10	10	10
	Mean	0.752 ▪	1.221	1.567	1.4570
	SD	0.178	0.581	0.855	0.460
	Range	O.5416-1.083	0.333-2.25	0.583-3.50	1.00-2.250
Live *E.coli* injected (heavy dose)	N	10	10	10	10
	Mean	0.675 ▪	1.167	2.167	0.974
	SD	0.195	0.592	1.344	0.437
	Range	O.333-1.00	0.333-2.08	0.916-5.08	0.500-1.91
Heat-killed *E.coli* injected (mild dose)	N	10	10	10	10
	Mean	0.946	1.602	1.567	2.469
	SD	0.482	0.917	1.545	1.731
	Range	O.500-2.083	0.500-3.00	0.583-6.00	0.666-6.33
Heat-killed *E.coli* injected (heavy dose)	N	10	10	10	10
	Mean	1.150	1.079	1.308	1.307
	SD	0.558	0.512	0.863	0.413
	Range	O.500-1.916	0.333-1.916	0.416-3.083	0.833-1.91

▪ Significantly lower than saline injected ($p<0.005$)

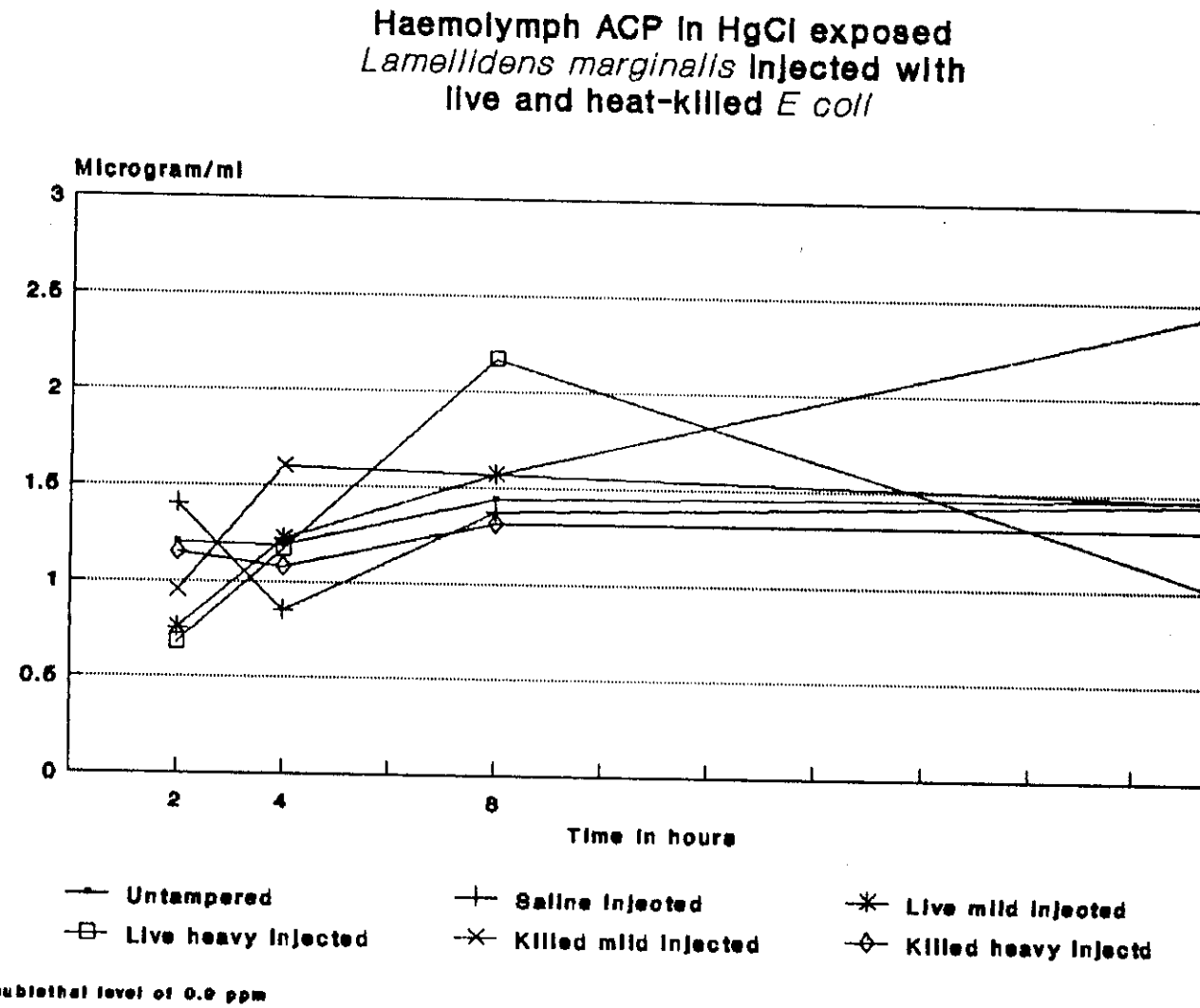

Haemolymph ACP in HgCl exposed
Lamellidens marginalis injected with
live and heat-killed E coli
Microgram/ml
3
2.5
2
1.5
1
0.5
0
2
4
8
Time in houre
Untampered
Saline injected
Live mild injected
Live heavy injected
Killed mild injected
Killed heavy injectd
At sublethal level of 0.6 ppm

FIGURE-8

TABLE-10

