HAEMATOLOGY OF SOME MARINE AND ESTUARINE MOLLUSCS OF COMMERCIAL IMPORTANCE

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TO MY PARENTS

CERTIFICATE

This is to certify that this thesis is an authentic record of the research work carried out by Shri.K.SURESH, under my scientific supervision and guidance in the Division of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the Cochin University of Science and Technology under the Faculty of Marine Sciences, 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.Suresh, do hereby declare that this thesis entitled "HAEMATOLOGY OF SOME MARINE AND ESTUARINE MOLLUSCS OF COMMERCIAL IMPORTANCE" is a genuine record of the research work done by me under the scientific supervision of Dr.A.MOHANDAS, Reader, School of Environmental Studies, Cochin University of Science and Technology, and has not previously formed the basis for the award of any degree, diploma, or associateship in any University.

4/1/88

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CONTENTS

PREFACE

Molluscs form valuable fisheries in various parts of India and for that matter throughout the world. They are being used as food, as a source of lime, pearls and decorative shells, and as constituents of medical preparations. Although the available resources are exploited at numerous places, the total production is not high compared to that from other countries. In India where malnutrition is widespread, aquaculture of oysters, mussels, clams etc. can very much augment production from fishing natural beds and provide protein and mineral-rich food. In recent years, following an increase in the fishing effort and greater awareness of the resources, there has been a steady rise in molluscan production, and newly developed methods are being adopted in mariculture programmes for increased production. But as is the case with other culture programmes, molluscan fisheries are also likely to get set-backs because of bacterial, viral, protozoan and metazoan parasitic outbreaks, and in studies designed to elucidate the mechanisms that may affect or influence the susceptibility and/or non-susceptibility of molluscs to invading organisms, it is essential that considerations be given towards understanding the internal defence mechanisms of molluscs when confronted with non-self substances, and the role of molluscan blood cells and haemolymph in internal defence.

Before detailed studies relative to the defence mechanisms are envisaged, it is necessary to obtain several details regarding haemocytes and haemolymph. For eg., the following very relevant questions need be answered- Are there different types of haemocytes in molluscs? Does age cause alterations in the total blood picture? Do pollutants cause change in the blood picture? What hydrolytic enzymes occur in blood? What is the state of a non-self organism that become degraded intracellularly? etc.

Such information is essential for programmes designed to the development of potential biological control agents for undesirable molluscs (those which act as intermediate hosts for larval trematodes, the adults of which are parasitic in human beings, domestic animals, birds etc.), to understand and prevent microbial and other parasitic diseases that weaken or destroy commercially important molluscs, and also to logically and rationally develop and design methods to depurate molluscs that have become biotically and abiotically polluted.

Yet another important aspect that needs an emphasis is the utilization of marine organisms as models for research directed at understanding the basic bio-medical problems that remain unresolved. Examples could be cited where modern bio-medicine has taken major strides as a result of international use of marine organisms as models: studies on Horse-shoe crab by Bang (1956) led to the discovery that all Gram-negative bacteria produce endotoxin which is lipopolysaccharide in nature and the toxin causes septicemia; the discovery of Hoffstein and Weissman (1975), (based on their work on dog-fish Mustelus canis) linking gouty arthritis with the uptake of monosodium urate (MSU) by phagocytes, and subsequent cell death causing inflammation. It is also an area of interest to invertebrate pathologists because, studies with this view as the guiding theme not only may help resolve human disease problems, but also those affecting fisheries and related areas, all induced by an altered environment. Yet another strong point favouring their increased use is the explosive growth of aquaculture in recent years. Additionally, the aquaculture facilities themselves, generally oriented toward propagating animals for food, can diversify by including the propagation of species that prove valuable in

medical research. Aside from its scientific advantages, the use of marine organisms has a practical advantage, viz., many species of marine animals are readily available, usually at low cost.

It is well known that under certain conditions, populations of oysters and clams are susceptible to destructive epizootics caused by pathogenic micro-organisms. It has also been shown that exposure of mammals to certain heavy metals causes increased susceptibility to and severity of microbial infections (Koller, 1980). Consequently, pollutants that affect haemocyte viability or interfere with internal defence functions of the haemocytes which are considered as the major means of defence in molluscs against invading foreign organisms and pathogens (Cheng, 1981) may have profound effect on long term survival of molluscan populations. All these justify the significance of the present study in the context of the current status on molluscan culture programme, and how the data on molluscan haematological studies could be taken as the reliable criteria for pollution monitoring studies.

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INTRODUCTION

INTRODUCTION

The earliest work in India on molluscan blood was that of Narain (1968,1972a,b;1973). His study was mainly on the circulating amoebocytes, formed elements of the blood and blood chemistry of Lamellidens corrianus. Pal and his group are working on blood cells associated with the digestive tubules of some marine and estuarine bivalves (Pal and Modak, 1981; Pal et al., 1983). Contributions from Patel and his group include the spectrophotometric studies on the haemoglobin of Anadara spp. (Patel and Patel, 1964), the effects of ionising radiation on haemoglobin of marine lamellibranchs (Patel and Patel, 1971), the effects of environmental parameters on tissue lysosomal marker enzymes in the tropical blood clam Anadara granosa (Patel and Patel, 1985), and the interactions of Mercury, Selenium and Glutathione on lysosomal enzyme response in A. granosa (Chandy and Patel, 1985). To my knowledge, apart from these published work, no other information is available on molluscan blood from India.

In India, molluscs have occupied a marked place in the affairs of man from time immemorial, in his affairs of state and economy, of mind and aesthetic values, of religion and rites of worship. From their pride of place in mythology and legend they have inspired countless tales in folklore, caused long-standing customs and traditions, and in more recent times come to occupy prominent position in heraldry and royal insignia, besides featuring conspicuously in the economy of vast sections of the people (Mukundan, 1968). Mussels and clams have considerable local importance. While mussels are an important molluscan fishery resources in India, in terms of total production clams are the foremost (Nayar and Rao, 1985). Besides, being a good source of protein from the nutritional point of view, they have got the advantage

of easy digestibility, and form good source of minerals. They are being regularly used as subsistence food by fishermen communities in some localities. From commercial point of view, there are extensive sub-fossil molluscan shell deposits in the Vembanad Lake, largely comprising of clams, the total resources of which has been estimated at 2 to 4 million tonnes (Nayar and Rao, 1985). The annual estimated production of shells from this lake is 198809t of which live clams form 26859t (Rasalam and Sebastian, 1976). The shells form the raw material for the manufacture of cement and calcium carbide. Lime shells are used for a number of purposes, such as preparing mortar and slaked lime, for neutralising acidic soil, and in rayon and paper industries. When they are exploited for these purposes indiscriminately, a large number of young ones are also caught, causing depletion in the recruitment.

The capacity of bivalves to accumulate potentially toxic heavy metals in their tissues far in excess of environmental levels is well known (Phillips, 1976a,b; 1977a; Roberts, 1976; Calabrese, et al., 1984), and has become the focus of increasing number of studies. Bivalves may be either suspension or deposit feeders, and these feeding strategies pose different problems with respect to threshold levels of environmental stress. The fact that bivalves can accumulate toxic heavy metals restricts their use as food, and hence of global concern. Goldberg (1975) has urged for a global mussel watch on the concentration of certain contaminants in the tissues of different species of Mytilus to provide an integrated index of environmental condition. The mussels continuously sample their environment and hence the average concentrations of the metals in the habitat medium will be reflected in their tissues. Several Indian workers have also reported that clams, mussels and

oysters can concentrate heavy metals in their tissues to significant extent (Lakshmanan, 1982; National Seminar on Mussel Watch, 1986).

Likewise, it is established that molluscs have the ability to concentrate micro-organisms from the environments in which it habits (Kelly, 1956; Metcalf and Stiles, 1965; Liu et al., 1966; Slanetz et al., 1968; Cabelli and Heffernan, 1970). There are reports stating that they have served as vehicles for the transmission of enteric diseases to man (Hart, 1945; Dougherty and Altman, 1962). Bivalves when placed in a sea water environment free of these organisms, will give off the enteric micro-organisms from the body. The phenomenon by which it is accomplished, and environmental factors affecting it are studied in the oyster (Wells, 1916; Wood, 1961; Hedstrom and Lycke, 1964; Mitchell et al., 1966), the soft clam (Arcisz and Kelly, 1955; Erdman and Tennant, 1956), and the mussel (Dodgson 1928; Crovario, 1958). This is mediated by physiological functions such as pumping rate, filtration efficiency, the rate of transport and elimination of materials through the epithelium, etc. Elimination of bacteria includes the phagocytic activities of cells in the haemolymph (Bang, 1967). These phagocytic cells are continuously lost to the exterior through epithelial borders (Cheng et al., 1969). This is an efficient mechanism to clear phagocytized bacteria and other materials (Malek and Cheng, 1974). The exteriorized phagocytes are carried away in mucus or faeces by the water stream set up by natural pumping action in the body of bivalves (Houser, 1964; Malek and Cheng, 1974).

Molluscan haemocytes have been implicated in diverse functions including an active function in internal defence against invading foreign materials. Before detailed studies relative to the defence mechanisms are envisaged,

it is necessary that the numbers and types of haemocytes as well as their normal behaviour be ascertained. Such studies are absolutely essential as preface to studies concerned with functions related to internal defence. Phagocytosis of foreign materials by haemocytes is an important aspect of internal defence mechanism and this aspect is particularly important in molluscan aquaculture programmes as haemocytes phagocytose both biotic and abiotic foreign materials. Hence, in the present study data on total and differential count as well as observations on the uptake mechanism of foreign materials by haemocytes have also been included. The study has been extended to four size groups of the two species of clams to find out whether there are differences in the total and differential blood count, organic composition of haemolymph, and activities of acid and alkaline phosphatases in different size groups.

Bivalve molluscs are found in aquatic habitats ranging from oceanic waters to fresh water. The open marine environment is relatively stable with respect to the physico-chemical composition, but in coastal and estuarine habitats significant fluctuation may occur. Thus, these bivalves are frequently exposed to a range of sub-lethal levels of environmental stresses such as salinity, temperature, oxygen etc., and to further stress, arising from man's activities such as offshore drilling, dredging or the release of pollutants, and to the stress caused by micro-organisms, protozoan and metazoan parasites. Bayne (1975) gave the following working definition of stress as applied to marine bivalves. "Stress is a measurable alteration of a physiological (behavioural, biochemical, or cytological) steady-state which is induced by an environmental change, and which renders the individual (or the population)

more vulnerable to further environmental change". The adverse effects of stress, in general, on aquatic organisms including bivalves have been generally identified with their acute and lethal impacts. Mortality is the end point that can be readily recognised and quantified, hence the standard assay for acute toxicity testing for pollutants in aquatic organisms measures the particular stress condition of concentration which causes 50% mortality over a standard period of time (LC_{50}). The sub-lethal effects can be induced at much lower levels than the LC_{50}. While not directly resulting in death, sub-lethal effects can affect survival through effects on behaviour, growth, physiology and reproduction (see Akberali and Trueman, 1985). The ultimate test of significance of a sub-lethal effect of environmental stress is whether it has an impact on the propagation of a species and on its population (Waldichuk, 1979, Bayne et al., 1979a; 1981).

The effects of environmental variables on bivalves have been studied on the organisms, tissue and at cellular levels. In the past, the main approach adopted by many has been through experiments where either a particular response or a multitude of the responses of the animals are monitored in relation to experimental variation of a single experimental variable, such as salinity, temperature or pollutant, while maintaining other factors as constant as possible. These studies were mainly at the organismal levels and as Akberali and Trueman (1985) has put it "It is now perhaps the right time for sub-lethal levels of the heavy metals to be examined in detail at tissue and cellular level". Sub-lethal effects can have far more serious long-term consequences on various processes which can ultimately affect the survival and propagation of species. In the past, in studies dealing with sub-lethal

effects of heavy metals, particular attention was given to specific organs such as mantle, gills, kidney, digestive gland,gonad etc. but haemolymph was seldom considered as an organ system. Therefore, in my studies, attention was given specifically to this system because bivalves have an open circulatory system and hence the body tissues are continuously bathed in haemolymph, and the transport of materials to the environment or to other organs from the site of entry occurs through circulating system, and it can be a reliable initial indicator of the type of metabolic response occurring within the organism under stress.

As mentioned earlier, biotic and abiotic factors cause stress and induce adverse effects on tissues, cells and cellular organelles. In many cases, the earliest detectable changes of "primary events" are associated with a particular type of subcellular organelle such as the lysosomes, endoplasmic reticulum and mitochondria. Many marine invertebrates, and molluscs in particular, have organs or tissues whose physiological functions are heavily dependent on a highly developed lysosomal system. Functional disturbance of these lysosomes, will lead to deleterious consequences for the organism (Moore and Farrar, 1985). The lysosomal-phagosomal complex forms a vacuolar intracellular digestive system capable of catabolising both endogenous cellular components and exogenous substances which are engulfed by the processes of autophagy and heterophagy, respectively. Under conditions where organisms are stressed, autophagy can function as a physiological survival mechanism or in severe cases, it can represent a pathological condition (Ericsson, 1969). In contrast, heterophagy involves the endocytosis, ie., pinocytosis and phagocytosis of materials, frequently nutritive, from the extracellular environment and their

subsequent transport into the lysosomal vacuolar system, thus providing the means for the intracellular digestion of external substances. Besides autophagy and heterophagy, the lysosomes are involved in other physiological activities such as storage, excretion, resorption, cell proliferation, immune mechanism, and in the control of cellular immunity (see Moore, 1982; Patel and Patel, 1985).

One of the fundamental biochemical properties of lysosomes is the structure-linked latency of their hydrolytic degradative enzymes which is a direct consequence of the impermeability of the lysosomal membrane to many substrates as well as the internal membrane bound nature of many of the enzymes which renders them inactive (Moore, 1980). But the membrane stability of the lysosomes can be altered under certain conditions which will subsequently activate and in some instances release, the previously bound enzymes (Moore, 1980). Lysosomal destabilisation has been demonstrated in the mussel Mytilus edulis in response to hypothermia and thermal death, starvation, reduced salinity and experimentally induced spawning, variety of polycylic aromatic hydrocarbons as well as hormones (Moore et al., 1979; Moore, 1980; Moore, 1982; Pipe and Moore, 1985; Patel and Patel, 1985; and others).

It has been established that molluscan haemocytes, especially granulocytes represent a major site of lysosomal enzyme synthesis although the head foot and visceral mass are also considered to be the sources for lysosomal enzyme synthesis (Cheng, 1983a). Under normal condition these acid hydrolases are restricted to within the lipoprotein lysosomal membrane; however, if the granulocytes are challenged with a variety of abiotic and biotic factors, the

lysosomes become unmasked or destabilised (Moore et al., 1979; Cheng, 1983b). Studies by Cheng and his associates have revealed that challenge with bacteria results in the hypersynthesis of lysosomal enzymes within haemocytes and the subsequent release of these hydrolases into serum through degranulation (see Mohandas et al., 1985; Mohandas, 1985). Obviously, during stress caused by abiotic as well as biotic factors, there is release of acid hydrolases from the lysosomes into the serum. This is yet another reason to choose haemolymph as an organ system to understand the effect of stress caused by the abiotic factor (copper) and the biotic factor (bacteria) in the present study.

Xenobiotic (toxic environmental chemical)-induced alteration of the permeability or fluidity of the lysosomal membranes could result in interference with the normal processes or turnover of intracellular proteins and organelles (autophagy) as well as the intracellular digestion of pinocytosed or phagocytosed food (Moore, 1982), and hence it was thought worthwhile to investigate the activities of protein, carbohydrate and glycogen in the haemolymph of clams exposed to copper and bacteria. By activation of a xenobiotic to a more toxic molecular species mediated by cytochrome-p-450, which is an integral component of the endoplasmic reticulam, damage is caused to DNA, protein or membranes (Moore, 1985). Moreover, changes in ATP content and in protein synthesis following sublethal exposure to copper have been reported by Viarengo et al. (1980a).

Since stress imposed by abiotic and biotic factors causes the release of acid hydrolases from lysosomes into the haemolymph, it was thought worthwhile to investigate the release pattern of acid phosphatase, the marker

lysosomal enzyme, in the haemolymph of clams dosed with copper, and exposed to bacterial challenge. It is known that heavy metals can inhibit the activity of many enzymes (Dixon and Webb, 1967; Mahler, 1961; Roesijadi, 1980-1981) or stimulate the activity (Iordachescu et al., 1978). The result may be a disturbance of metabolic integration leading to damage to cellular function. It is also known that many toxic substances or their metabolites cause cell injury by reacting primarily with biological membranes, and among the damages are included, changes in the content or activity of enzymes or other membrane components (Moore, 1985). It is noted that alkaline phosphatase is considered as a marker enzyme of plasma membrane (Bogitsh, 1974). Since granulocyte plasma membrane is involved in phagocytosis also (Mohandas et al., 1985), it was thought worthwhile to investigate the activity pattern of this marker enzyme in the haemolymph of clams dosed with copper, and exposed to bacterial challenge.

While many molluscs are well adapted to survive anoxic or hypoxic conditions, there are only limited uniformity in the biochemical pathways in the energy metabolism that are employed (Kluytmans et al., 1980). Further, in bivalves anaerobic glycogen breakdown results in multiple end-products whose formation differs considerably among species, fluctuates quantitatively with time and is dependent on several factors (Zurburg and Kluytmans, 1980; Meinardus and Gäde, 1981). Lactate, which is the classic end-product of carbohydrate fermentation, is normally not considered by some as a main anaerobic product during anoxia/or hypoxia in bivalves where accumulation of succinate or alanine occurs. As exception to this was reported by a few (Gäde, 1980; Zurburg and Ebberink, 1981), an experiment was designed to

determine the lactic acid levels in the haemolymph of S.scripta exposed to sublethal concentrations of copper and mercury with a view to find out whether (i) lactic acid could be one of the end-products, if not the major one, in bivalves under environmental hypoxic conditions prevailing within the shell, and (ii) whether haemolymph could be taken as an organ system in studies related to end-product accumulation in specific tissues of bivalves.

So, in general, the four important rationales for studying the various aspects of molluscan blood are: (i) to understand how the economically important molluscan species defend themselves against infectious agents as this is particularly important in molluscan culture programmes, (ii) to understand the various aspects of molluscan blood for academic reasons as very little work has been done in India on molluscan blood, (iii) to understand the internal defence mechanisms of undesirable molluscan species so that some methods may be devised to reduce or suppress their ability to recognize and to degrade pathogens, and as a consequence render them more vulnerable to the attack by microbial biological control agents, and (iv) to monitor pollution in marine and brackish water environments as it has been suggested that quantitative determination of the levels of the lysosomal enzymes can be employed as a reliable indicator of the stress by environmental pollutants.

In the present study, the two species of clams used were Sunetta scripta (Linné) and Villorita cyprinoides var. cochinensis (Hanley). While S.scripta is a typical marine form, showing a salinity tolerance of 25‰ to 35‰ (Latha Thampuran, personal communication), V.cyprinoides var.cochinensis is considered to be a brackish water form showing a salinity tolerance of 4.73‰ to 27.11‰ (Sivankutty nair and Shynamma, 1975). Specimens of S.scripta

were collected from off Fort Cochin, and V.cyprinoides var.cochinensis from the backwaters at Nettor, 5 km south of Ernakulam.

The thesis is arranged in eight chapters. In the first chapter, opinions of different workers on the various aspects of bivalve haemolymph and haemocytes, and their functional role including the part they play in defence mechanisms are brought together in the form of a review. The second chapter is on the haemocytes, and the third one deals with the variation of haemolymph glycogen and carbohydrate concentrations in the four size groups of the two clam species, and also when subjected to abiotic and biotic challenges. The fourth chapter deals with variations in protein concentrations in the haemolymph under the same conditions as mentioned above. Variations in the haemolymph acid and alkaline phosphatases activities in clams of the four size groups, and subsequent to abiotic and biotic challenges are outlined in the fifth and sixth chapters, respectively. The seventh chapter contains observations on the end-product accumulation study in S.scripta exposed to sub-lethal concentrations of copper and mercury. Summary of the work forms the eighth chapter, followed by the list of references.

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CHAPTER I

REVIEW OF LITERATURE

The cells of the haemolymph -haemocyte- in bivalves are distributed throughout the vascular system, and since the bivalves possess an open circulatory system the haemocytes are also found in the tissues. Earlier literature indicates the presence of different types of haemocytes in bivalves and the parameters used to differentiate them were cell size, nuclear size, presence or absence of granules, differential staining of the cells and their inclusions, etc. Recent studies, however, show that many of the different types of haemocytes can be grouped into one or two categories. A brief review of literature given below lays emphasis on the types of cells, haemopoiesis, ontogeny, cell behaviour and the functional role of haemocytes, such as shell repair, nutrient digestion and transport, excretion, wound repair, and internal defence.

In this thesis, and particularly in the review part, the term, leucocyte, haemocyte, blood cell, haemolymph cell, and amoebocyte are used rather loosely but convey the same idea, and mean only the haemocytes, ie., the cells of the haemolymph.

CELL TYPES

The classification of the types of cells that occur in haemolymph of molluscs originated with Cuenot (1891). Numerous subsequent investigators have followed this line of research. The earlier studies as well as many recent ones have employed static morphological features, staining properties of nucleus, cytoplasm and granules etc., to classify these cells, but in most cases the interpretations have been conflicting. Cheng (1981) has given a detailed account of the designations given to the various types of bivalve haemocytes, and the characteristic features of these cells. When the

classification systems for the molluscan haemocytes employed by the various authors are analysed, it is apparent that there are only two principle patterns. The first, originated by Cuenot (1891), recognises three categories of cells; finely granular haemocytes, coarsely granular haemocytes and cells with very little cytoplasm. The second system attributable to Takatsuki (1934a), involves classifying these cells into two categories-granular cells i.e., those with conspicuously large numbers of cytoplasmic granules, and hyalinocytes, i.e., those with less cytoplasm and including only a few granules or none. All subsequent classification systems may be considered modifications of one of these two systems.

The formerly employed system of distinguishing between several types of cytoplasm-homogenous, granular, alveolar, fibrilar, etc., (Jordon, 1952)-is no longer considered valid since morphology of cytoplasm at the light microscope level is easily altered by employing different fixatives. Moreover, electron microscope studies on blood cells of several species of molluscs have revealed that the cytoplasmic granules are functional organelles such as the mitochondria, lysosomes, phagosomes, Golgi etc. (Cheng, 1984). Variations in the numbers of one type of organelle over another coupled with the methods of fixation could result in morphological differences at light microscope level. Also, differences in the dimensions of the cytoplasmic granules could reflect ontogenies of different stages during the physiological cycle of a cell.

Cell dimensions also have been employed as a criterion for distinguishing between types of haemocytes, but the recent contributions on the behaviour of molluscan haemocytes particularly their spreading behaviour, give clear indications that dimensions of haemocytes measured in vitro vary considerably.

The cell sizes by themselves are not a totally reliable criterion. It is, however, noted that differences in granulocytes and other interchangeably designated agranulocytes are based on cell dimension but this is not the only difference. It should be emphasized that real size differences may occur among structurally similar cells as has been pointed out by Renwrantz et al. (1979). Rather than classifying the different size-classes of granulocytes as being distinct, Cheng (1984) considers them to be of one kind, but at different developmental stages. The same holds true for the different types of granular cells of Crassostrea gigas as described by Tanaka et al. (1961), of Mytilus coruscus as described by Feng et al. (1977), of Mercenaria mercenaria as described by Moore and Eble (1977) and for the different types of lymphocytes of Arca inflata as described by Ohuye (1937).

Considering that the classification system initiated by Cuénot (1891) based on finely or coarsely granular cytoplasm is not natural, Cheng (1981, 1984) accepted Takatsuki's (1934a) system which considers molluscan haemocytes to be of two categories, granulocytes and hyalinocytes. But this system has been modified because of the differences in the staining affinities of granulocytes. Dundee (1953), Ruddell (1971b, c), Narain (1969, 1972a) Hazleton and Isenberg (1977), among others, considered the granulocytes with acidophilic granules to be distinct from those with basophilic and sometimes neutrophilic granules.

But critical microscopical studies on a large number of identically treated cells of clams and oysters by Galtsoff (1964), Feng et al. (1971), Foley and Cheng (1972, 1974), and others, have revealed that granulocytes may include two or three combinations of acidophilic, basophilic and

neutrophilic granules. Thus the demarkation between types of granulocytes based on the staining affinities of the granules seems unnatural, especially when it is known that most of the cytoplasmic granules in some species are true lysosomes (Yoshino and Cheng, 1976a; Moore and Eble, 1977; Mohandas et al., 1985), or as in the case of oysters, are secondary phagosomes (Cheng and Cali, 1974; Cheng, 1975). The occurrence of both alkaline and acid phosphatases in the cells and serum of Crassostrea virginica and M.mercenaria (Cheng and Rodrick, 1975) indicates that both basophilic and acidophilic granules may be present in the granulocytes. This indicates that one of the two phenomena mentioned below or both may be taking place: (1) There are vesicles that have an alkaline milieu in which alkaline phosphatase can function, and there are other vesicles that have an acidic milieu in which acid phosphatase and other acid hydrolases can function, and (2) All of the granules or vesicles go through an alkaline phase prior to becoming acidic. Evidence favouring the first interpretation was provided by Yoshino and Cheng (1976a) when they demonstrated that not all of the granules in the granulocytes of M.mercenaria include acid phosphatase activity. Evidence for the second hypothesis again comes from the work of Yoshino and Cheng (1976a) which is interpreted to mean that the cells they examined were entering the acid phase. Supportive evidence for the second hypothesis includes the finding of several workers that all of the hydrolases associated with bivalve granulocytes are lysosomal acid hydrolases (see Cheng, 1981, 1984).

Another characteristic that has been employed to distinguish between types of haemocytes is the acidophilic and basophilic nature of the nucleus (Feng et al., 1977). It is, however, noted that the basophilic or acidophilic

nature of the nucleus is a function of the intranuclear RNA synthesis which changes with age and/or primary function at the time, and hence cannot be relied as a diagnostic characterisitic.

The basophilic or acidophilic nature of the cytoplasm has also been pointed out as a diagnostic criterion (Dundee, 1953). It is interesting to note the finding of Foley and Cheng (1972) who have demonstrated that staining of the cytoplasm of haemocytes, specifically the granulocytes, of C.virginica is dependent on the fixative used. Also, the basophilic or acidophilic nature of the cytoplasm may depend on the staining characteristic of the nucleus at that time, and hence this diagnostic criterion is also not reliable.

In view of the reasons for rejecting certain commonly employed criteria to distinguish the so-called types of molluscan haemocytes, it now appears that there are only two categories of cells, granulocytes and hyalinocytes or agranulocytes (Cheng, 1984). However, there are detectable staining and other morphological alterations that occur during aging and functionalships during the life of cells. In this context the reports of Renwrantz et al. (1979), Yoshino et al. (1979), and Cheng et al. (1980b) that there are sub-populations of granulocytes in C.virginica, serve to point out that subtle differences, most yet unexplored, do occur among molluscan haemocytes, but these differences most probably reflect functional differences during the ontogeny of cells of the same line.

HAEMOPOIESIS

In vertebrates there are definite erythropoietic and leucopoietic areas. As to the haemopoietic sites of bivalve haemocytes, there are no definitive answers. In the case of bivalves, Cuénot (1891) suggested that haemocytes

may have their origin in special "glande lymphatiques" at the base of the gills. Wandering phagocytic cells have been described as originating in connective tissues, from the epithelial layers of various tissues, and from other sources (Wagge, 1955). According to Narain (1973), K. Tanake in Japan considered the alimentary tract to be the center of haemopoiesis in bivalves and suggested that the breakdown products of food may be utilised for haemocyte production by the ciliary lining of the intestinal wall. Wagge (1951) has reported that in the gastropod Helix aspersa certain haemocytes originate from the mantle epithelium and others from the connective tissue of the mantle. Millott (1937) in Jorunna tomentosa described the formation of haemocytes by budding from the hepatopancreas. Müller (1956) reported that blood cells of the gastropod Lymnaea stagnalis are formed continuously in connective tissue, particularly in the lung. Pan (1958) suggested that the haemocytes of the gastropod Biomphalaria glabrata may be formed from fibroblasts situated in the trabeculae of the mantle blood sinuses and from a cellular reticulum found in the wall of the kidney near the pericardium. Kinoti (1971) in Bulinus truncatus; Lie et al. (1975) and Joky et al. (1985) in B. glabrata have identified what they consider as amoebocyte-producing organ (APO). In B.glabrata it is situated between the pericardium and the posterior epithelium of the mantle cavity. The ultrastructure of the amoebocyte-producing organ in B.glabrata as reported by Jeong et al. (1983) consists of small clusters of primary amoeboblasts resting on the epithelial cells lining the pericardium. The amoeboblasts are held in a loose reticulum formed by extensions from smooth muscle and few fibroblastic cell. Secondary amoeboblasts and amoebocytes constitute further stages of this cell line.

Amoebocytes resembling cells in the snail circulation, appear in the blood sinus passing through the interior of the amoebocyte-producing organ.

Gatenby and Hill (1934), Haughton (1934), Bourne (1935) and Crawford and Barer (1951) who examined tissues of H.aspersa maintained in culture, have reported that haemocytes differentiate from connective tissue, particularly that found in the mantle. Cheng and Rifkin (1970) hesitate to accept this as irrefutable fact because certain types of molluscan cells maintained in culture, particularly epithelia and connective tissue elements, such as fibroblasts, are capable of becoming dissociated from surrounding cells, rounding up and even becoming phagocytic. This can be interpreted as dedifferentiation, so common among certain types of cells maintained in culture.

In addition to the formation of leucocytes at presumably specific haemopoietic sites, various investigators have reported that these cells are capable of multiplying by division. Bohuslav (1933a, b) has expressed the opinion that haemocytes migrating from tissues maintained in culture can divide both by mitosis and amitosis while Gatenby (1932), Gatenby and Hill (1934), Gatenby et al. (1934), and Bourne (1935) have described only amitotic division of haemocytes associated with cultures of the mantle of the snail H.aspersa. Gresson (1937) in Modiolus, and Fretter (1939) in Philine asperta reported leucocytes undergoing division. In addition to nuclear division, Bourne (1935) described cytoplasmic fragmentation as another mechanism for the formation of new cells.

No satisfactory account has yet been published as to where haemopoiesis actually occurs in bivalves. It is generally believed that haemocytes arise from differentiation of connective tissue cells (Cheng, 1984).

In vertebrates, apart from the recognition of erythropoietic and leucopoietic areas, the sequences of development of blood cells are also worked out. The sequence of development is called ontogeny. The ontogeny of bivalve haemocyte is also not clearly understood, and there is difference of opinion.

ONTOGENY

If haemocytes are formed primarily from the differentiation of haemopoietic tissue cells, it would appear essential to recognize that ontogenic stages occur between the youngest blast cells and the mature circulating cells.

The two detailed accounts of the ontogeny of molluscan haemocytes are those of Mix (1976), and of Moore and Lowe (1977). Their accounts are mostly based on interpretive evaluations. In addition, Cheney (1969), in an unpublished dissertation, has presented some information. Mix (1976) based on the data of several investigators, postulated the occurrence of a leucoblast which is the stem cell. It differentiates into a hyalinocyte. Subsequently these hyalinocytes are believed to differentiate into "granular hyalinocytes" or two classes of "intermediates". Following this, the granular hyalinocytes are believed to differentiate into basophilic and acidophilic granulocytes, while one of the "intermediates" is believed to give rise to fibroblasts and myoblasts, while the second group of "intermediates" is postulated to differentiate into pigment cells.

Moore and Lowe (1977) recognised two developmental series: (1) the basophilic haemocyte growth series, and (2) the acidophilic haemocyte growth series. The basophilic haemocyte growth series is comprised of a variety of

structural types. The youngest in this series are small (4-6 μm in diameter), and include a small volume of basophilic hyaline cytoplasm. They are generally spherical and include a spherical nucleus. As the cytoplasmic volume increases, it becomes strongly basophilic and with a high RNA content. Interpretatively, it was proposed that as these small basophilic hyaline cells develop, they become larger basophilic haemocytes (7-10 μm in diameter). The RNA content later decreases in these cells, and the cells frequently take on an irregular appearance, produce pseudopods, and develop cytoplasmic granules and vacuoles. Nuclei also become irregular in outline.

The second growth series, the acidophilic line, is represented only by the eosinophilic granular haemocytes. These cells measure 7-12 μm in diameter, and the degree of acidophilic nature of the cytoplasmic granules increases with growth. They regarded this as a separate line, as no morphologic forms were observed which might represent cells intermediate between macrophages and granular haemocytes.

Cheng (1984), based on accumulated data, has given another ontogenic line. He postulated a hypothetical granuloblast. From this cell arises young granulocytes designated as progranulocytes characterised by relatively small size, few cytoplasmic granules and basophilic nucleus. They are the smallest of the circulating cells of the granulocyte line, are capable of phagocytosis, though not actively, and also produce few pseudopodia when spread.

The progranulocyte gives rise to granulocyte I which is a medium-sized cell with numerous cytoplasmic granules some of which are basophilic, others acidophilic, and still others refractile. They are actively phagocytic, with pseudopodia of filopodial type, and have increased numbers of organelles such

as Golgi, rough endoplasmic reticulum, liposomes and lysosomes, and have relatively high levels of acid hydrolase activity. They are capable of contributing to clumping.

The granulocyte I matures into a granulocyte II which is the largest of the granulocyte line, containing large numbers of cytoplasmic granules, most of them being acidophilic. They have cytoplasmic vacuoles, Golgi, smooth and rough endoplasmic reticulum, liposomes and lysosomes. They are actively phagocytic, produce large numbers of semi-permanent filopodia when spread, have the highest levels of acid hydrolase activity, and actively involve in clumping.

If a granulocyte II has phagocytosed foreign materials and intracellular degradation has occurred, it becomes a spent granulocyte (earlier designated as fibrocyte). Such a cell is characterised by a few filopodia commonly at two poles, a few cytoplasmic granules, larger number of cytoplasmic vacuoles of various shapes and sizes, less lysosomal and hydrolase activity, and their phagosomes commonly include digestive lamellae and amorphous, partially digested materials. In their cytoplasm, clumps of glycogen granules will be present.

Under certain pathological conditions, several granulocyte IIs may become fused to become a multinuclear giant cell, designated as a macrocyte.

A hypothetical hyalinoblast is proposed for the hyalinocyte line also by Cheng (1984). According to him, hyalinoblast is capable of dividing and differentiating into young hyalinocytes, designated as prohyalinocytes. Such a cell is characterised by a relatively large nucleus surrounded by a small volume of cytoplasm, absence of cytoplasmic granules, none or a few lobopodia,

and the cell is essentially basophilic.

The prohyalinocyte matures into a hyalinocyte which has a relatively small volume of cytoplasm surrounding a large nucleus. It includes few cytoplasmic granules and moves by producing a small number of lobopodia.

There is a third line comprised of serous cells, brown cells or pigmented cells. Although these cells do occur in the general circulation, they are formed in Keber's glands.

The molluscan haemocytes are highly variable in behaviour, ie., spreading, migrating, endocytosing foreign materials, dimensions, staining affinities and in other morphological features (Cheng, 1984). So, further studies are needed to derive any conclusive opinion on the ontogeny of haemocytes.

Having reviewed the ontogeny of haemocytes, it is only appropriate to look into the behaviour of these haemocytes in vivo and in vitro.

CELL BEHAVIOUR

Feng (1965a) has observed that in C.virginica, the number of circulating leucocytes is dependent upon the amount of turbulence produced by cardiac action. He has also proposed that number of leucocytes may also vary with the feeding and excretion cycles since leucocytes participate in both these activities.

When living cells of C.virginica are first placed on a glass slide, they are mostly irregularly oval (Foley and Cheng, 1972). When freshly examined, the cells include a single or several tufts of filopodia-like extensions. It is noted that fresh cells commonly form clusters. However, if the preparation is permitted to stand, there is an exomigration of cells from each cluster in a defined pattern. The migrating cells commonly become crescent-shaped

and the activity generally commences at about 5 min. after freshly drawn haemolymph is placed on slides maintained at 22° C. When permitted to stand for several minutes they commence to spread against the substrate. This begins within 15 min. post preparation. The spreading process is initiated when the cells become more or less spindle-shaped, and with the filopodia irregularly situated along the surface. As time progresses the peripheral cytoplasm of each cell tends to flatten out against the substrate so that the cytoplasmic granules and vacuoles become confined to the endoplasm, and at this time unbranched pseudopod generally occur at the periphery. The adherence of cells to the substrate is attributed to positive thigmotaxis (Foley and Cheng, 1972). By the time the spreading process terminates, in 15 to 25 min., fine rib-like cytoplasmic rays extend towards the periphery from the region of endoplasm and terminate along the outer margin of the cell as fine cytoplasmic extension.

When haemocytes of M.mercenaria are placed on a slide, mostly they are irregularly oval- or spindle-shaped (Foley and Cheng, 1974). Upon contacting the slide, both agranular and granular leucocytes adhere and commence to spread. Haemocytes will adhere to and spread on both the upper and lower glass surfaces. Spreading of individual cells begins with adherence to the slide. Cells that do not adhere do not spread. Haemocytes not only adhere to glass but also to one another. In M.mercenaria, agglutination of the haemocytes occur in vitro, and is more pronounced if the haemocytes are mixed with sea-water or with homologous serum. The spreading behaviour of M.mercenaria leucocytes was employed by Foley and Cheng (1974) to identify the cell types.

In the living state, haemocytes of M.mercenaria take up vital dyes (Foley and Cheng, 1974). The cytoplasmic granules take up Neutral Red from a concentration of 0.02% within 15 sec. The refractile inclusions do not take up the dye. When exposed to Janus Green B at a concentration of 0.002% nearly all of the cytoplasmic granules take up the dye rapidly. After 15 min., some granules appear violet or purple, thus indicating possible reduction of this dye to diethyl Safranin. Refractile granules do not take up this dye. When exposed to a mixture of Neutral Red and Janus Green B at a final concentration of 0.001% for each stain, the cytoplasmic granules appear red.

FUNCTIONS OF HAEMOCYTES

Haemocytes are known to serve a variety of functions, and are involved in shell repair, nutrient digestion and transport, excretion, wound repair, and internal defence against foreign materials.

I. SHELL REPAIR

The blood cells of lamellibranchs have been implicated in the transfer of shell material to the site of shell formation during the repair of shell damage (Dunachie, 1963; Beedham, 1965; Saleuddin, 1967; Bubel, 1973). In almost all cases amoebocytes migrate on to the outer surface of the mantle epithelium at the early stage of shell repair (Watabe, 1983), but in Musculus and Anodonta the amoebocytes adhered to the basal part of the outer epithelium during shell repair (Kawaguti and Ikemoto, 1962; Tsujii, 1976). But Saleuddin (1970) rarely observed amoebocytes in the mantle of Helix.

The reports on the function and time of migration of amoebocytes are controversial (Saleuddin, 1980). Beedham (1965) has reported that amoebocytes pass through breaks in the damaged epithelium and form a protective barrier

for the mantle before the formation of organic membrane in Anodonta. But Kapur and Gupta (1970) in Euplecta indica, Watabe and Blackwelder (1976) in Brachydontes exustus, and Watabe and Blackwelder (1980) in Pomacea paludosa showed that the migration of amoebocytes occurred after the formation of organic membrane at the beginning of the repair. Abolins-Krogis (1963) has made observations in Helix pomatia similar to that of Beedham (1965) but barrier formation was not mentioned.

Amoebocytes have been reported to transport calcium and other repair materials to the sites of shell repair (Wagge, 1951; Dunachie, 1963; Abolins-Krogis, 1963,1972,1973; Kapur and Gupta, 1970; Tsujii, 1976). However, McGee-Russel (1957), and Durning (1957) did not assign them any role in the repair process. Timmermans (1973) and Watabe and Blackwelder (1980) did not detect significant amounts of calcium in the amoebocytes. In their experiment, Bubel et al. (1977) could not find the deposition of shell materials by granulocytes in M.edulis, however, they presumed that granulocytes play a role in this process. Their presumption was strengthened by the report that in M.edulis shell repair is a slow process (Wagge and Mittler, 1953; Meenakshi et al., 1973)

There is, however, general agreement that during shell repair, amoebocytes are involved in the transport of materials from the hepatopancreas to the region of shell repair. Based on the observation of Moore and Lowe (1977) of a close association between granulocytes and Leydig and digestive tubule cells in M.edulis, Bubel et al. (1977) indicated that granulocytes may transport materials to the region of shell repair. Despite several investigations on molluscan shell repair, the evidence for the role played by haemocytes in this

process is somewhat conflicting. As Wilbur (1964), and Beedham (1965) assumed, the amoebocytes may play a limited role in the early stage of shell repair, but the majority of the repair process is accomplished by the mantle epithelium (Watabe, 1983). Abolins-Krogis (1976) has also indicated the significant role played by amoebocytes in shell repair but added that the bulk of the substances needed for the complete restoration of materials of the damaged shell came from other sources in the body.