Alkaline phosphatase activity (μ moles/ml haemolymph/minute) pattern in haemolymph of *Lamellidens marginalis* challenged with mild and heavy dos of live and heat-killed *E. coli* under sublethal exposure to $HgCl_2$.

		2 hr	4 hr	8 hr	24 hr
Un-	N	10	10	10	10
tampered	Mean	1.389	1.100	1.070	1.042
	SD	0.235	0.220	0.465	0.397
	Range	0.916-1.830	0.750-1.500	0.333-1.875	0.583-2.08
Saline	N	10	10	10	10
injected	Mean	0.884 a	0.847	1.776	0.846
	SD	0.243	0.393	0.44	2.620
	Range	O.50-1.250	0.25-1.333	1.00-2.833	0.50-1.416
Live	N	10	10	10	10
E.coli	Mean	1.083 a	0.982	1.083 c	0.875
injected	SD	0.323	0.249	0.337	0.193
(mild dose)	Range	O.583-1.666	0.50-1.416	0.583-1.50	0.583-1.25
Live	N	10	10	10	10
E.coli	Mean	0.766 a	1.375 b	1.06 c	0.81
injected	SD	0.25	0.592	0.423	0.291
(heavy dose)	Range	O.50-1.083	0.541-2.75	0.583-1.875	0.50-1.416
Heat-killed	N	10	10	10	10
E.coli	Mean	1.208	0.917	1.542	0.917
injected	SD	0.361	1.338	0.278	0.229
(mild dose)	Range	O.660-1.916	0.25-1.75	1.290-2.080	0.583-1.62
Heat-killed	N	10	10	10	10
E.coli	Mean	1.125	0.857	1.506	0.885
injected	SD	0.341	0.353	0.274	0.322
(heavy dose)	Range	O.583-1.916	0.25-1.50	1.00-1.916	0.541-1.50

a Significantly lower than untampered ($p<0.05$)

b Significantly higher than saline and Heat-killed injected ($p<0.05$

c Significantly lower than saline heat-killed mild and heavy inject

($p<0.05$

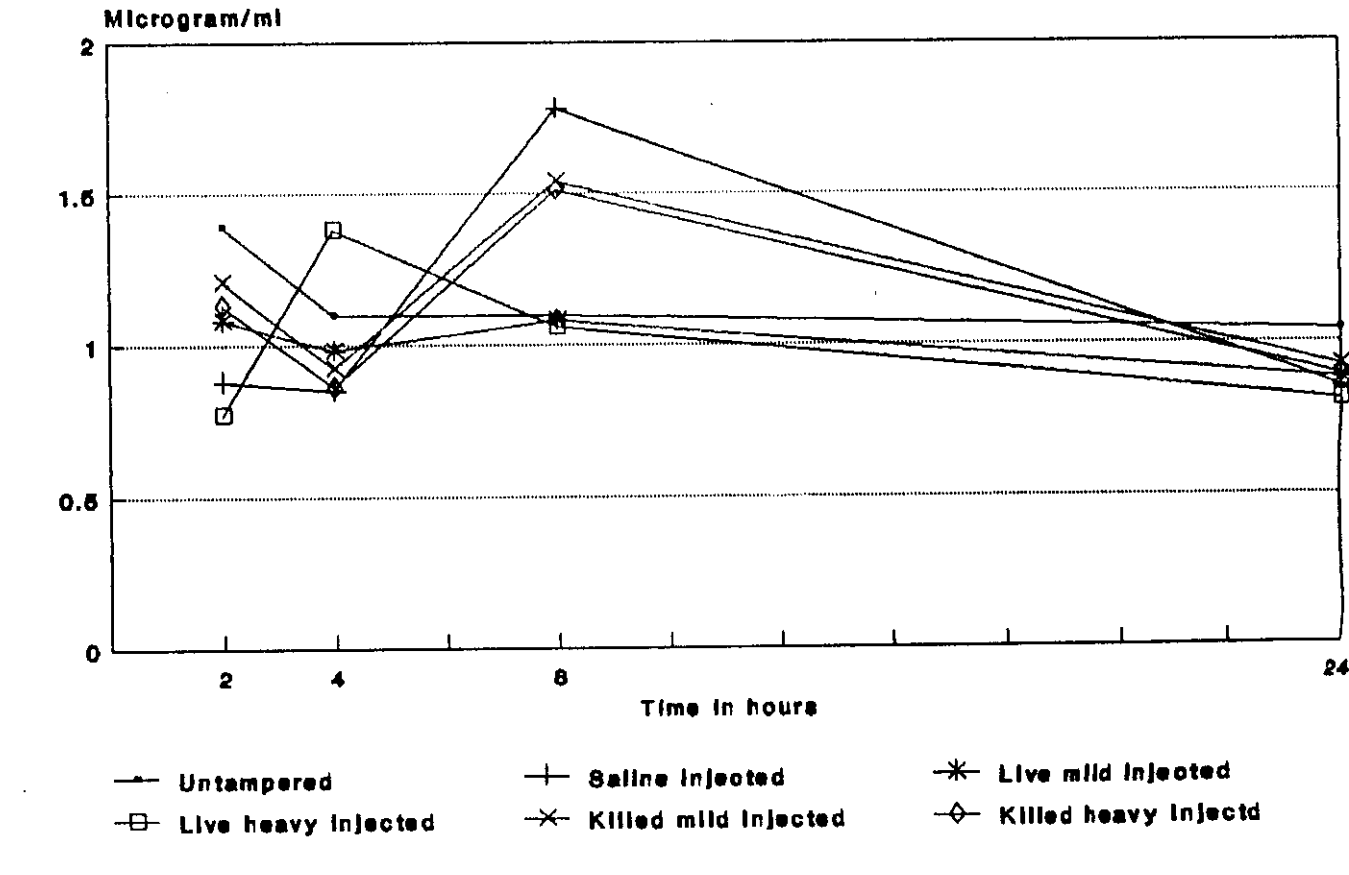

Haemolymph ALP in HgCl exposed
Lamellidens marginalis injected with
live and heat-killed E coli
Microgram/ml
2
1.5
1
0.5
0
2
4
8
24
Time in hours
Untampered
Saline injected
Live mild injected
Live heavy injected
Killed mild injected
Killed heavy injectd
At sublethal level of 0.9 ppm

FIGURE-9

In the case of 24 hour period, statistically no significant change was seen in activity between all test groups.

5.3.2.2. Comparison between time-periods

In the case of saline injected, heat-killed mild and heavy dose groups there was signficantly higher activity at 8 hour compared to those at 2, 4, and 24 hours post-challenge ($P<0.05$). In live mild injected animals no change has been noticed, but in live heavy injected animals 2 hour and 24 hour sets showed lower activity compared to the activity of 4 hour set ($P<0.05$).

5.3.3.ACP activity in Cu exposed groups

Table 11 gives n, mean value, standard deviation, and range of enzyme activity for all test groups.

The Figure 10 outlines the general trend in ACP activity pattern at all time-periods for all test groups.

From the results it has been observed that variation in the activity pattern of ACP in controls and in bacteria challenged groups was insignificant.

In general, however, it was observed that all the bacterial challenged groups showed lesser enzyme activity compared to those in untampered and saline injected groups at 2 and 24 hour post-injection.

5.3.4 ALP activity in Cu exposed groups

Table 12 gives n, mean value, standard deviation, and range in all test groups.
Figure 11 outlines the general trend in ALP activity pattern at all time-periods for all test groups.

From the result it was observed that the over all activity pattern of ALP in controls and in bacteria challenged groups showed comparatively little change at all time-periods. But live mild and heavy challenged sets showed relatively lower mean values compared to those of untampered and saline injected group at 2 and 4 hour post injection.