II. NUTRIENT DIGESTION AND TRANSPORT

The role of haemocytes in digestion has been comprehensively reviewed by Takatsuki (1934a), Wagge (1955), Owen (1966), Purchon (1968) and Narain (1973). In molluscs, digestion of food is both extra and intracellular. Extracellular digestion takes place in the lumen of the intestine by enzymes released from dissolution of the crystalline style. Intracellular digestion takes place within two categories of cells; digestive cells of the digestive diverticula, and haemocytes. As bivalves have an open circulatory system, haemocytes are found migrating through tissues. Some migrate into the lumen of the alimentary tract and pinocytose soluble nutrients and phagocytose particulate food stuffs (Yonge, 1923, 1926; Takatsuki, 1934a; Yonge and Nicholas, 1940; Zacks and Welsh, 1953; Wagge, 1955; Zacks, 1955; Owen, 1966; Purchon, 1968; Narain, 1973; and others). Once inside the cell, digestion commences and the haemocytes pass back into the deeper tissues of the body and in this manner transport nutrients to various tissues (Yonge, 1926). George (1952), however, was unable to show that amoebocytes in the gut were able to pass into the tissues. The finding of Feng et al. (1977) of carotenoids, specifically flavenoids, β-carotene, canthaxanthin and other unidentified xanthophylls,

which can only be synthesized by plants, in haemocytes indicates that the haemocytes must have acquired them by phagocytosing pigment-bearing unicellular algae which comprise the diet of these filter feeders.

The haemocytes are also capable of reversing this process and capturing unwanted materials in the haemolymph and transporting the material for disposal in the faeces (Stauber, 1950; Tripp, 1958a, 1960; Morton, 1980; Fisher, 1986). Pal and Modak (1981) have suggested that amoebocytes make frequent characteristic, topographic association with the digestive cells of the digestive diverticula implying that their role in digestion, assimilation and waste removal is more important than is at present realised.

In view of the recent findings, nutrient digestion and transport cannot be considered as an isolated function, and it is so intimately associated with internal defence that in many ways the two are indistinguishable.

III. EXCRETION

Durham (1891) reported that degenerated pigments are excreted from Anodonta and Unio via the exodus of laden haemocytes. Canegallo (1924) in Unio and Orton (1923) in oysters reported the excretion of oil globules and metals, respectively. Stauber (1950), Tripp (1960) and Feng et al. (1977) have reported that indigestible particles and macromolecules are voided via, the migration of foreign material-laden phagocytes across certain epithelial borders. Cheng et al. (1969) have demonstrated that Indian ink experimentally injected into Littorina scabra are rapidly phagocytosed by haemocytes, most of which are subsequently eliminated from the body via exodus through the epithelia of the nephredial tubules.

Another aspect of the excretory function of haemocytes involves the

pericardial glands which are also known as Keber's glands. These glands which comprise a part of the excretory system, are prominent in most bivalves occurring in two locations, on the auricle and/or mantle and empty into the pericardial cavity. The Keber's glands are also the sites for the production of the so-called brown or serous cells. Takatsuki (1934b) who gave the most detailed description of brown cells has reported that they are amoeboid and capable of phagocytosing carmine particles. He proposed that they are modified leucocytes. The most important function attributed to serous cells is related to their role in the removal of degradation products of dead or moribund parasites and metabolic by-products of successful parasites (Cheng, 1981).

IV. WOUND REPAIR

It is known that haemocytes are involved in wound repair (Pauley and Sparks, 1965; Des Voigne and Sparks, 1968; Pauley and Heaton, 1969; Ruddell, 1971a; Sparks, 1972). As to the exact mechanism and the extent of involvement, opinions differ. It is said that the clumping of haemocytes is intimately involved in wound healing. Clumped haemocytes have been reported to delineate and plug wounds and arrest haemorrhage (Dakin, 1909; Drew, 1910); Goodrich, 1919; Orton, 1923; Des Voigne and Sparks, 1968, 1969; Ruddell, 1971a; Foley and Cheng, 1972). From these studies, the sequence of events during wound repair is as follows (Cheng, 1981).

1. Infiltration (at least in oysters) of the wounded area by large number of haemocytes.
2. The aggregate haemocytes form a plug as well as delineate the area.
3. Healing proceeds from the interior of the lesion toward the surface.

4. Collagen is deposited between the cells comprising the plug and later both the haemocytes and collagen are replaced by Leydig cells.
5. Meanwhile phagocytic haemocytes infiltrate and phagocytose the necrotic cell debris.

It is noted that there is no fibrin or any other plasma involvement, and the fusion between the haemocytes is not permanent as they can reverse the process and dissolve the clot by returning to an individual existence (Narain, 1973; Fisher, 1986).

But Pauley and Heaton (1969) in Anodonta oregonensis reported little cellular response associated with repair. According to them, the edge of the wound invaginates in an apparent attempt to close the incision and this is the only response noted during the first eight days. A clot is formed only rarely in the wound channel and this results from infiltration and aggregation of haemocytes, and these do not completely fill the wound channel. An extremely weak cellular response also occassionally develops in the surrounding tissues. Initially, a few fibroblasts are intermingled with the haemocytes and these deposit scanty collagen-like material. Subsequently, the haemocytes are almost completely replaced with fibroblasts which are connective tissue cells. Ruddell (1971a) has indicated that fibroblast-like cells are normally associated with wound repair sites. Replacement of the surface ciliated epithelium results from division and migration of adjacent healthy epithelial cells, and this occurs without the formation of a cellular wound plug. Thus, the lack of a pronounced initial cellular response and subsequent lack of involvement of large quantities of connective tissue fibres categorizes wound healing in A.oregonensis.

It has been proved beyond doubt that the haemocytes play an important role in internal defence against foreign materials. In recent years, research on molluscan haemocytes has been directed mainly to understand their role in internal defence against non-self materials. Stauber (1961), in his review on immune mechanisms of invertebrates, informally defined cellular defence as being of three categories, viz., Leucocytosis, Encapsulation and phagocytosis. This scheme has been adopted by several subsequent workers, and has been extended to include Nacrezation also (Cheng and Rifkin, 1970). Like vertebrates, the internal defence mechanisms of invertebrates are also of two types: Innate and Acquired. Innate mechanisms are those that are assumed to be genetically mediated and represent the native capabilities of the host to act against an invader. Acquired mechanisms are those that develop in the host in response to previous exposure to non-self material. Theoretically, both innate and acquired internal defence mechanisms can be of two types, Cellular and Humoral.

INNATE CELLULAR INTERNAL DEFENCE MECHANISMS

Innate mechanisms are of prime importance in host-parasite relationships in invertebrates. The known types of phenotypic manifestations of innate cellular internal defence mechanisms in bivalves comprise such mechanisms as Leucocytosis, Encapsulation, Nacrezation and Phagocytosis.

LEUCOCYTOSIS

Leucocytosis is defined as an increase in the number of leucocytes or haemocytes and is a forerunner of phagocytosis and/or encapsulation since the increased number of cells contributes to these active processes

(Cheng, 1981). Leucocytosis as associated with parasitism, including helminth parasitism, has been conveniently summarised by Farley (1968). It is still not known whether these cells are formed de novo or represent cells released from sequestration sites in tissues.

ENCAPSULATION

Encapsulation involves the enveloping of an invading organism or experimentally introduced tissue too large to be phagocytosed by host cells. Studies directed at molluscs, in general, and bivalves in particular, have been reviewed by Cheng and Rifkin (1970). They have also attempted to classify the different capsules formed in terms of both the parasite and host material used in the capsule. When a parasite on entering the host, is recognised as non-self, usually the first reaction is leucocytosis. Many of these cells migrate towards the parasite and form a capsule of discrete cells around it. It is believed that this migration of cells is the result of chemotaxis in response to molecules incorporated in parasite's body surface and in other instances to secreted molecules which in the case of trematodes and cestodes, are usually mucopolysaccharides, in the case of nematodes it was reported to be the exsheathing fluid (Cheng, 1986), and in the case of protozoa and bacteria, it could be a relatively small peptide (Howland and Cheng, 1982). Rifkin and Cheng (1968) reports another mechanism in C.virginica infected by the larva of the tapeworm Tetragonocephalum. Here, the parasite compresses the surrounding connective tissue cells and this triggers the host cells to synthesize the precursor of the fibrous material which becomes deposited intercellularly, and gradually become concentrically deposited in layers around the parasite (Rifkin et al., 1969). After the parasite is encapsulated by

fibres, the granulocytes and the brown cells migrate into the matrix of the capsule. This migration is followed by the death of the parasite, and eventually the parasite's tissues are completely disintegrated and the fragments become phagocytosed by host granulocytes. Besides these two types of encapsulation, other minor types are also known which also involve blood cells and connective tissue fibres (Cheng and Rifkin, 1970). According to Gotz (1986), amoebocytes, mainly granular, infiltrate to the region where the foreign body is introduced. The amoebocytes which are directly attached to the foreign body surface flatten and extend protrusions into the irregularities of the foreign surface. The cellular envelope solidifies by the interdigitation of pseudopodia. Within the flattening cells microtubules and bundles of microfilaments appear. The intercellular spaces between the middle and outer layer become filled with connective tissue fibrils in an amorphous ground substance which is synthesized by amoebocytes transformed into fibroblasts. The extent of capsule formation differs depending upon the degree of compatibility of the host and the parasite. The capsules are thick in non-susceptible hosts whereas in susceptible ones there is no or a very little reaction. The activity of encapsulating haemocytes is not restricted to the formation of a solid capsule but also for the formation of a cellular envelope. They infiltrate and phagocytose the foreign tissue until only a small "granuloma", mainly of connective tissue, is left behind.

Cheng and Rifkin (1970) have proposed that encapsulation represents aborted attempts at phagocytosis, and this concept is based on the observation that the first cells to approach a foreign body too large to be phagocytosed become initially flattened against the surface of the foreign body in a fashion

which could be interpreted to be unsuccessful attempts at phagocytosis.

It is of interest to note that not all parasites too large to be phagocytosed become encapsulated. This is suggestive of co-evolution of the host and the parasite (Cheng, 1986; Fisher, 1986). The fates of encapsulated parasites differ, while some are destroyed, others continue to develop normally. The reason for this difference remains unresolved.

NACREZATION

Nacrezation (a term coined by Cheng, 1967) or pearl formation, is another type of cellular defence mechanism known in molluscs. When zooparasites or grains of sand occur between the inner surface of the shell (nacreous layer) and the mantle of bivalves, the mantle is stimulated to secrete nacre that becomes deposited around the object. The result is pearl formation.

PHAGOCYTOSIS

Phagocytosis is a well known type of internal defence mechanism in invertebrates. It involves the uptake of foreign materials by certain types of host cells and thus prevents direct contact of such materials, biotic or abiotic, with the host's tissues. That molluscan haemocytes are capable of phagocytosis has been known since Haeckel's (1862) report on Helix and Thetis. In vitro experiments have demonstrated that haemolymph cells of eleven species of fresh water mussels phagocytose carbon and carmine particles (Dundee, 1953) and haemolymph cells of C.virginica phagocytose various marine bacteria (Bang, 1961), yeast (Eble and Tripp, 1969), and viruses (Fries and Tripp, 1970).

It is noted that not all foreign organisms inside the body of molluscs are phagocytosed. It is reported that the mutualistic zoochlorellae in Lymnaea,

Anodonta and Unio are seldom found within host cells (Yonge and Nicholas, 1940; Goetsch and Scheuring, 1962; Buchner, 1965), and are presumed to lead an extracellular existence. Similarly, it is noted that all invading organisms will not induce phagocytosis. It has been reported that in C.virginica the presence of the sporozoan Minchinia nelsoni and virus Staphylococcus aureus phage 80 induces little or no phagocytosis (Feng, 1966a, 1967; Feng and Stauber, 1968). Again, it is noted that not all phagocytosed foreign materials are eliminated; some are retained within the cytoplasm of host cells for longer periods while others, specifically, a number of micro-organisms and the fungus Perkinsus marina, grow and multiply therein (Prytherch, 1940; Mackin, 1951; Michelson, 1961).

CELLS INVOLVED IN PHAGOCYTOSIS

In the early 70's, it was very uncertain which cell type is the most active from the stand-point of phagocytosis, though qualitative observations had indicated that in C.virginica, the granulocyte is the active phagocyte (Bang, 1961; Galtsoff, 1964; Cheng and Rifkin, 1970). Recent evidences have piled up to show that in C.virginica, M.mercenaria and Mytilus campechiensis, it is the granulocytes that are the most active from the stand-point of phagocytosis (Foley and Cheng, 1975; Rodrick and Ulrich, 1984). But in Pinctada radiata (Nakahara and Bevelander, 1969), in C.gigas (Ruddell, 1971a,c), and in Tridacna maxima (Reade and Reade, 1972), the agranular cell has been reported to be the actively phagocytic cell. Moore and Lowe (1977) considered these haemocytes as functionally analogous to macrophages in Mytilus but did not consider them as agranular as they contained granular inclusions. In a critical review of the existing literature on bivalve haemocytes, Cheng (1981)

concluded that the smaller phagocytic macrophages described by Moore and Lowe (1977) are younger (smaller) granulocytes, and Rasmussen et al. (1985) comparing their studies on the leucocytes of M.edulis with those of Moore and Lowe (1977) opined that the macrophages are similar to the large granular leucocytes.

ENERGY REQUIREMENTS DURING PHAGOCYTOSIS

Phagocytosis is an energy requiring process. Cheng (1976a) has reported that there is no increase in oxygen consumption by M.mercenaria haemocytes actively phagocytosing Bacillus megaterium, indicating that glycolysis is the energy-providing pathway. This conclusion was strengthened by the fact that KCN does not inhibit phagocytosis. It was also reported that nitroblue tetrazolium reduction, charecteristic of mammalian phagocytosis, is absent in M.mercenaria haemocytes, and the myeloperoxidase -hydrogen peroxide- halide antimicrobial system of mammalian phagocytes is also wanting.

FACTORS AFFECTING PHAGOCYTOSIS

Various factors affect phagocytosis, many of which are not well understood. In all the in vivo studies, the rate of clearance of bacteria from the body of bivalves has been shown to be slower (Feng, 1966b; Feng and Stauber, 1968) or less efficient (Feng and Feng, 1974; Foley and Cheng, 1975) at lower temperatures ie., 5-9° C. From the available data of in vitro studies, it is now known that phagocytic activity is enhanced by higher temperatures (Foley and Cheng, 1975; Rodrick and Ulrich, 1984), and this finding is in general accord with in vivo experiments on clearance rates performed by others. Preliminary evidence from on-going research implies that the influence of temperature may be, atleast partially, due to its effect on haemocyte

locomotion which has a Q_{10} of about 2 (Fisher, 1986). Lower particle clearance in vivo at lower temperatures in oysters may be because of the decreased ability of the cells to phagocytose heterologous materials at lower temperatures, and also due to the effect of lower temperature on haemocyte locomotion, as the elimination of bacteria include the phagocytic activities of haemocytes (Bang, 1967) and continued loss of phagocytic cells laded with bacteria to the exterior through epithelial borders (Houser, 1964; Cheng et al., 1969; Malek and Cheng, 1974).

Studies by Cheng and Rudo (1976a), Cheng and Howland (1979), Font (1980), and Cheng et al. (1981) indicate that chemotactic response of the haemocytes to foreign substances vary depending upon the nature of the non-self substances, and it is interpreted to mean that the chemical configuration of the non-self substance affects chemotaxis of the haemocytes which in turn affects phagocytosis. In a subsequent study, Cheng and Howland (1982) showed that colchicine and cytochalasin interfere with microfilaments and microtubules formation in C.virginica haemocytes and produced significant increase in percentage of inhibition of chemotaxis. This implies the necessary need of an intact cytoskeletal system for chemotactic response. Such pharmacological substances which interfere with the process of chemotaxis also affect phagocytosis.

Cheng and Sullivan (1984) have shown that heavy metal ions also affect phagocytosis. Exposure of haemocytes to Hg^{2+} resulted in increased phagocytosis at 0.1 ppm, and at higher concentrations of 0.5,1 and 5 ppm resulted in increasing inhibition of phagocytosis and increasing cell death.

Besides temperature, chemical configuration of the non-self substances,

certain pharmacological substances and heavy metal ions, other factors such as pH, size, and species of molluscs, nature of challenge, lectin binding sites on haemocytes, phagocytosis enhancing factors in serum, integrity of the cell coat or glycocalyx etc. also influence phagocytosis (Mohandas and Cheng, 1985a).

UPTAKE MECHANISM DURING PHAGOCYTOSIS

Currently it is accepted that the mechanism of phagocytosis, actually consists of four distinct stages: (a) Attraction of phagocytes to the non-self material, (b) Attachment of the non-self material to the surface of the phagocyte, (c) Internalization, and (d) Intracellular digestion.

(a) Attraction of phagocytes to the non-self material.

Both the processes, phagocytosis and encapsulation, involve the migration of haemocytes towards the non-self substances. This migration was first demonstrated by Cheng et al. (1974) and was attributed to chemotaxis. Subsequently, Cheng and Rudo (1976a), and Cheng and Howland (1979) demonstrated chemotaxis of C.virginica haemocytes to several species of live Gram-positive and Gram-negative bacteria (but not to heat killed ones). Font (1980) demonstrated chemotaxis of haemocytes of C.virginica to dead (but not live) cercaria of several species of marine trematodes. Cheng et al. (1981) have also demonstrated that haemocytes collected from C.virginica 2hr. post challenge with live B.megaterium were significantly less chemotactic to this bacterium. They attributed this to: (i) qualitative differences in recognition sites on haemocyte surfaces which are capable of recognizing chemotactic signals, (ii) quantitative differences in surface recognition sites on sub-populations of molluscan haemocytes, and (iii) altered composition of serum and other changes subsequent to an insult. In support of all the three, there

are evidences. It is noted that sub-populations of haemocytes occur in C.virginica (Renwrantz et al., 1979; Yoshino et al., 1979; Cheng et al., 1980b) and in gastropods (Sminia and Knaap, 1986). Recent studies on haemocyte lectin receptors and E-rosetting properties of molluscan haemocytes, and studies on their surface antigens by the application of monoclonal antibodies suggest that the population of circulating molluscan haemocytes may actually be composed of molecularly and antigenically distinct cell sub-populations potentially capable of some degree of functional compartmentalization (Yoshino and Granath, 1985). It is possible, as suggested by Cheng (1983a) that the different sub-populations of granulocytes are quantitatively and/or qualitatively differentially stimulated by exogenous factors. In support of the third, it is noted that alterations occur in molluscan serum as a result of infection, and specifically qualitative and quantitative changes were observed in serum protein fractions, in the composition of free amino acid pools, and carbohydrates (Cheng et al., 1981). Also, alterations in the internal defence mechanisms of molluscs as a result of previous challenge are well known (Cheng et al., 1981).

(b) Attachment of the non-self materials to the surface of the phagocyte.

This represents the second phase of cellular response. Invertebrates do not synthesize antibodies, and one class of non-immunoglobulin molecules that may be involved in non-self recognition is agglutinins, and the invertebrate agglutinins that have been characterised, have been shown to be lectins (Sharon and Lis, 1972; Lackie, 1980). Lectins are divalent or multivalent carbohydrate binding non-immunoglobulin proteins that agglutinate cells and/or precipitate glyco-conjugates through interaction with glycoproteins or glycolipids (Boyd and Shapleigh, 1954; Barondes, 1981). Although carbohydrate binding

specificity is unanimous for binding by lectins, secondary forces such as hydrophobicity and charge interaction, possibly operating through multivalent binding, may also stabilise the bond (Olafsen, 1986). In molluscs, lectins have been described from the tissues and sera. A limited number of studies have been conducted to ascertain whether binding sites occur on bivalve haemocytes and also for serum lectins and haemocyte lectins. From studies conducted on these aspects in bivalves by Jenkin and Rowley (1970), Baldo et al. (1977), Hardy et al. (1978), Yoshino et al. (1979), Cheng et al. (1980b, 1984), Vasta et al. (1982, 1984), Renwrantz and Stahmer (1983), and others, a few generalisations could be made: (1) binding sites occur on cell surfaces, (2) haemocyte lectin is an integral plasma membrane protein, (3) the haemocyte lectin is located on the outer surface of the cell membrane and its distribution is irregular, suggesting that "patching"-and "capping"-like phenomena occur, (4) lectins are present in serum also, (5) the haemocyte membrane lectin is serologically related to the serum lectin, (6) distribution of the surface lectin on haemocytes is heterogeneous, ie., not associated with a specific sub-population of cells alone, (7) lectins bind specifically to polysaccharides and glyco-conjugates (a protein-protein recognition system associated with chemotaxis also occurs, (Howland and Cheng, 1982), (8) serum lectins facilitate endocytosis, and (9) different sub-populations of haemocytes show differences in specific surface binding sites. From these findings, it can be surmised that: (1) the non-self particle or molecule revealing a compatible saccharide on its exposed surface can become bound to a membrane lectin, or (2) a non-self molecule or particle with the appropriate sugar moiety on its exposed surface can become bound to the surface of a phagocyte bearing the same sugar by a serum lectin.