5.4.1. Comparison of ACP activity between Hg and Cu exposed groups

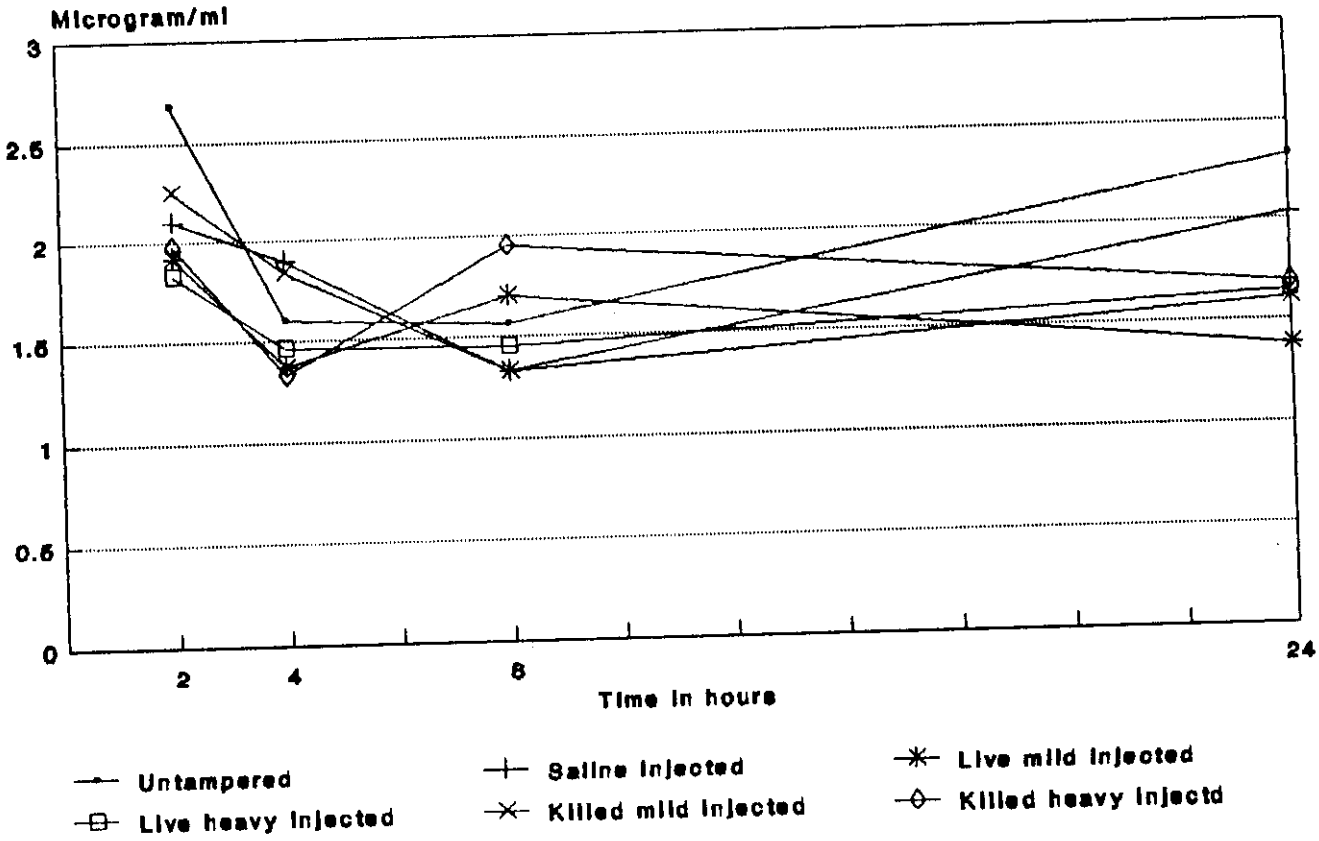
From the results it was observed that the trend in ACP activity of all the experimental sets exposed to Cu was higher than that Hg exposed groups. Further, it was also observed that in Cu

TABLE-11

Acid phosphatase activity (μ moles/ml haemolymph/minute) pattern in the haemolymph of *Lamellidens marginalis* challenged with mild and heavy dose of live and heat-killed *E. coli* under sublethal exposure to $CuSO_4$ $2H_2O$.

		2 hr	4 hr	8 hr	24 hr
Un-	N	5	5	5	5
tampered	Mean	2.683	1.600	1.558	2.333
	SD	0.764	0.461	0.170	0.679
	Range	1.83-3.75	1.08-2.416	1.416-1.875	2.330-2.75
Saline	N	5	5	5	5
injected	Mean	2.10	1.90	1.333	2.033
	SD	0.3118	0.3779	0.190	0.433
	Range	1.50-2.50	1.416-2.333	1.00-1.583	1.583-2.833
Live	N	5	5	5	5
E.coli	Mean	1.9166	1.367	1.700	1.383
injected	SD	0.2838	0.319	0.422	0.554
(mild dose)	Range	1.50-2.25	1.080-1.833	1.290-2.50	3.916-2.250
Live	N	5	5	5	5
E.coli	Mean	1.825	1.458	1.450	1.650
injected	SD	0.244	0.371	0.336	0.111
(heavy dose)	Range	1.583-1.875	1.083-2.160	0.916-1.916	1.50-1.833
Heat-killed	N	5	5	5	5
E.coli	Mean	2.250	1.842	1.333	1.608
injected	SD	0.497	0.253	0.5986	0.438
(mild dose)	Range	1.66-3.083	1.416-2.166	0.583-2.416	1.290-2.458
Heat-killed	N	5	5	5	5
E.coli	Mean	1.983	1.325	1.950	1.692
injected	SD	0.2603	0.119	0.327	0.318
(heavy dose)	Range	1.583-2.333	1.66-1.50	1.416-2.333	1.08-2.00

Haemolymph ACP in CuSo4 exposed *Lamellidens marginalis* injected with live and heat-killed *E coli*



At sublethal level of 2.30 ppm

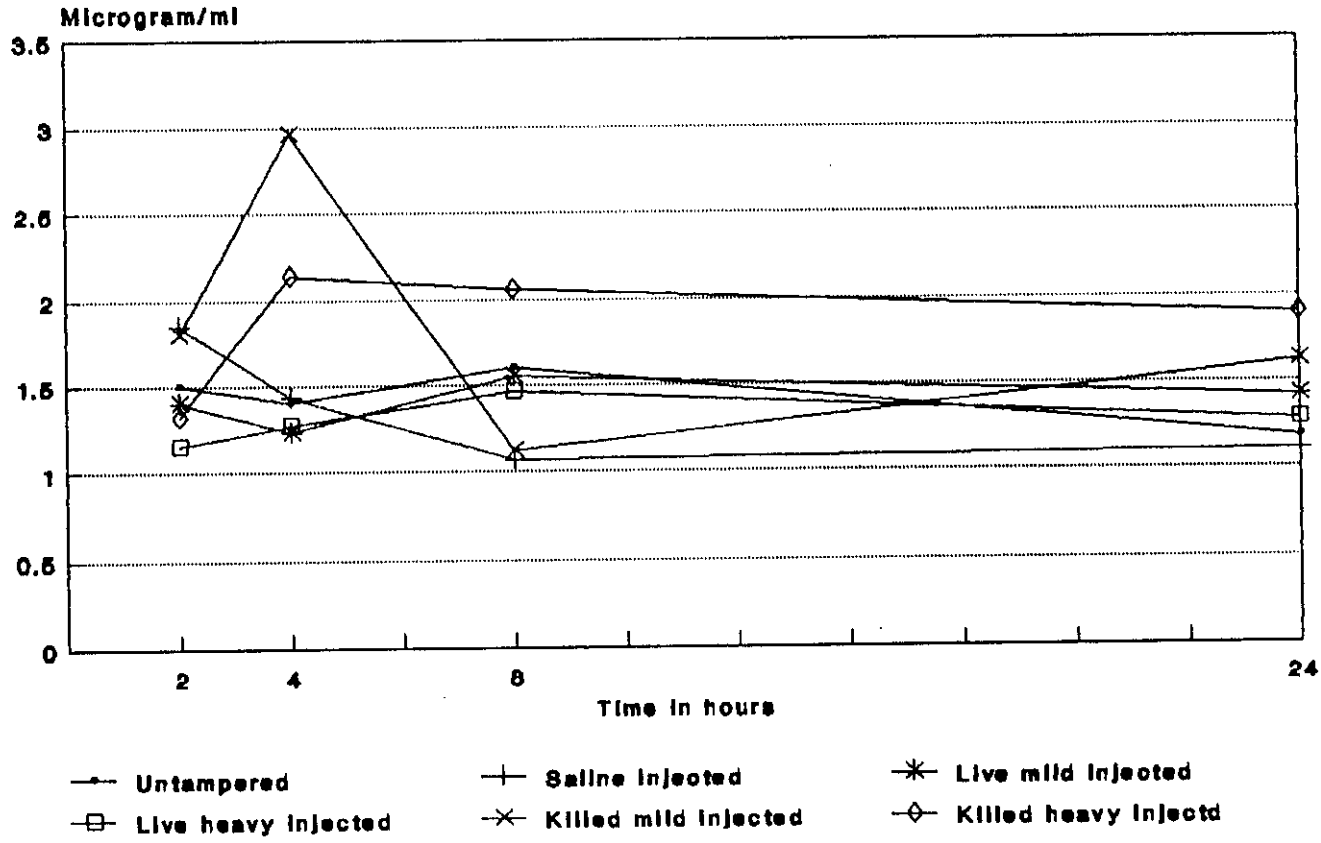
FIGURE-10

TABLE-12

Alkaline phosphatase activity (μ moles/ml haemolymph/minute) pattern in haemolymph of *Lamellidens marginalis* challenged with mild and heavy doses of live and heat-killed *E. coli* under sublethal exposure to $CuSO_4$.