In either case, there is specific binding during attachment involving lectins (Cheng, 1985).

In the light of current knowledge of induction phenomena at cellular level what have been stated thus far suggests that atleast three categories of binding or recognition sites occur (Cheng, 1983a). Specifically, there must be one category on the surface of granulocytes that receives the signal generated by the insult, the second category of binding sites on the nuclear surface to initiate the signalling of intranuclear events, and a third category of signal receiving sites on the surface of lysosomes, the activation of which induces degranulation. The occurrence of the first category of binding sites has been demonstrated clearly. The nature of the second and third categories of recognition sites is yet to be investigated.

It has been suggested by several investigators that naturally occurring serum lectins in invertebrates may serve the function of opsonins, although these lectins are chemically quite different from vertebrate opsonins (Robohm, 1984; Cheng et al., 1984). The term "opsonin" is now used to represent any agent in serum which acts on particles to increase their ingestion by phagocytes (Robohm, 1984). Although a limited degree of specificity is reported in molluscan opsonins, it is noted that Mytilus haemocytes apparently can recognize non-self materials in the absence of serum lectins (Bayne et al., 1979b). Renwrantz and Stahmer (1983) have recently shown that Ca^{++} alone could stimulate in vitro phagocytosis of yeast by Mytilus haemocytes in the absence of serum opsonins. On balance, it appears that the efficacy of the recognition function of haemocyte surface receptors can be enhanced by plasma components.

All these studies make one thing clear that the specific chemical interaction of molluscan haemocyte lectins with external ligands can trigger responses which, in many cases, can be directly related to internal defence. As yet, however, it is still unclear how the lectins operate in vivo and the nature and extent of any regulatory mechanism which modulates their reactivity in cellular resistance.

(c) Internalization.

In bivalves, there are three morphologically distinguishable uptake mechanisms. Bang (1961) in C.virginica, and Mohandas (1985) in M.mercenaria have reported that motile bacteria about to be phagocytosed by granulocytes, initially stick to the surface of the granulocyte, generally to the surface of the filopod, and subsequently they are taken into the cytoplasm by gliding along filopodia and become enclosed in a phagosome. The ectoplasm that forms a web between adjacent pseudopods serves as a trap, and the uptake of bacterial cell is effected when they are trapped in the web (Bang, 1961; Mohandas, 1985).

Cheng (1975) in C.virginica, and Mohandas (1985) in M.mercenaria granulocytes observed a second uptake mechanism in which no filopodia is involved. This mechanism involves the formation of invaginations on the cell surface, and the bacteria are endocytosed into vacuoles. In the two mechanisms mentioned above, microtubules are involved as it was shown that the filopods in C.virginica and in M.mercenaria include microtubules, and they extend internally into the cytoplasm (Foley and Cheng, 1972; Cheng, 1975; Mohandas, 1985). The ecdoplasmic web and the lining of the channel through which bacteria pass and become endocytosed also contain a rich array of

microtubules. This finding supports that microtubules are involved in these types of uptake mechanisms.

The third mechanism as reported in C.virginica by Renwrantz et al. (1979) involves the formation of funnel-shaped pseudopodia, and the rat erythrocytes are endocytosed by gliding into a phagosome in the ectoplasm.

In addition to phagocytosis of particulate non-self materials, the pinocytosis of soluble molecules has also been reported (Feng, 1965b). The mechanism(s) involved in pinocytosis by molluscan cells, however, remains uninvestigated.

(d) Intracellular digestion.

The process of intracellular digestion is apparantly the same, whether it is food stuff or engulfed foreign organisms (Cheng, 1981), and has been clarified by electron microscope and biochemical studies. Cheng and Cali (1974), employing C.virginica as the model demonstrated that bacteria are initially taken into large membrane lined vesicles, the primary phagosomes, where the development of digestive lamellae occurs. The partially digested bacteria are transferred to secondary phagosomes where the bacteria are subjected to further degradation. Eventually, the digestive lamellae disappear, and meanwhile, glycogen is synthesized from the degradation product, ie., glucose. Concurrent with the appearance of glycogen granules, primarily as rosettes, the wall of the enclosing phagosome begins to break up and eventually disappear. Clumps of glycogen are set free in the cytoplasm where they coalase. Later the cytoplasmic glycogen is discharged into serum enveloped by the plasmalemma of the granulocytes (Cheng and Cali, 1974; Cheng, 1975). There are now ample evidence that the amount of glycogen

in haemolymph increases after the phagocytosis of bacteria, and the synthesis of glycogen is from sugar of bacterial origin (Cheng, 1975, 1977a,b; Cheng and Rudo, 1976b; Rodrick and Ulrich, 1984).

In M.mercenaria, formation of secondary phagosome appears to be absent, and phagosomal wall do not appear to breakdown so as to release glycogen into the cytoplasm, but the occurrence of phagosomes containing clumps of glycogen granules and nondigestible materials close to the granulocyte plasma membrane with sprout-like extensions merging with the plasma membrane suggests the process of expulsion of these glycogen granules and nondigestible materials from the phagosomes directly to the exterior of granulocytes, obviously into the serum (Mohandas, 1985). The non-carbohydrate constituents are expelled from primary phagosomes to the exterior of the phagocytes as residual bodies (Cheng, 1975; Mohandas, 1985).

Cytoplasmic granules of granulocyte as revealed by light microscopy in M.mercenaria, M.edulis and Mya arenaria have subsequently been shown to be lysosomes (Cheng, 1975; Yoshino and Cheng, 1976a; Moore and Lowe, 1977), and in C.virginica, they were found to be secondary phagosomes (Cheng and Cali, 1974; Cheng, 1975) by electron microscope studies. In all species of molluscs studied so far, lysosomes were found to be involved in the degradation of non-self materials, and the next step was obviously to identify the lysosomal enzymes. Several lysosomal hydrolases in both haemocytes and serum have been identified by employing biochemical and cytochemical methods in M.mercenaria (Cheng and Rodrick, 1975; Cheng 1976b; Moore and Eble, 1977; Yoshino and Cheng, 1976a), M.campechiensis (Rodrick and Ulrich, 1984), C.virginica (Eble, 1966; Cheng and Rifkin, 1970; Feng

et al., 1971; Rodrick and Cheng, 1974; Cheng, 1976b; Yoshino and Cheng, 1976b; Rodrick and Ulrich, 1984), M.edulis (Moore and Lowe, 1977), Mytilus californianus (Bayne et al., 1979b), M.arenaria (Cheng and Rodrick, 1974; Cheng and Yoshino, 1976a; Huffman and Tripp, 1982), and in A.granosa (Chandy and Patel, 1985; Patel and Patel, 1985).

(2) INNATE HUMORAL INTERNAL DEFENCE MECHANISMS

The contribution of innate humoral factors to invertebrate defence mechanisms has not been studied extensively. There are, however, some evidences for their existence in molluscs. In invertebrates they fall under two functional categories: (i) Those that are directly parasitocidal:- Tripp (1960) has reported that bacteria innoculated into C.virginica were rapidly destroyed extracellularly. A constituent in the tissue extracts of several species of marine pelecypods, especially that of C.gigas, that will induce the ectopic immobilization and encystment of the cercariae of the echinostome Himasthla quissetensis, preventing their penetration into the second intermediate host, has been reported by Cheng et al. (1966). Wittke and Renwrantz (1984) have shown that a haemolytic substance is secreted by M.edulis haemocytes. (ii) Those that enhance cellular reaction:- Agglutinins or lectins are the bivalve humoral factors that enhance cellular reactions, and these glycoproteins enhance phagocytosis, and are believed to function as opsonins in bivalves. In C.virginica, Tripp (1958b), and Feng (1959) reported the occurrence of both agglutinating and properdin-like properties. Johnson (1964) reported that saline extracts of the butter-clam Saxidomus giganteus will agglutinate human A_1 and A_1B erythrocytes, and Tripp (1960; 1966) reported that the mantle fluid, serum and pericardial fluid will agglutinate erythrocytes of chicken and a

variety of mammals, and the fact that oyster fluids agglutinate the human blood cell types A, B, AB and O indicates a lack of specificity.

(3) ACQUIRED CELLULAR INTERNAL DEFENCE MECHANISM

Nothing is known about this in bivalves.

(4) ACQUIRED HUMORAL INTERNAL DEFENCE MECHANISMS

The two categories of acquired humoral factors are (i) Syntheized antimicrobial factors, and (ii) Secreted lysosomal enzymes.

(i) Synthesized antimicrobial factors

In bivalves, to date, antimicrobial property of only one lysosomal enzyme is well established. McDade and Tripp (1970) and Rodrick and Cheng (1974) in C.virginica, and Cheng and Rodrick (1974) in M.arenaria, have reported that lysozyme from the haemolymph will lyse several Gram-positive and Gram-negative bacteria. But Rodrick and Cheng (1974), and Cheng and Rodrick (1974) found no lytic effect on Staphylococcus aureus by C.virginica and M.arenaria haemolymph lysozymes. These studies show that the lysozyme is active only against certain bacteria, and the reason for it is not well understood. But it can be generally stated that the lysozyme in serum can serve as defence molecules against susceptible organisms.

(ii) Secreted lysosomal enzymes

Lysosomal hydrolases as mentioned earlier, play a significant role in intracellular degradation. Lysosome occur abundantly in certain types of bivalve haemocytes-the granulocytes. The pH optima of these lysosomal hydrolases are normally in the acid range, although some may be active in neutral pH also (Dean, 1977). These hydrolases include, lysozyme, acid and alkaline phosphatases, β-glucuronidase, amylase, lipase, aryl sulphatases, etc.

These enzymes are reported both from the haemocytes and also from the serum. From the studies of Cheng and Rodrick (1974), Rodrick and Cheng (1974), Cheng et al. (1975, 1977, 1978a), Cheng and Yoshino (1976 a,b), Cheng and Butler (1979) and also of others, it is well established that in bivalves and gastropods during phagocytosis, there is hypersynthesis of lysosomal enzymes in granulocytes, and subsequently they are released into the serum. Yoshino and Cheng (1977), Cheng et al. (1980a), and Cheng and Rodrick (1980) have also shown that in gastropods, besides haemocytes, tissues of the head-foot and visceral mass are also the sources of lysosomal enzymes and they are released into the haemolymph.

The information regarding the release of lysosomal enzymes into serum comes from the study, employing M.mercenaria as the model, by Cheng (1975), Cheng et al. (1975), and Foley and Cheng (1977). They demonstrated that degranulation in molluscan granulocytes is the morphological reflection of the release of enzymes into the haemolymph, and that degranulation is not restricted to actively phagocytosing cells but is enhanced during phagocytosis.

Scanning electron microscope and transmission electron microscope studies by Mohandas et al. (1985), and Mohandas and Cheng (1985b) on M.mercenaria granulocytes, have revealed that the lysosomes protrude from the membrane surface of granulocytes or form distended segments along filopodia, and at this time they are attached to the granulocytes by means of stalks. Later they get detached and are released into serum. The released lysosome is bound by two membranes, the inner lysosomal membrane and the outer granulocyte membrane. The mechanisms of detachment of lysosomes from the granulocyte plasmamembrane and the subsequent rupture of the lysosomes

releasing the enclosed hydrolases are not well understood. An autocatalytic mechanism (Mohandas et al., 1985), or differences in the internal osmotic pressure within the released lysosomes and in the serum caused by calcium influx (Mohandas and Cheng, 1985b), may be operating.

In general, during a parasitic challenge, there is hypersynthesis of certain lysosomal enzymes and they are released into the serum. Consequently, when parasites make contact with the elevated levels of lysosomal enzymes, atleast some are killed. The killing mechanism may be direct, ie., one or more of the enzymes attack and destroy the foreign invader, or indirect, ie., one or more of the enzymes cause chemical alterations of the parasite's body surface so that it becomes recognised as foreign and is attacked by the host haemocytes. Cheng (1977a), and Mohandas et al. (1985) have demonstrated that some bacterial species that are endocytosed have altered surfaces resulting from lysosomal enzyme action. Relative to the possible role of lysosomal enzymes acting as defence molecules against incompatible helminths, Cheng et al. (1978b), reviewing the earlier literature, have suggested that the elevated levels of serum aminopeptidase may alter the surface proteins of secondarily introduced parasites and thus act as a form of acquired humoral immunity. It is noted that several other workers have provided evidences of enhanced cellular and humoral reactions as a result of previous challenge with foreign materials. It needs to be pointed out, however, that enhancement of host reaction does not appear to be the case always. Exposure to non-self materials may enhance, suppress or not affect molluscan internal defence mechanisms (Cheng, 1983a). If an invading organism is not deleteriously affected by serum lysosomal enzymes, the reasons may be (i) the enzymes are

incompatible with the surface structure of the invader, (ii) the host fails to recognise it as foreign or the invader fails to trigger the release of enzymes, and (iii) the invader secretes a substance that inhibits or inactivates the enzyme (Cheng, 1977a).

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CHAPTER II

HAEMOCYTES OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

The concept of haematological manifestation in response to a stress, either biotic or abiotic, is old, and is used rather widely for the identification of stress factors. In medical and veterinary sciences, this is used as an indispensable tool for diagnostic purpose of various diseases including those caused by stress factors. In lower forms, characterisation of haematological manifestations is not codified upto the extent seen in medical and veterinary sciences. The reason for this situation is due to the great diversity of the lower forms, and also to the great variation encountered in them. But much of the information regarding the haematological variations are gathered from the study of lower forms; particularly insects, crustacea, and mollusca.

Metchnikoff (1968, page 76; quoted from Cheney, 1971) in 1891 defined leucocytes as "colourless cells, possessing one or rarely two nuclei, and a protoplasmic body capable of amoeboid movement. In many invertebrates there is only one variety of mobile blood corpuscles containing a few sparse granules, whereas in certain others, such as many insects and molluscs, two varieties occur- granular leucocytes, with a large number of coarse granules, and hyaline leucocytes, with a few or no granules". Within the broad range of this definition, the literature has been abundantly supplied with an array of names- haemocyte, amoebocyte, phagocyte, blood cell etc.,- all assigned to leucocytic cells. In the present study I have used the terminology haemocyte for the cells found in the haemolymph of clams. As indicated in the review of literature, different morphological varieties of cell types were mentioned by different workers, and in one paper (Betances, 1922) up to 76 different

varieties of cell types were classified from lamellibranchs. Although the recent classification adopted by Cheng (1981, 1984) indicates the presence of two varieties of haemocytes, granulocyte and hyalinocyte in bivalves, the occurrence of different sub-populations of haemocytes as reported by Renwrantz et al. (1979), Yoshino et al. (1979), Cheng et al. (1980b), the detectable staining and other morphological alterations that occur during aging and functionalships during the life of the haemocyte, may, in future, give clues for reclassification of haemocytes in a different way.

Regarding the number of circulating haemocytes and the factors that affect their distribution, not much data are available, except perhaps the contributions made by Feng (1965a, 1966b), Cheney (1971), Gurski and Eble (1973), and Foley and Cheng (1974). Besides the cytology of the molluscan haemocytes, some contributions have also been made on the cytochemical aspects. Zacks and Welsh (1953) demonstrated cholinesterase and lipase in M.mercenaria, and Zacks (1955) published the first extensive cytological and cytochemical study of blood cells of this animal. Feng et al. (1971) described the cytology, fine structure and cytochemistry of C.virginica haemocytes while Martin (1970) studied the cytochemistry of Spisula haemocytes. Moore et al. (1972), and Foley and Cheng (1974) reported on the cytochemistry of various enzymes in the haemocytes of M.mercenaria. Subsequently, Yoshino and Cheng (1976a) reported the fine structural localisation of acid phosphatase in this species. Some more contributions on the cytochemical aspects of the haemocytes of M.mercenaria and M.edulis were made by Moore and Eble (1977), and Moore and Lowe (1977). In a recent review, Moore (1980) has discussed the use of cytochemical procedures in the determinations of cellular responses

to physical and chemical environmental stressors.

The immediate fate of soluble and particulate materials introduced into molluscs is well known. Specifically, injected particulate materials are phagocytosed, unless they are too large, in which case they are encapsulated (Cheng, 1967; Feng, 1967; Cheng and Rifkin, 1970; Foley and Cheng, 1974).

Before detailed studies relative to various functions of molluscan haemocytes are undertaken, it is necessory that the number and types of haemocyte cells, as well as their normal behaviour be ascertained. In this chapter the morphological and behavioural aspects of the circulating cells of the haemolymph of S.scripta and V.cyprinoides var. cochinensis are described. In addition, an attempt has been made to study certain cytochemical aspects of the haemocytes of these two species of clams, and also to establish haematological parameters in four size groups of the two clams species in order to determine their normal values and to ascertain whether these are interrelated. Further, an attempt has also been made to find out whether there is any difference in the total number of circulating haemocytes in the most commonly available size groups of clams of the two species when subjected to biotic and abiotic stress factors.

MATERIALS AND METHODS

Specimens of Sunetta scripta were collected off Fort Cochin, and those of Villorita cyprinoides var. cochinensis from Nettoor, near Panangad. They were brought to the laboratory immediately after collection and while transportation, they were kept in water taken from the collection sites. In the laboratory, specimens of S. scripta were gradually brought to 30‰ salinity, and V.cyprinoides var. cochinensis to 15‰ salinity. They were acclimated in

the respective salinities mentioned above until used for experiments. The acclimation period ranged from 4 to 5 days. In the laboratory they were reared in large plastic tubs fitted with biological filters kept outside the tub. To facilitate the establishment of the bacteria in the filter, circulation of water through biological filters was done at least two days prior to transferring the specimens to the tubs. Particular care was taken to ensure that in none of the tanks was crowding and oxygen acting as limiting factors. All the experiments were carried out at room temperature ($29\pm1^{\circ}$C).

An algal culture of Synechocystis species was maintained in the laboratory and specimens were fed regularly with this during the entire acclimation period. Generally, feeding was done in the morning and at this time the circulation through the filters was stopped for about 2 hrs. as a precaution to prevent the trapping of the cells in the filters.

SELECTION OF ANIMAL GROUPS

To obtain information on the haemocyte number and the relative proportions of the types of haemocytes in different size groups of clams, four size groups were selected for S.scripta and V.cyprinoides var. cochinensis. They were 30-32mm, 36-38mm, 38-40mm and 42-44mm for S.scripta, and 24-26mm, 34-36 mm, 38-40mm and 44-46mm for V.cyprinoides var.cochinensis. To minimise variations, if any, within the same size group, the range was reduced to 2 mm in all the size groups of the two clam species. To find out the total counts in experiments where specimens of the clam species were exposed to copper and bacteria, the size group 38-40 mm was chosen, because of (1) their abundance in natural collection, and (2) the required quantity of haemolymph could be collected from individual specimens.

COLLECTION OF HAEMOLYMPH AND ESTIMATION OF TOTAL HAEMOCYTES

After acclimating the clams for 4 to 5 days, specimens were taken out of water and dried with a cloth. With the help of a scalpel, the shells were opened a little, taking care not to tear the adductor muscle. Keeping the shells open with the thumb, the mantle water was poured off and excess water, if any, in the mantle cavity was removed with a blotting paper. Haemolymph from the posterior adductor muscle sinus was withdrawn with a tuberculin syringe fitted with a 23-gauge needle, and rinsed with 40% formalin. Rinsing of the syringe with formalin prevented the adherence of haemocytes to the syringe and their clumping. A 0.3 ml sample of haemolymph was withdrawn into the syringe and shaken well. The first few drops were expelled out, and the next drop was discharged to the haemocytometer. The cells were allowed to settle for 1 to 2 minutes. The cells in the corner four squares were counted, and the number of cells per cubic millimeter was calculated and expressed as haemocytes per mm^3. Sampling was done from 32 specimens in 30-32mm size group, 39 from 36-38 mm size group, 26 from 38-40 mm size group, and 41 from 42-44 mm size group of S.scripta, and 13 each from the four size groups of V.cyprinoides var. cochinensis.

In the experiment designed to study the effect of copper as a stress factor, the haemolymph samples for total count were taken every 24 hrs. and continued for 5 days, and in the other experiments where the clams were injected with bacteria, samplings were done at 3, 6, 12, 24, 48, 72, 96 and 120 hrs. post-injection.