		2 hr	4 hr	8 hr	24 hr
Un-	N	5	5	5	5
tampered	Mean	1.50	1.40	1.573	1.183
	SD	0.175	0.363	0.483	0.300
	Range	1.333-1.75	0.916-1.75	0.908-2.25	0.667-1.5
Saline	N	5	5	5	5
injected	Mean	1.85	1.433	1.067	1.108
	SD	0.932	0.391	0.207	0.186
	Range	1.00-3.50	1.00-2.166	0.750-1.33	0.833-1.3
Live	N	5	5	5	5
E.coli	Mean	1.40	1.233	1.55	1.417
injected	SD	0.3779	0.367	0.651	0.506
(mild dose)	Range	1.00-2.083	0.833-1.750	1.08-2.830	0.916-2.2
Live	N	5	5	5	5
E.coli	Mean	1.146	1.267	1.458	1.283
injected	SD	0.168	0.359	0.239	0.611
(heavy dose)	Range	0.833-1.291	0.833-1.916	1.166-1.833	0.916-2.5
Heat-killed	N	5	5	5	5
E.coli	Mean	1.80	2.957	1.117	1.617
injected	SD	0.5286	1.635	0.410	0.795
(mild dose)	Range	1.16-2.666	1.250-5.125	0.750-1.916	0.833-2.6
Heat-killed	N	5	5	5	5
E.coli	Mean	1.323	2.142	2.058	1.896
injected	SD	0.321	0.705	0.650	0.588
(heavy dose)	Range	1.00-1.916	0.916-2.916	2.291-3.25	0.833-2.5

Haemolymph ALP in CuSo4 exposed *Lamellidens marginalis* injected with live and heat-killed *E coli*



At sublethal level of 2.30 ppm

FIGURE-11

exposed groups, there was no change or reduction in activity in live bacteria challenged sets, and it was not the case with Hg exposed groups.

But in the case of ALP there is no such marked change was noticed between the two exposed groups and was equivocal.

5.4.0 DISCUSSION

The oyster haemocytes are known to accumulate heavy metals far higher than in the environment, and the presence of these heavy metals is correlated with acidophilic and basophilic nature of haemocytes (Orton,1923; Ruddel,1971b). Later, it has been found that these heavy metals are present within the membrane limited vesicles inside the cytoplasm thereby bringing down the concentration of these heavy metals in the body tissues and the level of toxicity. Further, these membrane limited vesicles vary in size and in their biochemical composition (George et al., 1978). The presence of Cu in acidophilic granulocytes, and Zn in basophilic granulocytes lead to the conclusion that there is variation in the localisation of different metals in different cells.

It has been reported that in *Ostrea sp.* Cu and Zn are confined to the amoebocytes and they accumulate pricipally in gills and mantle whereas in *Mytilus spp.* Fe and Pb ions are

transported via amoebocytes in membrane limited vesicles to kidney (George et al., 1976b, 1978; Coombs, 1977). Cadmium in both *Ostrea spp.*and *Mytilus spp.*was not seem to be immobilised in membrane limited vesicles (George et al., 1976a) but was found accumulated in digestive gland and kidney. Thus, the mechanism of detoxication vary for different metals as well as for different organisms (George et al., 1978). In the case of *C.virginica*, cadmium, both in ionic form and in EDTA chelated form, was found to induce haemopoesis, and cause reduction in hyalinocytes (Cheng, 1988).

There are findings that many of these xenobiotics (heavy metals) at lower concentrations entered into lysosomes (membrane-limited vesicles) subsequently transformed into biologically inactive forms (Chandy and Patel, 1985). Further, reports suggest that these heavy metals far in excess induce alterations in the bounding membrane of the lysosomes leading to destabilisation (Moore and Lowe, 1985). This destabilisation causes release of hydrolytic enzymes from lysosomal compartment (Moore, 1976; Baccino, 1978; Roesijadi 1980; Suresh, 1988). Secondly, these lysosomes are also involved in cellular defense against invading bacteria and parasite through intracellular fusion with phagosome or through the release of increased lysosomal hydrolytic enzymes into extra cellular fluid thereby rendering the invader non-viable or causing lysis (Cheng, 1984a,b,1985; Mohandas et al., 1985;

Sparks, 1972,1985).