HAEMOCYTE DIFFERENTIAL COUNT

A 0.3 ml sample of haemolymph was withdrawn from the posterior adductor muscle sinus of the clams, and 2 or 3 drops were discharged on to a previously cleaned glass slide. The slides were then kept in a humidified chamber (A large petri dish with a fitting cover, at the bottom of which was kept a damp filter paper with provision to keep the slides off the damp filter paper) for 10 minutes to allow it to spread. After 10 minutes, the slides were taken out of the humidified chamber and flooded with 10% formo-calcium (10 cc of conc. formalin plus 90 cc distilled water plus 1 gm of anhydrous calcium chloride) and kept for 10 minutes. The formo-calcium was drained, and the slides were then rinsed in buffer (phosphate buffer, 0.05 molar, pH 6.8), and post-fixed in 95% ethyl alcohol for one minute. They were stained with Wright's stain in absolute methyl alcohol for one minute. An equal quantity of buffer was then added. Two minutes later the slides were flooded with fresh buffer, then with distilled water, and finally air dried. Although it was not necessary to cover blood smears, after initial observation they were mounted in DPX.

The slides were also stained with Giemsa stain. The haemolymph placed on the slides was permitted to settle on the slide for 10 minutes in a humidified chamber, after which the adhered cells were fixed by flooding the slids with 3% seawater-glutaraldehyde. Subsequently, the attached cells were rinsed in distilled water for 2 minutes, dehydrated in 95% ethanol for 1 minute, and air dried. The cells were then stained with 4% Giemsa's stain in Sörensen's buffer, pH 6.5, for 20 minutes, rinsed with fresh buffer followed by distilled water, and air dried.

Some of the slides were stained with lead haematoxylin-basic fuchsin stain following the method of Guida and Cheng (1980). The haemolymph placed on the slide was permitted to settle on the slide for 10 minutes in a humidified chamber. The resulting mono layers were fixed by one of the three following methods:

1) Complete replacement of the serum with 1% glutaraldehyde in Sörensen's buffer, 0.05M, pH 7.4 for 30 minutes,

2) Complete replacement of serum with 10% buffered formalin for 15 minutes, or

3) removal of serum followed by immersion in absolute methanol for 5 minutes.

Cells fixed in glutaraldehyde and formalin were washed for 10 minutes in running tap water, while those fixed in methanol were washed for 5 minutes. Following fixation and washing, the haemocyte monolayers were stained according to the following regimen:

Reynolds (1963) lead citrate mordant	-	30 minutes
Wash in running tap water	-	1 minute
Stain with haematoxylin (Guida and Cheng,1980)	-	15 minutes
Wash in running tap water	-	5 minutes
Expose to Scott's solution (Humason, 1967)	-	3 minutes
Wash in tap water	-	5 minutes
Counter stain with 0.25% aqueous basic fuchsin	-	20 seconds
Dehydrate with acetone, 2 changes	-	3 minutes each
Clear with xylene, 2 changes	-	3 minutes each
Mount with DPX		

The preparations were examined under oil immersion, and the percentage composition was determined by averaging differential counts of 100 cells using oil immersion. Measurements of cells and nuclei were made on fixed preparations using calibrated occular micrometer.

For experiments on phagocytosis and pinocytosis, ten specimens each of the size group 38-40 mm of the two clam species were acclimated for 4 to 5 days and used for experiments.

PHAGOCYTOSIS OF BACTERIA

A 0.3 ml sample of haemolymph was withdrawn from the posterior adductor muscle sinus of each clam, and two to three drops discharged on to previously cleaned glass slides. Two to three drops of live bacterial suspension (1×10^{12} cells of Vibrio species) were also discharged over the haemolymph drops, throughly mixed, and the slides were then kept in a humidified chamber. After two hrs., the serum was drained and the adhered haemocytes were fixed in 10% formo-calcium. Ten min. post fixation, the fixative was drained, the slides were washed with buffer, and stained with Wright's stain as mentioned above.

Three more sets of slides prepared in the same way were used for the cytochemical localisation of glycogen, alkaline and acid phosphatases in the haemocytes, subsequent to phagocytosis. Fixation and staining procedures are described elsewhere.

PINOCYTOSIS OF PROTEINS

To study the pinocytosis of protein molecules by haemocytes, the procedure for the preparation of the slide was the same as mentioned above. A 10% solution of bovine serum albumin, stained with Congo red was prepared.

Two to three drops of this solution were discharged over the haemolymph drops on the slide (1:1 proportion). The slides were kept in a humidified chamber. Two hrs. later the serum was drained and the smear was fixed in 10% neutral Formo-Calcium. Ten min. post-fixation, the fixative was washed off, leaving a layer of fixative over the smear, and the slides were examined immediately.

PINOCYTOSIS OF OIL

Two drops of oil (Tween 80) were mixed with two drops of haemolymph on a slide and the preparations were kept in a humidified chamber for 2 hrs. Subsequently, the serum was drained off, and the smear was fixed in 10% Formo-Calcium for 10 min. The preparations were stained with Sudan Black and observed immediately.

DOSING THE ANIMALS WITH COPPER

Clams were acclimated for 4 to 5 days in 60-litre seawater tanks (30‰ salinity for S.scripta; 15‰ salinity for V.cyprinoides var. cochinensis) fitted with biological filters. Clams of 38-40 mm size group were selected for the experiments. On the fifth day, 80 specimens of S.scripta were transferred to each of the three 20-litre tanks. The three tanks were filled with filtered seawater (30‰ salinity), and dosed with 1,3 and 5 ppm of Cu^{2+}. These three batches of clams constituted the experimental group. For Cu^{2+}, the salt used was $CuSO_4.5H_2O$ (Glaxo labs., Bombay). The 80 clams changed to the fourth 20-litre tank filled with filtered seawater served as controls. The water was changed daily, and the metal ion concentrations in the experimental tanks were maintained at their respective levels, throughout the experimental period of five days. Feeding was stopped during the experimental

period, and particular care was taken to ensure that in none of the tanks were crowding, oxygen, temperature and/or salinity acting as limiting factors.

At every 24 hrs. for 5 days, a 0.3 ml sample of haemolymph was withdrawn from the adductor muscle of each of the 15 clams from each experimental batch by employing a 1-ml syringe attached to a 21-gauge hypodermic needle. The total haemocyte count was made as mentioned earlier. Collection of haemolymph samples from the control group was identically carried out.

In the case of V.cyprinoides var. cochinensis, the methodology was slightly different. In this species, each experimental batch consisted of 60 specimens, and the three batches of clams dosed with 0.15, 0.30, and 0.45 ppm. copper constituted the experimental group, and the 60 clams of the fourth 20-litre tank filled with filtered seawater served as controls. The salinity of seawater was adjusted to 15‰ for this species. The experiment lasted 5 days, and haemolymph sampling was done every 24 hrs.

In this experiment more clams than the required number were reared as experimentals and controls anticipating mortality during the course of the experiment.

CHALLENGING OF CLAMS WITH LIVE BACTERIA

Live Vibrio species were used for studying the animal's reaction towards a biotic challenge. Vibrio sp. was cultured on hard agar, and 24 hrs. after plating, it was harvested using 2% sterile saline. Specifically, 2% sterile saline was poured over the agar and with a loop, it was mildly agitated to detach the cells from the agar. These cells in saline formed a suspension, and it was transferred into a sterile 20 ml test tube. The required number

of cells, i.e., $1x10^8$ cells/0.02 ml, was obtained through serial dilution. The exact number was calculated through a standard graph prepared for the purpose by plotting the number of cells against the optical density.

The bacterial suspension ($1x10^8$ cells/0.02 ml) was injected into the clam in the following way. The clams were taken out of water and the surface was dried with a towel. The shell was opened in the same way as done for taking the haemolymph. A 0.02 ml of bacterial suspension was injected into the foot-gonad region with a sterile 1-ml tuberculin syringe, attached to a 21-gauge hypodermic needle. In the control group, the untreated clams were left alone. Clams consisting the second group of controls were each injected with 0.02 ml of sterile 2% saline.

In S.scripta the experimental group consisted of 280 clams divided into eight batches of 35 clams each. From these batches of clams, haemolymph was collected in the same way, as mentioned earlier, at 3, 6, 12, 24, 48, 72, 96, and 120 hrs. post-injection with bacteria to study the haemocyte number. Appropriate numbers of untampered and saline injected controls were also maintained, and collection of haemolymph samples from clams comprising these control groups was identically carried out. Methods of rearing the clams and other pre-requisites usually followed in these sorts of experiments were the same as described in detail earlier.

In V.cyprinoides var. cochinensis, the only difference was in the number of specimens used. Here, the experimental group consisted of 200 clams divided into eight batches of 25 clams each. Appropriate numbers of untampered and saline injected controls were also maintained.

In all the experiments, S.scripta was reared at 30‰ salinity, and

V.cyprinoides var. cochinensis at 15‰ salinity; feeding was stopped during the experimental period, and particular care was taken to ensure that in none of the tanks, crowding, oxygen, temperature and/or salinity acting as limiting factors.

STAINING FOR GLYCOGEN

For localisation of glycogen in the haemocytes, PAS technique was employed (Humason, 1979).

Fixation:

Gendre Fluid :

Ethyl alcohol (95%) saturated with picric acid	-	80.0 ml.
Concentrated formalin	-	15.0 ml.
Glacial acetic acid	-	5.0 ml.

Solutions :

Periodic acid :

Periodic acid (HIO_4)	-	0.6 gm.
Distilled water	-	100.0 ml.
Concentrated nitric acid	-	0.3 ml.

Schiff reagent :

Basic fuchsin	-	0.5 gm.
Distilled water	-	85.0 ml.
Sodium metabisulphite ($Na_2S_2O_5$)	-	1.9 gm.
N HCl	-	15.0 ml.

Sodium metabisulphite

Sodium matabisulphite ($Na_2S_2O_5$)	-	0.5 gm.
Distilled water	-	100.0 ml.

A thick smear of haemolymph was prepared on a clean glass slide, and allowed to spread for 10 min. in a humidified chamber. The serum was drained after 10 min. and the thick film of haemocytes was fixed in Gendre fluid for 20 min. It was then washed 3 times in 80% ethyl alcohol. Following fixation and washing, the haemocytes were stained according to the following regimen:

Treat with periodic acid solution	- 5 minutes.
Wash in running water	- 5 minutes.
Treat with Schiff reagent	- 10 minutes.
Transfer through sulphite solutions, 3 changes	- 1.5 minutes each.
Wash in running water	- 5 minutes.

Dehydrate, clear and mount.

The control slides, after fixation in Gendre fluid and washing away the fixative with 80% ethyl alcohol followed by distilled water, were treated with a 1% diastase solution in 0.2M phosphate buffer, pH 6.0, for 1 hr. at 37° C. After 1 hr. the control slides were stained as described above.

STAINING FOR ALKALINE PHOSPHATASE

Gomori method (Frankel and Peters modification, 1964, cited from Humason, 1979) was followed for the cytochemical localisation of alkaline phosphatase in haemocytes. The haemolymph was placed on a clean slide. After two hrs when the haemocytes had spread, the serum was drained off, and the adhered and spread cells were immediately fixed in chilled absolute acetone for two min. It was then hydrated by dipping twice in 80% acetone, then in 50% acetone, and finally in distilled water. The slides were put in the incubating solution at 37° C for 45 min., the composition of which is as follows:

0.8% paranitrophenyl phosphate	-	2.0 ml.
2% sodium barbitol	-	2.0 ml.
Distilled water	-	1.0 ml.
2% calcium chloride	-	4.0 ml.
5% magnesium sulphate	-	0.2 ml.

After 45 min., they were washed well in distilled water, treated with 2% cobalt nitrate for 5 min., again rinsed in distilled water, then treated with 2% yellow ammonium sulphide for 2 min., rinsed in distilled water, dehydrated, cleared and mounted in DPX.

Control slides were incubated in an incubating solution lacking in 0.8% paranitrophenyl phosphate. All other procedures regarding the preparation and staining of these slides were the same as described above.

STAINING FOR ACID PHOSPHATASE

Gomori method (Humason 1979) was followed for the cytochemical localisation of acid phosphatase in haemocytes. The haemolymph was placed on a clean glass slide. After two hrs when the haemocytes had spread on the glass slide, the serum was drained off and the adheared and spread cells were immediately fixed in chilled absolute acetone for two min. It was then hydrated by dipping twice in 80%, then in 50% acetone, and finally in distilled water. The slides were placed in the incubating solution at 37° C for one hour, the composition of which is as follows:

Acetate buffer (0.2M, pH 3.6)	-	2.5 ml.
Distilled water	-	10.0 ml.
Lead nitrate	-	0.012 gm.
3% Sodium glycerophosphate	-	1.0 ml.

After one hour, the slides were rinsed in distilled water for a few seconds, washed briefly in 1% aqueous acetic acid for 1 min., washed in distilled water for 1 min., treated with 1% aqueous ammonium sulphide for 2 min., again washed in distilled water for several min., and then dehydrated, cleared and mounted in DPX.

Control slides were incubated in an incubating solution without the substrate. All other procedures regarding the preparation and staining were the same as described above.

VITAL STAINING WITH NEUTRAL RED

A solution of 0.01% neutral red in 30‰ or 15‰ filtered seawater (for S.scripta and V.cyprinoides var. cochinensis, respectively) was mixed with haemolymph in 1:4 proportion on a glass slide, and allowed to spread for 10 min. in a humidified chamber. After this period, the serum was drained and the preparation was viewed under a microscope before drying up.

STATISTICAL ANALYSIS

The two-tailed 't' test (Zar, 1974) was employed to determine possible statistically significant differences in the number of haemocytes in the haemolymph of clams of the four size groups, and of the control and experimental batches.

RESULT

TOTAL HAEMOCYTE COUNT IN THE FOUR SIZE GROUPS OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

Table 1A gives the average, standard deviation and range of the total haemocyte count in the four size groups of S.scripta. Statistical analysis of the data revealed the following: The average value of the total haemocyte

Table 1A. Total Haemocytes/mm^3 in the four size groups of *Sunetta scripta*

Size group	30-32 mm	36-38 mm	38-40 mm	42-44 mm
N	32	39	26	41
Mean value	4556	4482	3812	2921
± SD	1464.9	1458.9	1198.5	799.0
Range	2230-7880	2540-8280	2010-7250	1800-4790

Table 1B.Total Haemocytes/mm^3 in the four size groups of *Villorita cyprinoides* var. *cochinensis*

Size group	24-26 mm	34-36 mm	38-40 mm	44-46 mm
N	13	13	13	13
Mean value	3345	3186	3187	3253
± SD	1187.9	857.8	693.9	696.5
Range	1920-6720	2100-5160	2140-4750	2040-4680

of the 42-44 mm size group was significantly lower than the average values of the three other size groups ($P < 0.01$). The average value of the total haemocytes of 38-40 mm size group was significantly lower than the average value of 30-32 mm size group.

Table 1 B gives the average of the total haemocyte count in the four size groups of V.cyprinoides var. cochinensis. Statistical analysis of the data revealed that there was no significant difference in the values when compared between the size groups in all possible combinations.

DIFFERENTAL COUNT OF HAEMOCYTES IN THE FOUR SIZE GROUPS OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

Tables 2A and 2B give the differential count of the four size groups of the two species of the clams. For the differential count, although three staining techniques were employed, as mentioned earlier, satisfactory results were obtained only by staining the haemocytes with Wright's stain. In a well-spread condition, two categories of cells could be distinguished, granulocytes and agranulocytes. Statistical analysis of the number (in percentage) of granulocytes and agranulocytes in the four size groups of the two clam species revealed no significant difference. However, the percentage of agranulocytes was roughly half of that of granulocytes in the four size groups of the two species of clams.

GRANULOCYTES: The granulocytes of four size groups of S.scripta did not show any significant difference in dimensions. The granulocyte of S.scripta measured $22.6 \pm 8.07 \times 17.2 \pm 6.65$ µm and that of V.cyprinoides var. cochinensis measured $22.4 \pm 7.84 \times 16.9 \pm 6.12$ µm. The measurement of the nucleus was $6.6 \pm 1.60 \times 5.4 \pm 1.43$ µm for S.scripta, $6.9 \pm 1.30 \times 5.5 \pm 1.47$ µm for V.cyprinoides

Table 2A. Differential count of haemocytes in the four size groups of Sunetta scripta

	30-32 mm (N=5)		36-38 mm (N=5)		38-40 mm (N=5)		42-44 mm (N=5)	
	Granulocyte	Agranulocyte	Granulocyte	Agranulocyte	Granulocyte	Agranulocyte	Granulocyte	Agranulocyte
Mean value	67.0	33.0	68.0	32.0	65.0	35.0	67.2	32.8
± SD	0.8944	0.8944	1.0954	1.0954	3.7417	3.7417	2.3152	2.3152
Range	66-68	32-34	66-69	31-34	60-69	31-40	65-70	30-35

Table 2B. Differential count of haemocytes in the four size groups of Villorita cyprinoides var. cochinensis

	24-26 mm (N=5)		34-36 mm (N=5)		38-40 mm (N=5)		44-46 mm (N=5)	
	Granulocyte	Agranulocyte	Granulocyte	Agranulocyte	Granulocyte	Agranulocyte	Granulocyte	Agranulocyte
Mean value	66.0	34.0	68.4	31.6	65.6	34.4	68.6	31.4
± SD	2.2804	2.2804	3.1369	3.1369	2.3324	2.3324	1.3565	1.3565
Range	63-69	31-37	64-73	27-36	63-69	31-37	67-71	29-33

var. cochinensis (Figures A and B). The staining reaction was found to be the same for the granulocytes of both the species of clams. During spreading, the cytoplasm was found to flow along the surface of the slide in an irregular fashion. At this time, the cytoplasm could be distinguished into an inner endoplasm and an outer ectoplasm. The granulocytes are characterised by the presence of large numbers of cytoplasmic granules, which are primarily restricted to the endoplasm. In a well spread condition, these cells produce thin filopodia each with a supporting rib-like structure that originates in the endoplasm (Figures E and F). Thus, the pseudopods produced by the granulocytes are semirigid structures, rather than temporary cytoplasmic protrusions. Occasionally, in both the species of clams, adjacent granulocytes were found to fuse with one another resulting in large multinucleated cells. The endoplasm contains the nucleus. The cytoplasmic granules may be totally basophilic, totally acidophilic or a combination of both. The cytoplasm and the nucleus were also found to be basophilic or acidophilic.

AGRANULOCYTE : The agranulocyte of S.scripta measured 25.7 $\pm$ 7.66 x 19.0 $\pm$ 5.72 µm, and that of V.cyprinoides var. cochinensis measured 25.0 $\pm$ 7.16x 18.5 $\pm$ 5.33 µm. The measurement of nucleus was 7.7 $\pm$ 1.46 x 6.9 $\pm$ 1.55 µm for S.scripta, and 7.7 $\pm$ 1.43 x 7.1 $\pm$ 1.42 µm for V.cyprinoides var. cochinensis (Figures C and D). The staining reaction was found to be the same for the agranulocytes of both the species of clams. During spreading, the cytoplasm could be seen flowing along the surface of the slide in an irregular fashion. It is difficult to differentiate between the inner endoplasm and the outer ectoplasm. These cells were also nucleated, but the cytoplasm contained none or a very few granules. In general, the cytoplasm appeared fibrous. These

cells also included one or several tufts of filopod-like protrusion each supported by rib-like structure that originates from within.

CELL ADHERENCE AND SPREADING

Haemocytes when taken from the adductor muscle sinus and placed on the glass slides were mostly irregularly shaped. Upon contacting the slides both granular and agranular haemocytes adhere and commence to spread. This generally begins 5-10 min. post preparation, although some commence sooner. The spreading of the cells was found to be induced by Ca^{2+} ions, and also when the slides were smeared with albumin. Spread granular and agranular cells are motile and show active cyclosis. The spreading process is initiated when each cell becomes more or less spindle-shaped and with filopodia situated irregularly on the surface. The number of these cytoplasmic extensions varies between cells. Contact between an attached and non-attached cell usually results in the adhesion of the two. Such cells may be of the same or of the different types.

As time progresses, the peripheral cytoplasm of each cell tends to flatten out against the substratum so that the cytoplasmic granules and vacuoles become confined to the endoplasm. At this stage, in unstained preparations, the cells could easily be differentiated into granular and agranular haemocytes. Unbranched pseudopods generally occur along the periphery. The adherence of the cytoplasm to the glass slide is not exclusively due to gravity, since cells have been observed identically adhering to the underside of a cover glass in hanging drop preparations, thereby indicating a positive thigmotaxis. Although several of these explanted cells appear to be subcircular in outline, some were elongated and others irregularly crescent-shaped. The cytoplasmic inclusions

become quite distinct at this stage, and vacuoles also become very clear in granulocytes. At this stage in granulocytes, the granules also become very distinct.

Within 15 to 20 min. post preparation, the spreading process terminates and fine rib-like cytoplasmic rays could be seen extending towards the periphery, from the region of endoplasm in granulocytes, and from within in agranulocytes, and terminating along the outer margin of the cells as fine ectoplasmic extensions. The spaces between adjacent pseudopods are gradually filled by flowing ectoplasm, ie., the ectoplasm forms a web between adjacent pseudopods. These rays appear semi-rigid and were found shifting laterally without marked bending. These cytoplasmic extensions are not limited to any specific region of the cell surface, and the frquency of their production is not different in granular and agranular cells.

The nuclei of both granular and agranular cells are essentially identical, being of the compact type with more or less evenly distributed chromatin.

As mentiond earlier, the haemocytes on the slide adhere together to form clumps (Figures G and H). These clumps are clusters of cells, generally composed of 2 to 15 cells, but occasionally large clumps were also seen. Formation of large clumps was found to be aided by the presence of air bubbles in the haemolymph during sampling. Also, when haemolymph samples of two individuals in the same size group of the same clam species were mixed, formation of large clumps was observed. However, the cells forming the clumps, when allowed to stand for 15 to 30 min., were seen to migrate away from the clups, in a more or less defined pattern, and the fusion of abutting cells have been observed. The smaller the clumps, the more complete will

be the exomigration of haemocytes. This clearly shows that during clump formation there is no cell fusion taking place. The formation of clusters of haemocytes is a serious problem while withdrawing the haemolymph for studying the total haemocyte counts. However, cluster formation can be entirely prevented if the haemolymph is withdrawn by a hypodermic syringe attached to a warm needle or using a hypodermic syringe rinsed with 40% formalin or any dilute acid. Since there was no cell death, this method was preferred to withdraw the haemolymph for studying the total haemocyte number. One drawback in this procedure was that the cells fail to spread on glass slides, and hence it was found unsuitable to study the cell behaviour.

PHAGOCYTOSIS OF BACTERIUM

Phagocytosis involves the uptake of foreign materials by a certain types of host cells, and prevents direct contact of such materials, biotic or abiotic, with the host's tissues. Two types of uptake mechanisms have been observed in S.scripta and V.cyprinoides var. cochinensis. In the first mechanism, the motile bacteria about to be phagocytosed by granulocytes of S.scripta and V.cyprinoides var. cochinensis initially stick to the surface of the molluscan cell, generally to the surface of the filopod and subsequently they are taken into the endoplasm by gliding along the filopodia and become enclosed into a phagosome (Figures I and J). The second uptake mechanism involves formation of invaginations on the cell surface and the bacteria are endocytosed into vacuoles. Here, the involvement of the filopodia is rather doubtful (Figures K and L).

In both the species of clams, more than 75% of the granulocytes were found to be involved in phagocytosis of bacterium. The involvement of

agranulocyte in this process could not be ascertained with certainty at light microscopic level.

PINOCYTOSIS OF PROTEIN AND OIL

Within 2 hrs, most of the haemocytes were found to uptake bovine serum albumin (Figures M and N) and tween 80 (Figures O and P). Although about 90% of the haemocytes were involved in this process, it is difficult to ascertain, the role played by agranulocytes in this uptake mechanism. However, it is believed that in both the species of clams, the agranular haemocytes are also involved in pinocytosis. The uptake mechanism is similar to the second type of uptake mechanism of bacterium by haemocytes, and involves the formation of invaginations on the cell surface and the protein molecules and oil globules are pinocytosed into vacuoles. In this uptake mechanism, there is no involvement of filopodia.

TOTAL HAEMOCYTE NUMBER IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS DOSED WITH THREE CONCENTRATIONS OF COPPER

The total number of haemocytes in S.scripta and V.cyprinoides var. cochinensis when exposed to three different concentrations of copper for 5 days is given in Tables 3A and 3B, and Figures 1A and 1B, respectively.

In S.scripta dosed with 1, 3 and 5 ppm of copper, the total number of haemocytes did not vary significantly in the control and the experimentals during the exposure period of five days. The data were analysed statistically in all possible combinations and 'P' values were greater than 0.05. However, in V.cyprinoides var. cochinensis exposed to 0.15, 0.30 and 0.45 ppm of copper, the values were found significantly lower in 0.15 ppm and 0.30 ppm copper

Table 3A. Total Haemocytes/mm^3 in Sunetta scripta dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	15	15	15	15	15
	Mean value	4015.3	4466.7	4049.3	3637.3	3945.3
	± SD	1650.0	1477.8	1357.8	1016.7	1085.1
	Range	1950-7250	2400-6900	2200-7300	2060-5900	2250-5650
1 ppm of Cu^{2+} dosed	N	15	15	15	15	15
	Mean value	2902.0	3764.0	3718.7	3600.0	3861.3
	± SD	865.8	1372.6	1037.3	1012.0	1184.4
	Range	1850-4850	2000-6540	2300-5550	2040-5100	2100-6250
3 ppm of Cu^{2+} dosed	N	15	15	15	15	15
	Mean value	3445.3	4226.0	3652.7	4214.0	4030.0
	± SD	948.2	1306.3	1420.8	1212.5	843.5
	Range	1710-5350	2490-6500	2020-7100	2300-6100	2650-5950
5 ppm of Cu^{2+} dosed	N	15	15	15	15	15
	Mean value	3412.0	3437.3	3450.0	4140.0	3333.3
	± SD	948.9	1122.5	1059.2	990.2	1058.6
	Range	2400-5910	2200-6500	1950-5400	2650-5400	1900-6100

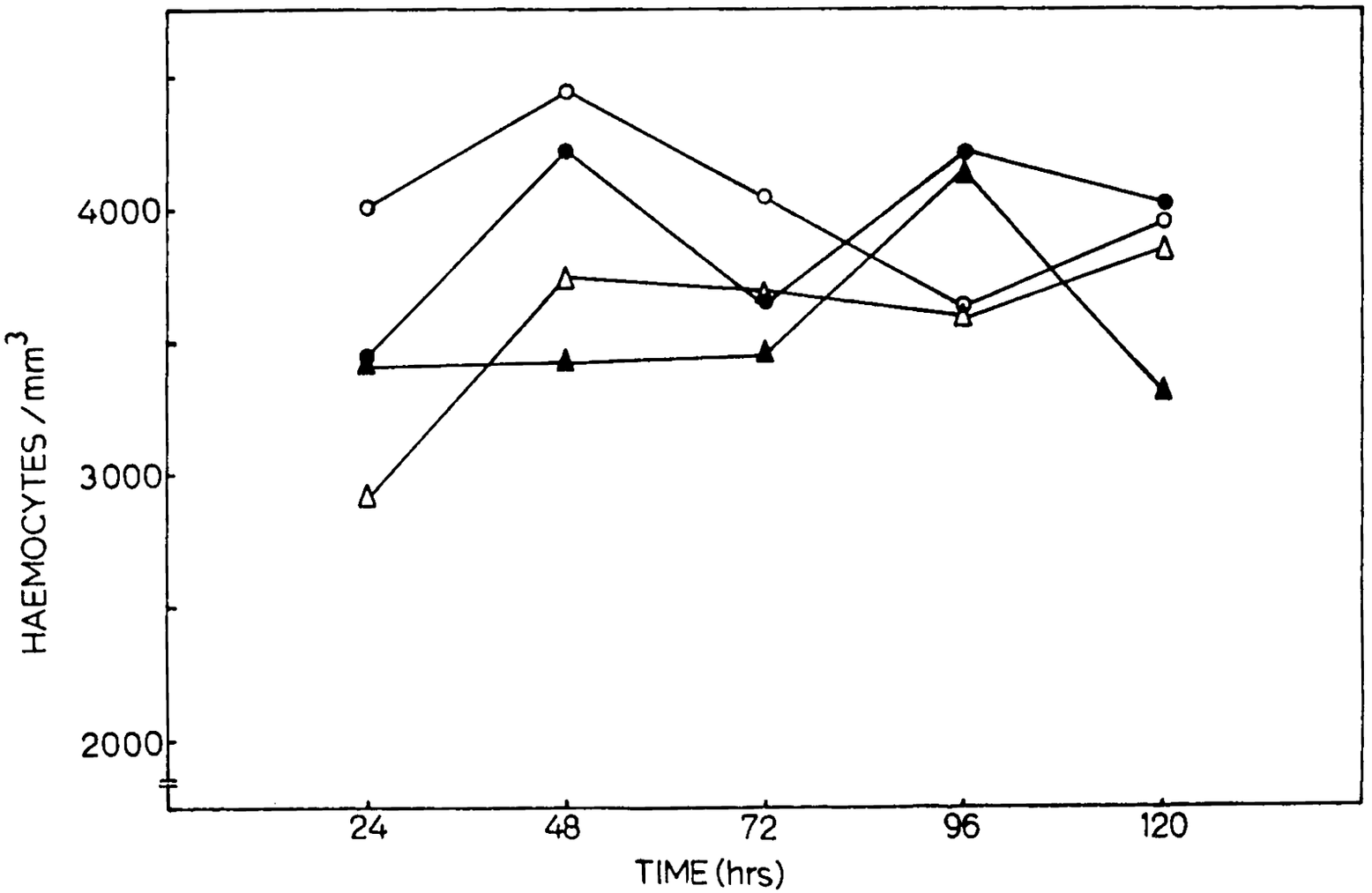

Fig. 1A. Total Haemocytes/mm ^{3} in Sunetta scripta dosed with three concentrations of copper. Control (o); 1 ppm copper (Δ); 3 ppm copper (●), 5 ppm copper (▲).

Table 3B. Total Haemocytes/mm^3 in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	3998.0	3431.0	3499.0	3359.0	3505.0
	± SD	1445.5	1040.5	1182.8	1020.5	722.0
	Range	1780-6550	2030-4910	1950-5700	2100-5170	2350-4700
0.15 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	3490.0	2318.0	2385.0	2402.0	2550.0
	± SD	967.6	375.0	525.4	423.9	820.1
	Range	2430-6050	1560-2930	1540-3160	1580-3090	1500-3820
0.30 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	3185.0	1553.0	1288.0	1729.0	1765.0
	± SD	1177.3	382.9	321.3	153.5	337.5
	Range	1870-5300	1030-2300	850-1780	1290-2600	1420-2440
0.45 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	2648.0	2104.0	2131.0	2412.0	2417.0
	± SD	739.1	866.8	491.6	449.0	706.4
	Range	1850-4440	1330-3930	1330-2930	1770-3390	1570-3910

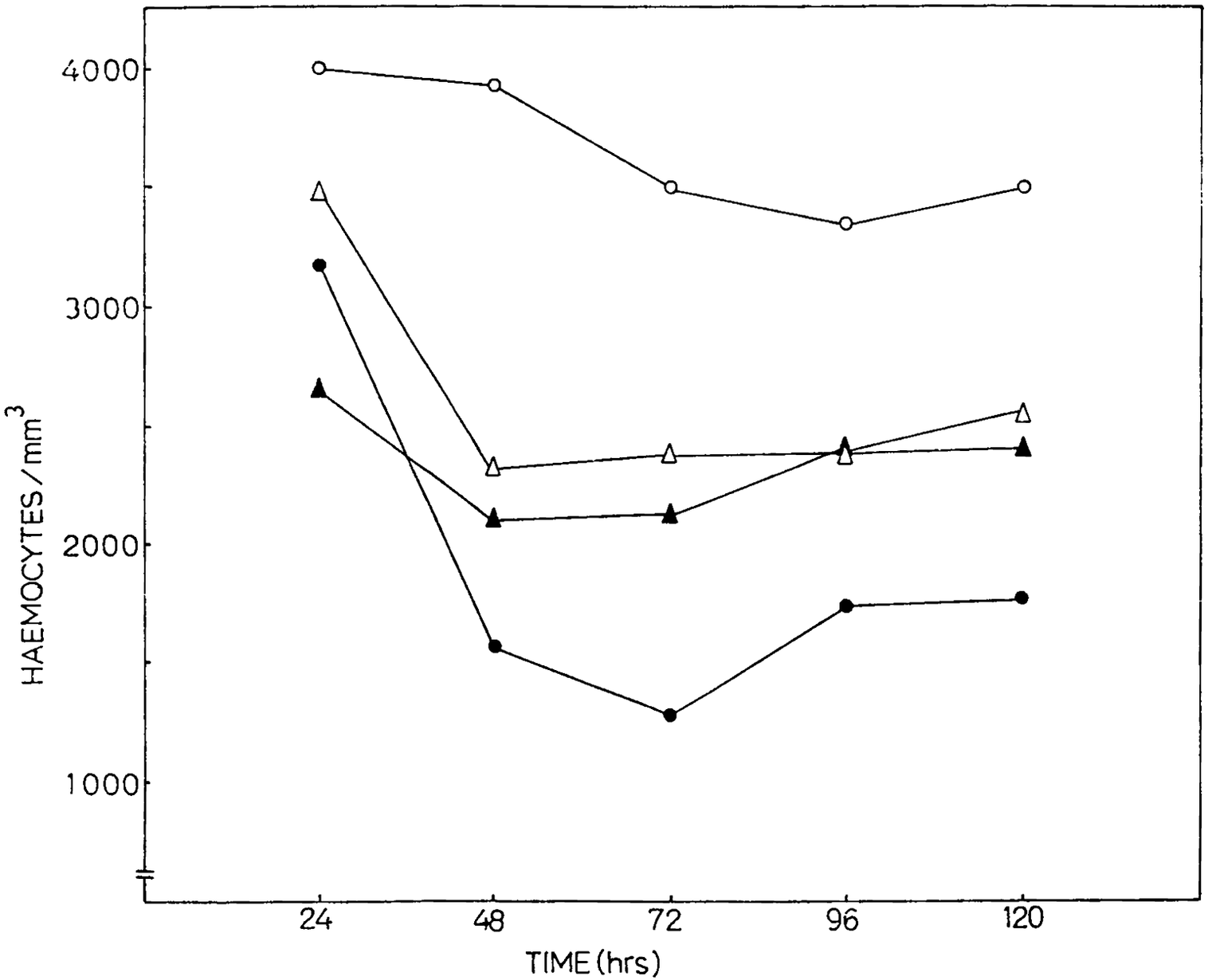

Fig. 1B. Total Haemocytes/mm^3 in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper. Control (o); 0.15 ppm copper (Δ); 0.30 ppm copper (●); 0.45 ppm copper (▲).

dosed clams at 48, 72, 96 and 120 hrs; and in 0.45 ppm copper-dosed clams on all the five days when compared with the values for the respective days of the respective controls ($P < 0.05$). When a comparison was made between concentrations on all the five days, the values in 0.15 ppm copper-dosed clams were found to be significantly higher from 48 hrs onwards when compared with the values of 0.30 ppm copper-dosed clams, and the values of 0.45 ppm copper-dosed clams were found to be significantly higher from 72 hrs onwards when compared with the values of 0.30 ppm copper-dosed clams in the respective days ($P < 0.05$). In the other combination between the values of 0.15 and 0.45 ppm dosed clams, there was no significant difference in the values ($P > 0.05$).

TOTAL HAEMOCYTE NUMBER IN THE HAEMOLYMPH OF *SUNETTA SCRIPTA* AND *VILLORITA CYPRINOIDES* VAR. *COCHINENSIS* INJECTED WITH *VIBRIO* SP.

The total number of haemocytes in *S.scripta* and *V.cyprinoides* var. *cochinensis* when injected with 1x10 cells/0.02 ml of *Vibrio* sp. is given in Tables 4A and 4B, and Figures 2A and 2B, respectively.

In *S.scripta*, the cell counts were significantly high ($P < 0.05$) in bacteria injected clams at 48, 72 and 96 hrs when compared with the respective control values, and the values were significantly high at 6 and 48 hrs when compared with the values of saline injected clams at the respective time periods. When a comparison was made between untampered controls and saline injected controls, the value of saline injected clams was significantly higher at 6 hrs only ($P < 0.05$). In the case of *V.cyprinoides* var. *cochinensis*, in bacteria injected clams, the haemocyte counts were significantly high ($P < 0.05$) at 3,

Table 4A. Total Haemocytes/mm^3 in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	35	35	35	35	35	35	35	35
Untampered control	Mean value	4318.0	4053.4	3577.1	4082.0	3871.4	3720.0	3850.0	3768.3
	± SD	1198.6	959.1	998.0	1433.6	1279.7	982.5	1001.7	1427.9
	Range	2010-6400	2250-6400	1900-6450	1950-7250	2400-6900	2200-5800	2400-5900	2050-6490
	N	35	35	35	35	35	35	35	35
2% Saline injected	Mean value	4700.0	4886.3	3848.0	4090.0	4264.0	4154.1	4239.1	4274.6
	± SD	2020.3	1059.1	834.9	1443.2	859.9	1515.9	1248.2	1245.0
	Range	2450-7790	2930-6850	2040-5850	1950-6950	2950-6100	2200-7350	2850-6950	2300-6550
	N	35	35	35	35	35	35	35	35
Vibrio sp. injected	Mean value	4138.9	3861.7	4068.0	3495.7	4968.6	4751.7	4777.4	4278.3
	± SD	1155.8	1056.3	1397.3	1084.5	1548.1	1714.7	1402.9	1289.4
	Range	1980-7150	2020-6510	1810-7090	1860-6700	2510-8550	2400-8800	2350-7650	2230-7450

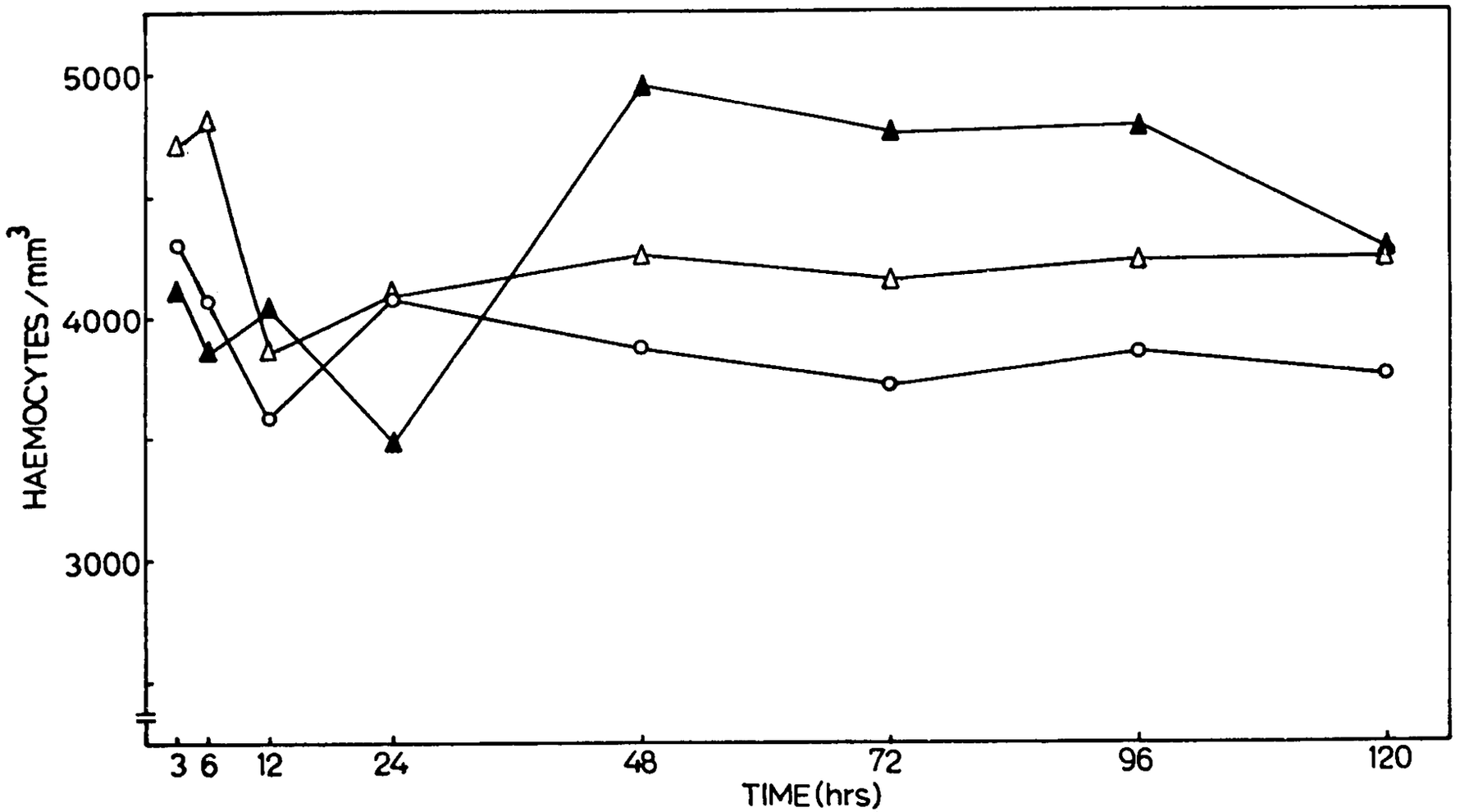

Fig. 2A. Total Haemocytes/mm^3 in Sunetta scripta injected with 1x10^8 cells/0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