But studies related to pollutant induced impairment or enhancement and subsequent bacterial challenge are fragamentary. Fries and Tripp (1980) reported that phenol exposure depressed phagocytic activity and caused cytoplasmic disorganisation and selective lysis of haemocytes in *Mercenaria mercenaria*. Anderson et al. (1981) reported that increase in haemocytic lysozyme was more pronounced in lower levels of pollutant exposure, and found no inhibition in the bacterial clearance compared to controls but higher levels of pollutants were found to impair clearance rate and release of lysozyme; probably due to increased tissue burden in *Mercenaria mercenaria*.

The effect of in vitro phagocytosis of haemocytes of *C.virginica* has been studied by Cheng and Sullivan (1984). In their study exposure to 1.00 ppm Cu^{+}, and 0.05 Hg^{+} resulted in no alteration in phagocytic activity. However, exposure to 5 ppm Cu and 0.1 ppm Hg enhanced uptake activity, but exposure to 0.5, 1.0, and 5 ppm Hg resulted in inhibition of phagocytosis and increased cell death. They are of the view that since Hg^{+} ions are known to show strong affinity to proteins bearing sulfhydryl groups (Pearse, 1980), the denaturation of enzyme or strucutural protein in the cell membrane or in cytoplasm due to Hg exposure might be responsible for impaired phagocytosis.

Failure of Cu^{+} to inhibit phagocytosis, though it is known to accumulate in the haemocyte lysosomes (George et al., 1978), may have been due to lower affinity for such binding sites. But the maximum of apparant stimulation of phagocytosis at 5.00 ppm Cu, and 0.1 ppm Hg has not been well elucidated.

Anderson (1988) reported that several of the immune variables such as total and differential haemocyte counts, titers of serum haemolysin, haemoagglutinin, and bacterial agglutinin did not show any change regardless of pollutant stress. Thus, the effect of heavy metals include both inhibition and incitation at different concentration levels through activation and inactivation reactions that are not fully understood.

In the present study in the case of Hg exposed groups the reduction in ACP and ALP activity in live injected sets at 2 hour time-period could be due to the enzyme-*E.coli* interaction that results in spheroplast formation similar to unexposed groups (Chapter-IV). But the general lower ACP activity compared to activity in Cu exposed group also indicates the intensity of toxicity of Hg and its influence on cellular protein denaturation. Thus, Hg induces inhibitory effect on the rate of lysosomal enzyme release pattern thereby affecting internal defense meachnism at 0.9 ppm level of exposure.

In the case of Cu exposed group, since a general elevated level of ACP activity is noticed in all test groups, the neutralisation of the enzymes by live *E.coli* through spheroplast formation might have taken place within 2 hours of post injection. Hence, at 2 hours no change in enzyme activity has been noticed. But the slight reduction in the activity compared to the activity in untampered and saline-injected ones give a clue to the role of live *E.coli* in neutralising the enzyme activity. Further, since Cu is known to accumulate in acidophilic granulocytes, the increased ACP activity in Cu exposed group could also be due to the acidophilic granules' availability in the haemocytes or to the occurrence of more haemocytes with acidophilic granules.

Further, 2.33 ppm Cu exposure poses stimulatory effect on lysosomal activity pattern despite the continued maintenence in Cu^{+} for 96 hour prior to challenge, and after challenge. In the case of Hg^{+} exposed groups, despite the toxicity of $Hg^{+}$the slow enhancement of ACP and ALP activity to the level of control value after 8 hours of post-challenge with bacteria indicates the slow recoup or the animal's ability to defend at such extremes of toxicated condition.

CHAPER-VI

SUMMARY AND CONCLUSION

6.1. SUMMARY

1. The present investigation was carried out on the haemocytes and haemolymph of the mussel *Lamellidens marginalis* .

2. Studies were confined to the following aspects: In vitro spreading activity pattern of haemocytes at different temperature periods, chemotaxis towards different bacterial strains, both Gram neagative and Gram positive, the role of serum in chemotaxis, in vivo phagocytic capability, activity pattern of selected lysosomal enzymes, and the effect heavy metals on the release pattern of selected haemolymph enzymes.