Table 4B. Total Haemocytes/mm^3 in Villorita cyprinoides var.cochinensis injected with 1x10^8 cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	25	25	25	25	25	25	25	25
Untampered control	Mean value	3414.0	3322.0	3202.0	3538.0	3431.2	3499.2	3359.2	3505.2
	± SD	1260.2	1174.7	780.7	1162.8	1040.5	1182.8	1020.5	721.9
	Range	1740-6080	1730-5920	2280-4710	1780-5950	2030-4910	1950-5700	2100-5170	2350-4700
	N	25	25	25	25	25	25	25	25
2% Saline injected	Mean value	5845.2	3114.0	2704.0	3264.0	2970.0	3577.2	3155.2	2930.8
	± SD	1429.9	1205.0	959.2	948.5	660.4	1029.8	800.6	1282.8
	Range	3360-9550	1560-5060	1220-4570	2390-5290	2580-4920	2060-5680	2110-4460	1580-5550
	N	25	25	25	25	25	25	25	25
Vibrio sp. injected	Mean value	5338.0	6182.0	5002.8	3845.2	4090.0	3591.6	3970.5	4081.2
	± SD	1526.7	2004.7	1820.4	1363.5	1231.7	996.5	1106.7	1447.5
	Range	2960-8800	3740-11600	2580-9750	2030-6800	2210-7300	2140-6300	2060-6530	2100-7390

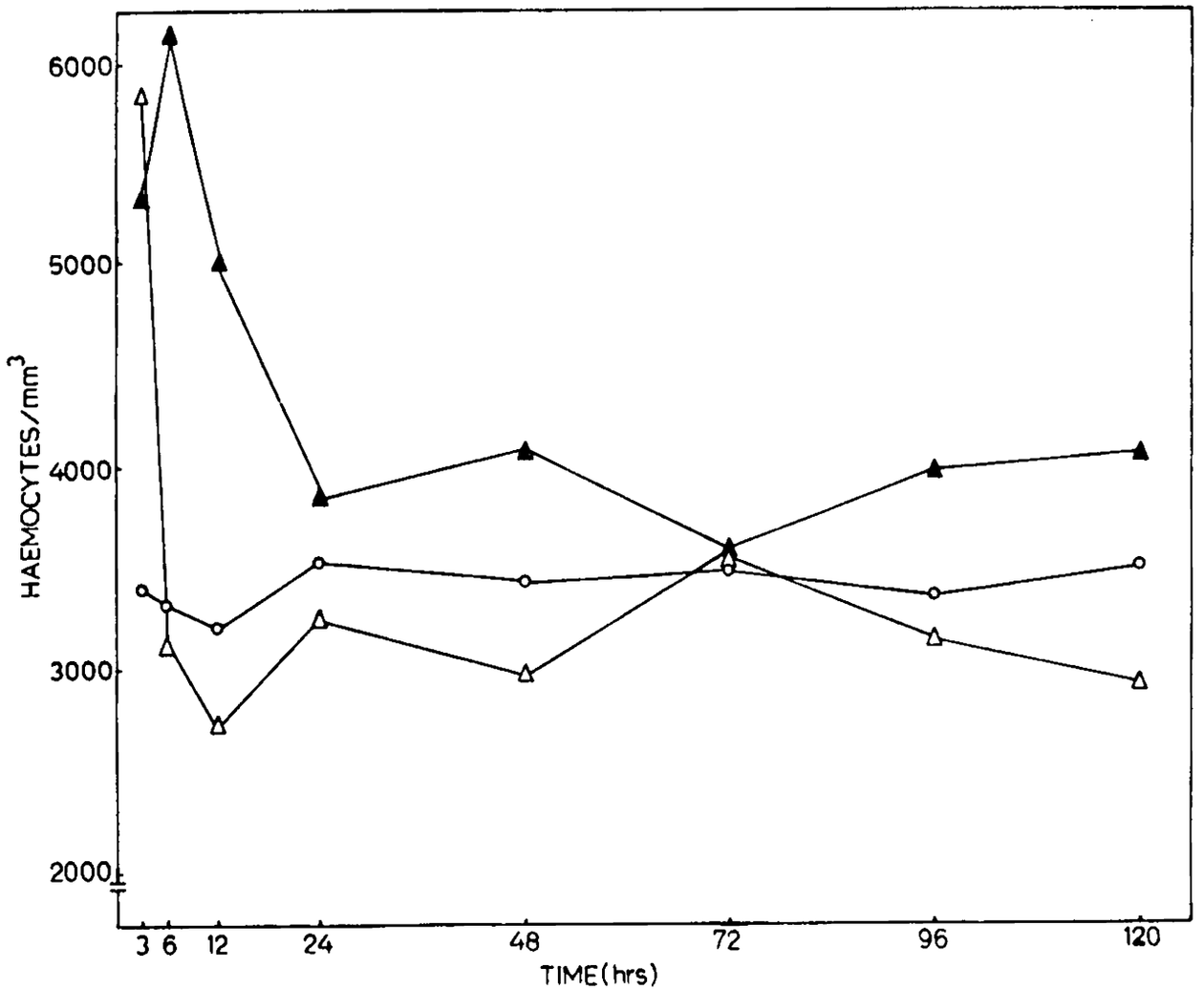

Fig.2B. Total Haemocytes/mm^3 in Villorita cyprinoides var.cochinensis injected with $1x10^8$ cells/0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

6 and 12 hrs when compared with the values of untampered controls at the respective time periods, and with saline injected clams the values were significantly high ($P < 0.05$) at 6, 12, 48, 96 and 120 hrs. When a comparison was made between the values of untampered control and saline injected control, the value of saline injected controls was significantly high at 3 hrs only ($P < 0.05$).

CYTOCHEMICAL LOCALISATION OF GLYCOGEN, ACID AND ALKALINE PHOSPHATASES IN THE HAEMOCYTES OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

In S.scripta, 2 hrs after the in vitro challenge with bacteria, when the haemocytes were stained for glycogen, 60 to 80% of the granulocytes showed intense reaction for glycogen (Figure Q), whereas in V.cyprinoides var. cochinensis, only 20 to 25% of the granulocytes showed the presence of glycogen (Figure R). Even in this 20 to 25% granulocytes, the staining was less intense than that of S.scripta.

In S.scripta and V.cyprinoides var. cochinensis, the acid phosphatase activity was intense in 70 to 80% of the granulocytes, 2 hrs after the in vitro challenge with bacteria whereas alkaline phosphatase activity was very less and usually gave a diffuse staining.

UPTAKE OF VITAL STAIN BY THE HAEMOCYTES OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

GRANULOCYTES : The cytoplasmic inclusions- the granules, took up Neutral Red from the concentration used, and appeared purple within three to four minutes (Figures S and T) in both S.scripta and V.cyprinoides var. cochinensis. Neutral Red in the concentration employed did not appear to be deleterious to these cells.

AGRANULOCYTES : No uptake of the vital stain was observed in these cells.

DISCUSSION

Molluscan haemocytes have been implicated in diverse functions such as wound repair, shell repair, nutrient digestion and transport, excretion and internal defence. The various functions of haemocytes have been described in detail in the review of literature, and hence a repetition is avoided. As a preliminary to detailed studies on the responses of haemocytes to experimentally induced biotic and abiotic stress conditions in S.scripta and V.cyprinoides var. cochinensis, it is necessary to determine the number of circulating haemocytes and to characterise the various types of haemocytes.

In the present study, the number of haemocytes in the four size groups of S.scripta was found to vary from a minimum of 1800 to a maximum of 8280, and in the four size groups of V.cyprinoides var. cochinensis it varied from 1920 to 6720. Regarding the number of circulating haemocytes and the factors affecting the number in circulating haemolymph, studies are very few. Feng (1965a) has observed that the number of circulating haemocytes in C.virginica is dependent upon the amount of turbulence produced by cardiac action but no correlation was found between wet weight and haemocyte number. The number of leucocytes was found to vary with the feeding and excretion cycles, since leucocytes are involved in both these activities. Later, Feng (1966b) has reported that there is a diurnal cycle of feeding activity in M.mercenaria also and the cycle can be disrupted by changes in the intensity of light, salinity and temperature or by the presence of organic materials; individual clams may remain inactive ie., not pumping for several days. Cheney (1971) has reported that the average cell count for all circulating haemocytes

for the Manila clam Tapes semidecussata as 1650 ± SD. 180 per mm^3. Foley and Cheng (1974) found no difference in total haemocyte counts of M.mercenaria from the two geographical locations, Buzzard's Bay (1954.9 ± SD. 1062.7) and Great Bay (1411.7 ± SD. 879.9). Thompson et al. (1978) observed the total number of circulating haemocytes in M.californianus as 7626 ± SD. 2569. They also observed that there was no consistency in the direction of change in the cell count made at 1 hr. and 6 hr. post bleeding. In the present study, the total haemocyte counts in the largest groups of clams (S.scripta) were found to be significantly lower. This observation, and the observations of Feng (1965a,b), Foley and Cheng (1974), and Thompson et al. (1978), undoubtedly show that the differences in the total numbers of circulating haemocytes are dependent upon the physiological state of the individual clams. Although it was marked in the case of S.scripta, it was only apparent in the case of V.cyprinoides var. cochinensis. In this context, it is worth mentioning the observation of Stumpf and Gibertson (1978) in the gastropod B.glabrata where two strains examined showed logarithmic increase in cell number with increasing snail size. They, however, have added that the association between snail size and haemocyte number does not necessarily suggest a cause and effect relationship, and alternatively explain that both these factors may be associated with a third factor, such as, age. Although Thompson et al. (1978) observed no significant difference in the total haemocyte number after bleeding in M.californianus, in the gastropod L.stagnalis, the number of blood cells was found to increase considerably after incision or haemolymph withdrawl (Muller, 1956; Sminia, 1972). In another gastropod, B.glabrata an increase in the total number of haemocytes subsequent to certain larval trematode infection was

observed by Michelson (1975); Stumpf and Gilbertson (1978, 1980), and Jeong et al. (1980), but Lie and Heyneman (1976) found no increase in haemocyte number in the same snail species infected with yet another larval trematode. Thus, besides the physiological state of the individual specimens, other factors that affect the haemocyte numbers of molluscs, in general, are: (1) age, (2) feeding-excretion cycles, (3) cardiac activity, (4) temperature, (5) infection, (6) wounding, and (7) host strain difference.

Although it has been generally agreed that there are only two type of haemocytes in bivalves, granular and agranular haemocytes, regarding the relative percentage of these cells, there is little agreement. Cheney (1971) reported the commonly designated two types of haemocytes in the Manila clam, but the percentage of different types of cells was based on the nuclear diameter and a clear picture is not evolving. In M.mercenaria, one of the best studied species, Foley and Cheng (1974) reported 58.4% granulocytes and 41.6% agranulolcytes in cells fixed immediately, but cells from the same animal fixed and stained after spreading on a glass slide yielded 67.7% granulocytes, 24.1% fibrocytes and 8.2% hyalinocytes. These specimens were collected from the Buzzard's Bay. From specimens collected from the Great Bay, cells fixed immediately showed 65.7% granulocytes and 34.3% agranulocytes. But fixed and stained preparations yielded 67.6% granulocytes, 19.6% fibrocytes, and 12.8% hyalinocytes. Results from different laboratories also showed wide variations.

In addition to the above data for M.mercenaria, other workers have reported the proportions of granulocytes:agranulocytes as 98:2 (More and Eble, 1977); 81:14 (plus 5% fibrocytes, Fries and Trip, 1980) and 30-50:50-70 (R.S. Anderson, quoted from Huffman and Tripp, 1982). Similarly, in C.virginica,

Galtsoff (1964) reported 40% agranulocytes, and presumably 60% granulocytes, while Feng et al. (1971) found 37.2% granulocytes, and presumably 62.8% agranulocytes, almost exactly the opposite. Renwrantz et al. (1979) reported 70% granulocytes and 30% agranulocytes, and more recently Rodrick and Ulrich (1984) found 54.6% granulocytes and 45.4% agranulocytes in C.virginica. In M.campechienasis, the percentages of the granulocytes and agranulocytes were 58.4 and 41.6, respectively (Rodrick and Ulrich, 1984), and in M.arenaria they were 76.5 and 23.5, respectively (Huffman and Tripp, 1982).

Examination of individual reports indicates that such variation may be due to different populations of animals examined, seasonasl variation, cell source (adductor sinus, heart or pericardial fluid), examination of live cells, cells fixed immediately or cells fixed after they have been allowed to adhere to glass, type of fixation and stain, and perhaps other factors. Thus, it is not possible now to generalise with confidence about the composition of molluscan haemocyte populations in individuals within a species or to compare similar cells among species (Huffman and Tripp, 1982).

In granulocytes, of both the species of clams, the granules were found to be acidophilic, basophilic or a combination of both. Feng et al. (1971) and Foley and Cheng (1972) have observed that in C.virginica granulocytes also such a situation occurs. Cheng (1975) concluded that, rather than treating them as being of two distinct types, they were to be considered of the same type; only the pHs of the mileu within the granules fluctuate. Although the functional reason for this fluctuation remains to be elucidated, the occurrence of different metabolic phases which are correlated with the pH changes, has been suggested by him. Yoshino and Cheng (1976a) have indicated that there

must be non-synchronised chemical cycle which occurs within these granules. According to Huffman and Trip (1982), this striking variation is due to age and/or the metabolic history of individual cells, with younger cells being more active metabolically and gradually loosing synthetic capability with age. They have also indicated that enzyme masking may account for some of this variation.

During adherence and spreading of haemocytes several filopods are formed, each with a supporting rib-like structure with fans of ectoplasm stretched between the filopods. Although the number of such filopods formed was more or less the same in the granulocytes and agranulocytes of both the species of clams examined, in M.mercenaria Foley and Cheng (1974) observed that these processes were more on granulocytes than on hyalinocytes (agranulocytes). In C.virginica, although agranulocytes are capable of producing filopods, they do not spread like granulocytes (Foley and Cheng, 1972). Basically, as in the case with S.scripta and V.cyprinoides var. cochinensis, the frequency of pseudopod projection is not different in agranular and granular haemocytes of C.virginica. Although rib-like structures were found in the granular and agranular haemocytes of both the species of clams examined, in C.virginica this was present only in granulocytes (Foley and Cheng, 1972). Subsequently, these rib-like structures have been demonstrated to be comprised of a fascicle of microtubules and it is believed that these organelles aid in maintaining the rigidity of the filopods (Cheng, 1975). The filopods of M.mercenaria also include microtubules which extend internally into the endoplasm (Mohandas, 1985).

Haemocytes not only adhere to glass but also to one another as observed in the present study, as well as in C.virginica (Foley and Cheng, 1972), , and in M.mercenaria (Foley and Cheng, 1974). In the present study agglutination

was observed also when haemolymph samples of two individuals of the same size group of the same clam species were mixed. Foley and Cheng (1974) have observed that agglutination was more pronounced if the leucocytes were mixed with seawater or with homologous serum. A few attempts have been made to investigate the causative factors of clumping in lamellibranchs. The studies made in this connection in lamellibranchs, such as, Cardium norwegicum (Drew, 1910), and L.corrianus (Narain, 1972a) suggest that contact and friction with foreign objects and mechanical agitation may act as promoting causes for agglutination and that haemorrhage may impart the power of amassing to the amoebocytic blood elements (Narain, 1973).

Fusion of adjacent granulocytes was observed in the present study in both the species of clams. Foley and Cheng (1972), however, did not observe multinucleated cells in C.virginica. But Foley and Cheng (1974) and Cheng (1975) observed the phenomenon in M.mercenaria. Sparks and Pauley (1964) have reported the occurrence of multinucleated cells in C.gigas, that have been subjected to injury. Cheng and Galloway (1970) have found similar cells in the gastropod Helisoma duryi normale that received allografts and xenografts. Though definite explanation could not be given for the occurrence of this process, Cheng and Galloway (1970) have suggested that they may serve as an additional line of defence against incompatible materials, such as allografts and xenografts, which are not lysed and are too large to be phagocytised.

Phagocytosis is an important type of internal defence mechanism in vertebrates as well as in invertebrates. Basically, phagocytosis and pinocytosis come under the broad category of endocytosis in which vesicles and vacuoles formed by the plasma membrane regulate the uptake of molecules in a cell's

environment. Phagocytosis or 'eating' is used to describe the uptake of large particles. This uptake occurs by the close apposition of a segment of plasma membrane to the particle's surface. Particle's size has been described as having the range of 0.01 μm to 10 μm, and may include micro-organisms as well as inert particles (Cohn, 1972). Pinocytosis is used to describe the vesicular uptake of everything else, which include small particles, soluble macromolecules and low molecular weight solutes.

A comprehensive review of early literature on the subject of phagocytosis by the haemocytes of the oyster was compiled by Takatsuki (1934a). Modern studies on molluscan phagocytosis commenced from the works of Stauber, who traced the ultimate disposition of Indian-ink particles experimentally injected into C.virginica. Since then, critical reviews of the fates of a variety of experimentally introduced foreign materials which are phagocytosed in molluscs, have been published, among others, by Cheng (1967), and Feng (1967). Briefly, it is now known that digestible particles which are macromolecules are degraded within oyster leucocytes (Tripp, 1958a,b;1960; Feng, 1959, 1965b), while indigestible particles and macromolecules are voided via the migration of foreign material-laden phagocytes across certain epithelial borders (Stauber, 1950; Tripp, 1960; Feng, 1965b).

That molluscan haemocytes are capable of phagocytosis is known since Haeckel's (1862) report on Helix and Thetis; but it was uncertain which cell type was the most active from the standpoint of phagocytosis. With the quantitative study on the haemocytes of C.virginica and M.mercenaria, performed by Foley and Cheng (1975) and similar types of work done subsequently by others, it is now considered almost certain that in most of the bivalves, the

granulocytes are more phagocytic than the agranulocytes.

Of the two types of the uptake mechanisms observed in the present study, the first one is comparatively less observed. Bang (1961) appears to be the first to report this kind of uptake mechanism by the granulocytes of C.virginica. Subsequently, it has been observed in M.mercenaria granulocytes, although less frequently (Mohandas, 1985). As mentioned earlier, the ectoplasm forms some sort of a web between adjacent pseudopods in S.scripta and V.cyprinoides var. cochinensis. In this uptake mechanism, the pseudopodia and the ectoplasmic web are mainly involved. It is noted that, in M.mercenaria the ectoplasmic webs contain a rich array of microtubules (Mohandas, 1985). Bang has also reported similar ectoplasmic web between adjacent pseudopods in C.virginica haemocytes, and has suggested that this cytoplasmic web serves as a trap, and the uptake of bacterial cells is effected when they are trapped in the web. The role of microtubules in the cytoplasm, besides aiding in maintaining the rigidity of filopods, is also assumed to be supportive to the ectoplasmic web itself to maintain rigidity (Mohandas, 1985). It has also been observed by him (Mohandas, 1985) in M.mercenaria granulocytes that the microtubules are arranged longitudinally as well as latitudinally, and this arrangement may be to restrict the lateral shifting and marked bending of pseudopods.

The main uptake mechanism that has been observed in S.scripta and V.cyprinoides var. cochinensis granulocytes is the one involving endocytosis. After contact has been achieved between a granulocyte and bacteria invaginations on the surface of phagocyte develop and the bacteria are taken into the endocytotic vesicle. The involvement of microtubules in this process in these two species of clams, is difficult to ascertain at light microscopic

level. This type of uptake mechanism has also been observed in C.virginica (Cheng, 1975), and in M.mercenaria (Mohandas, 1985). Electron microscopic studies of endocytosis mechanisms in M.mercenaria (Mohandas, 1985) have revealed the role of microtubules in this type of uptake mechanism also. Here, the microtubules are believed to be involved in trapping the bacteria into the ectoplasmic web and help them glide through the channel into the primary phagosome. The presence of bacteria in the web, microtubules all along the channel, and bacteria inside the channel, all are supportive of this contention (Mohandas, 1985).