3.Spreading activity of haemocytes at different temperature was studied. It was found that at $32^{0}C$ higher number of haemocytes was spread than at $24^{0}C$, $26^{0}C$, $28^{0}C$, and $30^{0}C$, both at 1 and 2 hour periods.

4. Chemotactic attraction towards the Gram negative *E.coli*, and *Vibrio alginolyticus*, and the Gram positive *Micrococcus sp.*, and *Bacillus sp.* was studied.

5. Of the four bacterial strains, the association with *E.coli* was higher than with both the Gram positive strains. Hence, *E.coli* was chosen for further experiments.

6.The results showed that there was greater chemotactic

attraction with live bacteria than with killed-bacteria, and higher association at 2 hour period than at 1 hour period.

7. These results confirm the ability of haemocytes to recognise nonself substances, and to be selective in attachment.

8. The time taken for selectivity reaction suggests that the rate of cellular defense mechanism increases with increase in of exposure time.

9. The attraction of higher number of haemocytes towards live bacterial cells shows the capacity of haemocytes to distinguish the nature of, foreign substances, and confirms the the role of as primary function

10. Higher number of haemocyte-bacteria association in the presence of serum (with-serum incubated haemocytes) than in the absence of serum (with-saline incubated haemocytes) indicates the role of serum in the activities of haemocytes.

11. In in vivo studies the number of phagocytosed haemocytes was higher in animals maintained at $27^{0}C$ than in those maintained at $32^{0}C$ and $22^{0}C$, and the number was found to decrease with increase in time.

12. The maximum phagocytic rate at $27^{0}C$ shows the optimum temperature suitable for maximum activity in vivo.

13. Haemolymph glycogen level in both live, and heat-killed bacteria challenged animals was higher at 4 hour post-injection.

14. The increase in haemolymph glycogen level at 4 hour post-injection suggests the possible digestion of bacteria both live and killed by haemocytes and subsequent release of glycogen in to haemolymph.

15.Activities of two enzymes, acid phophatase and alkaline phophatases origin were assayed in haemolymph.

16. The result indicated lower ACP activity at 2 and 4 hour post-challengein live-bacteria injected animals, and higher at 24 hour, but little change was noticed between untampered, saline, and heat-killed bacteria injected groups. ALP activity pattern showed no significant change amoung test groups.

17. The reduction in ACP activity at early time-periods in live *E.coli* injected animals suggests neutralization of enzyme with *E.coli* cell wall resulting in the formation of spheroplast. The increase at 24 hour suggests the increased synthesis of ACP by the animal to counter the bacterial challenge.

18. The no change in ALP suggests that the release of lysosomal enzymes not be through membrane destabilisation.

19.Effect of exposure to sublethal concentrations of heavy metals on the activity pattern of haemolymph ACP and ALP in animals challenged with *E.coli* was investigated.

20.Heavy metals selected for the experiments were mercury ($HgCl_2$) and copper ($CuSO_4$ $2H_2O$).

21. Results of ACP activity in Hg exposed group showed the following . At 2 hour post-injection live bacteria injected

animals showed lower activity but no significant change was noticed in other groups at any time-period.

22.Regarding haemolymph ALP activity in Hg exposed animals there was no change in activity in the untampered, and in those injected with heat-killed bacteria. In live bacteria injected animals at 2,and 8 hour post-challenge, reduction in enzyme activity was noticed., but increase at 4 hour.

23. In Cu exposed animals, there was, in general higher haemolymph ALP activity at all time-periods, and no change was noticed between the test groups.

24. Haemolymph ALP activity in Cu exposed animals showed no significant change.

6.2. CONCLUSION

Haemocytes of *Lamellidens marginalis* show reaction to non-self substances. They are also selective, showing more reactivity to live bacterial strains than to dead ones and greater attraction towards *E.coli*. This could be due to presence of specific receptor sites on live *E.coli*. Thus, variation exists in the degree of chemotaxis by haemocytes to different bacteriadue to variation in the chemical nature of receptors on them. It was also found that serum plays a major role in recognition process by haemocytes to bacteria.

In in vivo studies maximum phagocytosis was noticed at ambient temperature indicating efficient defense mechanism However, the results indicate that activity of haemocytes on Gram negative bacteria is often influenced by the formation of a complex between the lysosomal hydrolases and bacterial cell wall which protects the bacteria from lysis at early time-periods.

The effects of Hg ions and Cu ion on enzyme activity exposure suggest the influence of different heavy metals on the internal defense physiology, in different ways. However, further investigation is required on this aspects to draw clear conclusions on the resistance potential of haemocytes, and the molecular mechanism of interaction between haemocytes and heavy metals.

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