In the present study, a high percentage of granulocytes was found to be actively involved in phagocytising the bacteria. Although morphologically different types of haemocytes have been previously described in several molluscan species, until recently, it had not been demonstrated whether there are functional differences correlated with the morphological ones. Most of these studies concerned with the quantification of phagocytosis and particle clearance have demonstrated the effect of some experimental parameters on phagocytosis, but did not involve differentiation between the activities of the different types of cells (Tripp, 1958a, 1960; Feng, 1966a; Feng and Stauber, 1968; Feng and Feng, 1974; Bayne, 1983). As a result, functional differences between types of cells could not be discerned. But studies by Bang (1961), Galtsoff (1964), Cheng and Rifkin (1970), Cheng (1975), Cheng and Foley (1975), Huffman and Tripp (1982), Rodrick and Ulrich (1984), Rasmussen et al. (1985) have revealed, beyond any shadow of doubt, that in several species of bivalves, there are only two types of haemocytes- granulocytes and agranulocytes/hyalinocytes, and of these, granulocytes in C.virginica, M.mercenaria and M.campechiensis were

found to be the most active from the point of phagocytosis. However, in P.radiata (Nakahara and Bevelander, 1969), in C.gigas (Ruddell, 1971a), and in T.maxima (Reade and Reade, 1972), agranular haemocyte were found to be phagocytic. The confusion in M.edulis regarding the type of cell involved in phagocytosis was solved by Cheng (1981), and Rasmussen et al. (1985) by changing the terminology of the cell type 'macrophages' to granulocytes.

Foley and Cheng (1975) performed a quantitative study on the haemocytes of C.virginica and M.mercenaria and ascertained that in C.virginica 87.28% of granulocytes as compared to 12.32% of hyalinocytes were associated with experimentally introduced Staphylococcus aureus, and 83.48% of granulocytes as compared to 16.80% of hyalinocytes were associated with experimentally introduced E.coli. In the case of haemocytes of M.mercenaria 12.82% of granulocytes as compared to 2.56% of hyalinocytes were associated with bacteria (Foley and Cheng, 1975; Cheng, 1981).

In another study on C.virginica, Renwrantz et al. (1979), have demonstrated that 2% of the hyalinocytes and 80% of the granulocytes exhibited phagocytic activity towards rat erythrocytes. Studies by Bayne et al. (1979b) on M.californianus revealed that both agranular and granular haemocytes are capable of phagocytising yeast or blood cells. Rodrick and Ulrich (1984) have demonstrated that in M.campechiensis, C.virginica and A.ovalis, the granulocytes are more highly phagocytic although figures were not given. Foley and Cheng (1975) and Rodrick and Ulrich (1984) have also demonstrated that the phagocytic activity varied with the nature of the challenge and temperature. An increase in temperature was found to influence favourably the phagocytic ability of haemocytes.

The term pinocytosis was first introduced by Lewis (1931). Feng (1965b) has demonstrated the in vivo and in vitro uptake of proteins by oyster leucocytes by employing serological techniques and fluorescent microscopy. It has also been reported by several workers that metals in a soluble micromolecular form such as chelated iron, lead, zinc, copper, and cadmium enter epithelial cells by pinocytosis (Simkiss and Mason, 1983). Since the mechanism of pinocytosis does not differ from the second type of uptake mechanism of phagocytosis where only plasma membrane takes active participation, the involvement of microtubules may be believed to be taking place here also, since it has been demonstrated at ultrastructural level (Mohandas, 1985) that microtubules are involved in the second type of uptake mechanism. Regarding the uptake of metal ions from solution, at present there appears to be no clear interpretation, although the specific involvement of ion pumps in the absorption of trace metals from solutions is indicated (Simkiss and Mason, 1983). Evidence in favour of this interpretation has been provided by George and Coombs (1977) who have shown that ligand binding of Cd, Fe or Pb ions increases the rate at which they enter M.edulis.

In copper-dosed clams, there was no significant difference between controls and experimentals in the case of S.scripta, however, in the case of V.cyprinoides var. cochinensis a significant drop was observed in the experimentals and to a certain extent it was dosage dependent. It is noted that the concentrations of copper used for S.scripta were well below the LC_{50} values, and those used for V.cyprinoides var. cochinensis were very near to its LC_{50} values. It has been shown that S.scripta can stand 10 ppm of copper for more than 7 days without mortality (Latha Thampuran, personal communication).

The importance of haemocytes in metal metabolism was pointed out by Ruddell and Rains (1975) who reported that basophilic granulocytes of C.gigas and C.virginica contain deposits of zinc and copper. These observations have been extended by George et al. (1978) who reported that zinc was associated with phospherous in the basophilic cells whereas copper occurred with a sulphur ligand in acidophilic cells. Iron and lead may also be contained in membrane bound vesicles of amoebocytes in M.edulis (George et al., 1976; Coombs, 1977). It is interesting to note that Pirie et al. (1984) have found different oyster species containing blood cells with different metal composition. As to the actual mechanisms of these metal deposition within haemocytes opinions differ, and currently there are at least three theories (Simkiss and Mason, 1983). The first theory suggests that metals either in a particulate form or in association with denatured proteins in the blood are phagocytosed by amoebocytes in an indiscriminate way. The metals are treated as any other foreign material, but being resistent to digestion, they accumulate in the cytoplasm of these cells. The second theory proposes that amoebocytes represent a specific detoxification system that removes metal ions from the haemolymph and thereby keeps the concentrations in the blood below toxic level. This theory proposed by George et al. (1976) explains the pinocytic uptake of some soluble metals and could account for the specificity in metal uptake. The third theory implies that the amoebocytes are involved in the specific transportation of metals around the body, to the kidney and other tissues for storage and eventual excretion. In C.virginica, Ruddell (1971b,c) has suggested that amoebocytes may have an important function in discharging copper at sites of injury or cellular stress. He envisages copper as aiding the phagocytosis

of bacteria and microbes by binding pathogenic material into a denatured form. Cheng and Sullivan (1984) have reported that C.virginica exposed to 5 ppm Cu^{2+} showed a statistically significant increase in the phagocytosis of polysterene spheres. In other situations, amoebocytes have been observed, leaving the body by diapedesis after traversing the epithelial layers of the heart and kidney (Brown and Brown, 1965). Ruddell (1971b) believes that this may be one of the methods of removing zinc- and copper- laden amoebocytes from the bodies of oysters.

Except perhaps the work of Pickwell and Steinert (1984) practically nothing is known about the haemocyte number in stressed bivalves. However, Thompson et al. (1978) observed an increase in the number of circulating haemocytes in M.californianus when exposed to air, and attributed this increase to a release of these cells from the digestive gland. In their studies with M.edulis, Pickwell and Steinert (1984) found a spectacular drop in macrophage counts (young granulocyte) specifically at higher concentration, indicative of a toxic effect due to copper stress. They have also observed that macrophage counts in copper stressed clams declined earlier than in controls. Another spectacular observation was the dramatic increase in granulocyte (mature granulocyte) numbers in mussels exposed to the highest concentration (267 ppb of Cu^{2+}). The reason for finding no significant difference in the total haemocyte count in the controls and experimentals of S.scripta is attributed to the fact that the concentrations of the toxicant used were far below the LC_{50} values. Apparently the concentrations of the metal ion never reached a threshold level above which the haemocytes may get involved in the specific transportation of metals to the kidney and other tissues for storage and

eventual excretion. In V.cyprinoides var. cochinensis, there is a clear indication that haemocyte counts in copper-dosed clams declined significantly from the control values. It is believed that in stressed clams there is migration of haemocytes, as metal-laden haemocytes, transporting these metals to the kidney and other tissues for storage and eventual excretion. The role of haemocytes in transporting metal ions to various tissues has been stressed by George et al. (1976) and Coombs (1977). As indicated earlier, when the haemocyte counts of clams exposed to the three concentrations of copper were analysed, in 0.15 ppm and 0.45 ppm copper-stressed V.cyprinoides var. cochinensis, the values were significantly higher than those of 0.30 ppm copper-dosed clams from 48 hrs onwards and 72 hrs onwards, respectively. Interestingly, there is no significant difference in the values of 0.15 ppm and 0.45 ppm dosed clams on any day. In the light of the study by Pickwell and Steinert (1984), although no differential count of haemocytes was made by me in Cu^{2+} stressed clams, the following explanations are given to my observations. In 0.30 ppm copper-dosed clams, there was mass migration of metal-laden ("mass mortality" of Pickwell and Steinert, 1984) young granulocytes, but there was no substantial increase in mature granulocytes, and this is reflected in the low haemocyte count observed from 48 hrs onwards. In 0.15 ppm copper-dosed clams, since the concentration was less, there was less mass migration of young granulocytes and no substantial increase in mature granulocytes. So, obviously, the haemocyte counts of 0.15 ppm copper-dosed clams were significantly higher than those of 0.30 ppm copper-dosed clams from 48 hrs onwards. In 0.45 ppm copper-dosed clams, there was mass migration of young granulocytes due to high concentration of the toxicant but there was also dramatic increase in mature

granulocytes, apparently from other sources. Hence, in 0.45 ppm copper-dosed clams, the counts were significantly higher than those of 0.30 ppm copper-dosed clams from 72 hrs onwards. In 0.15 ppm copper-dosed clams as mentioned earlier, there was less mass migration of young granulocytes and no substantial increase in mature granulocyte, but in 0.45 ppm copper-dosed clams, although there was mass migration of young granulocytes, there was dramatic increase in mature granulocytes. Obviously, the net result is an apparent stable haemocyte count with no significant statistical difference. In this context, it is interesting to note the observation of Cheng and Sullivan (1984) on the effects of heavy metals on phagocytosis by C.virginica haemocytes. They have observed that in these oysters, haemocytes exposed to 1 ppm copper, did not show any alteration in the phagocytic ability, but those exposed to 5 ppm copper, showed a statistically significant increase in phagocytic ability. It is known that Cd^{2+}, Pb^{2+} and Zn^{2+} bind to negatively charged ionogenic groups on the plasma membrane on mammalian macrophages, resulting in a measurable decrease in the net negative surface charge of these cells (Kiremidjian-Schumacher et al., 1981). Assuming that the same principle may be operative in oyster haemocytes also, Cheng and Sullivan (1984) have indicated that the decreased surface charge may enhance binding and subsequent phagocytosis of non-polar particles, such as latex spheres, simply by facilitating hydrophobic interactions. To me, it appears, that besides this phenomenon, the increased phagocytosis at higher concentration of copper may be due to the involvement of more number of granulocytes at higher concentration of copper.

Since the cellular processes involved in stressed condition caused by biotic and abiotic factors are entirely different, the variations in haemocyte number,

in clams injected with Vibrio sp. are explained in a different way. As indicated in the review, there are several reports that haemocytes phagocytose bacteria. However, there is difference in the resident time of bacteria in the body depending on the route of entry. Hartland and Timoney (1979) have reported that oysters and clams innoculated via the intracardial route, generally exhibited a slightly greater persistence of bacteria than when exposed in the surrounding water. The fact that in S.scripta and in V.cyprinoides var. cochinensis, the response to the presence of bacterium in the system, varies with time clearly indicates interspecific difference. The significant increase in the number of haemocytes in the Vibrio injected S.scripta started at 48 hrs and continued till 96 hrs, whereas in V.cyprinoides var. cochinensis, it started at 3 hrs and continued upto 12 hrs. Obviously, to combat the microbial challenge, more haemocytes were mobilised into the system from other sources or more number of haemocytes were produced within 3 hrs in V.cyprinoides var. cochinensis, and within 48 hrs in the case of S.scripta. At this stage, however, it is difficult to differentiate whether, the increase in the number of haemocytes, subsequent to bacterial challenge was due to mobilisation or due to leucocytosis. The higher values in saline treated S.scripta at 6 hrs, and in V.cyprinoides var cochinensis at 3 hrs when compared with the respective controls, are explained as immediate stimulation/response to the presence of an abiotic factor (saline) in the system. This also shows that the stimulation is not as great as that resulting from challenge with bacteria. Interestingly, in S.scripta, at all time periods, there were increased cell counts in saline treated individuals when compared with the controls. But in V.cyprinoides var. cochinensis except at 3 hr- and 72 hr- time periods the values in saline treated individuals were less

than the control values. Whereas, in S.scripta, the bacteria injected clams recorded significantly higher counts than those of the untampered controls only from 48 hrs onwards, in V.cyprinoides var. cochinensis this was observed from 3 hrs onwards upto 12 hrs and beyond this time period, though the values were not statistically significant, the counts were always on the higher side. Although, the bacteria-challenged V.cyprinoides var. cochinensis had significantly higher counts than those of saline treated ones at certain time periods, and in S.scripta, the saline treated ones had higher values than the bacteria-challenged ones at one time period, and the vice-versa at another time period, at this stage it is difficult to give a definite explanation. From these investigations, it is however, clear that the number of haemocytes varies (1) between species, (2) cell counts will be affected by challenge, biotic or abiotic, and (3) the response may be immediate or delayed.

Although comparable data are not available on bivalves, Michelson (1975), in the gastropod B.glabrata, has reported a two-fold increase in haemocytes two weeks post infection with Schistosoma mansoni, and interestingly Stumpf and Gilbertson (1978) reported two-fold increase in haemocytes 2 hrs post infection of the same snail sp. with miracidia of S.mansoni. But Lie and Heyneman (1976) found no increase in haemocyte number of B.glabrata infected with Echinostoma lindoense. Jeong et al. (1980) have also reported increased haemocyte count in B.glabrata sensitized and re-sensitized to E.lindoense.

Cytochemical studies on molluscan haemocytes have been made by a few workers. Huffman and Tripp (1982) have reported that glycogen is present in small amounts in the cytoplasm of granular haemocytes of M.arenaria. Moore and Lowe (1977) made a similar observation in M.edulis cells. In the same

year, Moore and Eble (1977) reported that the glycogen is not present in agranular cells but occurred in small and large granulocytes of M.mercenaria. Electron microscope studies of C.virginica haemocytes performed by Cheng and Cali (1974) to ascertain the fate of phagocytosed bacteria, have revealed that glycogen is situated initially within secondary phagosomes, but later as the phagosome disintegrates, the granules become distributed in large clumps throughout the cytoplasm. Further studies carried out by Cheng and Rudo (1976b) in C.virginica injected with ^{14}C-labelled Bacillus megaterium have also revealed that bacterial degradation results in the synthesis of more glycogen from sugar of bacterial origin. Transmission electron microscopic studies by Rodrick and Ulrich (1984) on M.campechiensis exposed to B.megaterium have also revealed numerous glycogen rosettes in numerous phagocytic vesicles. In M.mercenaria granulocytes, electron microscope studies performed by Mohandas (1985) have revealed that along with the degradation of bacteria within the phagosomes more glycogen granules appear within the phagosome and these phagosomes containing clumps of glycogen granules and non-digestible materials are expelled from the phagosome to the exterior, obviously into the serum. These studies, thus indicate that glycogen is present in small amounts in the cytoplasm of haemocytes in several bivalve species, and that subsequent to phagocytosis of bacteria there is increase in the deposition of clumps of glycogen granules within the cytoplasm.

The present study has demonstrated alkaline and acid phosphatase activities in the haemocytes of S.scripta and V.cyprinoides var. cochinensis, and that the activities of these enzymes increase upon challenge with bacteria. Lysosomal enzymes such as non-specific esterase, and acid and alkaline phosphatases have

been demonstrated in the haemocytes of M.mercenaria (Yoshino and Cheng, 1976a; Moore and Eble, (1977) and of C.virginica (Feng et al., 1971), as well as acid phosphatase and B-glucuronidases in M.californianus (Bayne et al., (1979b). Moore and Lowe (1977) observed that macrophages (granulocytes) of M.edulis were strongly reactive for acid hydrolases activity. Huffman and Tripp (1982) have demonstrated non-specific esterase, and acid and alkaline phosphatases in M.arenaria haemocytes. As observed in the present study, some cells have great activity of acid phosphatase, some have great activity of alkaline phosphatase while others have no apparent enzyme activity. Huffman and Tripp (1982) have also made similar observations, regarding the hydrolytic enzymes in the haemocytes of M.arenaria. From these studies, it is obvious that the phagocytes of different species of bivalves vary widely in their enzymatic content. In vertebrates, for example, neutrophils of cats and chicken are devoid of alkaline phosphatase activity, while those of hamsters and ginea pigs, have a great deal of alkaline phosphatase activity (Kaplow, 1973). Further, within a single species, the cellular enzyme content may vary with physiological condition, for example, humans with bacterial infections have elevated alkaline phosphatase activity, while those with viral infections have normal or below normal enzyme activity (Kaplow, 1973). In invertebrates, the variations in cell enzyme content have been attributed to age, metabolic history of individual cells and physiological state of the individual. The finding of Yoshino and Cheng (1976a) that not all of the lysosomes in each cell include acid phosphatase suggests a non-synchronised chemical cycle occurring within this organelle or the lysosomes represent a chemically heterogenous population as suggested by Dean (1977), Schellens et al. (1977). Dean (1977)

has indicated that lysosomes even in single cell types are quite variable in their enzymatic constitution, and the heterogenity in size and shape of this organelle (Cheng and Foley, 1975) reflects the divergent functional activities of lysosomes in different types of cells as indicated by Schellens et al. (1977). Enzyme masking has also been accounted for some of these variations (Huffman and Tripp, 1982).

Regarding the vital staining of inclusions in haemocytes, what was considered as specific granules in the granulocytes of M.mercenaria by Zacks and Welsh (1953) was later treated as atypical mitochondria by Zacks (1955). Zacks (1955) has also stated that those vacuoles in leucocytes that take up Neutral Red are not the same as the granules that take up Janus Green B. Zacks (1955) has also reported that Neutral Red vacuoles are not performed in the granulocytes, but form as the dye accumulate in these cells. But Foley and Cheng (1974) have stated that these specific granules in the granulocytes of M.mercenaria take up both Janus Green B and Neutral Red. They did not observe large populations of granules, selectively staining with Janus Green B when a mixture of Janus Green B and Neutral Red was used, and opined that there is only one type of cytoplasmic granule in M.mercenaira and it is stained with both these stains. Interestingly, in the same species Moore and Eble (1977) observed four types of granules of which blunt granules (identified as mitochondria) and dot-like granules (identified as lysosomes), were found to take up both Janus Green B and Neutral Red. It is noted that the electron microscope studies performed by Cheng (1975), and Cheng and Foley (1975) on the haemocytes of M.mercenaria have revealed that these granules are membrane-bound vesicles containing a homogeneously

electron-dense substance, and these granules have subsequently been demonstrated to be true lysosomes (Yoshino and Cheng, 1976a). Recently Mohandas (1985) has demonstrated that in M.mercenaria granulocytes, these granules may appear as electron dense or as electron lucid vesicles depending upon the phase of activity.

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CHAPTER III

GLYCOGEN AND TOTAL CARBOHYDRATE IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

Early studies on the biochemical composition of bivalves have shown that, in most cases, changes in body weight are mainly due to changes in carbohydrate or glycogen content, and correspond to seasonal changes, or in some instances to the reproductive cycles or in certain other instances to a combination of both (Gabbott, 1976). For example, in M.edulis, the seasonal cycle of storage and utilisation of glycogen reserves is closely linked to an annual reproductive cycle (Gabbott, 1976), but in Meretrix casta this was attributed to seasonal changes (Salih, 1979). In exceptional cases as in Ostrea edulis, the glycogen storage cycle and the annual reproductive cycle are not clearly separated (Gabbott, 1976). In almost all living things stored energy is used up (1) when the animal faces a depletion in the food supply, (2) when it is made unavailable by physical, chemical or physiological agents, (3) when it becomes necessary to direct much energy to a particular cause, such as, burst activity during escape from adverse conditions, or gametogenesis, and (4) when the animal faces environmental or physiological stress. In almost all these circumstances, the major share of stored energy comes from the carbohydrate or glycogen reserves. Thus, carbohydrates form the central point in energy production because of its great mobility in the living systems, together with its capacity to get compartmentalized within the cells and tissues. The mobility is provided by the glucose, and compartmentalization by glycogen, glucose-6-phosphate, etc.

In bivalves, it is interesting to note that there are differences in the energy reserves from which the energy is drawn to combat stress, depending

on the seasons. For example, there are clear differences in the balance of carbohydrate and protein reserves in M.edulis between summer, autumn and winter (Bayne, 1973; Gabbott and Bayne, 1973). In the autumn, during more prolonged starvation, there was a marked increase in the utilisation of lipid reserves (Bayne, 1973). Bayne and Scullard (1977) have shown that in winter, nitrogen excretion rates increased as a result of starvation (indicative of mainly protein catabolism). Also, in winter, anaerobic metabolism may take place largely at the expense of protein reserves and this, in turn, will involve the conversion of amino acids into succinate (Gabbott, 1976). But during summer, the energy loss was accounted for by the utilisation of carbohydrate and lipid (Gabbott and Bayne, 1973; Bayne and Scullard, 1977). The measured loss of carbohydrate was for free sugars in the non-mantle tissue (presumably from the digestive gland). But information on the metabolic balance between the glucose utilisation and gluconeogenesis in marine bivalves is lacking (Gabbott, 1983), although this is probably a common feature of all organs (de Zwaan, 1983). Similarly, in the tropics, where there is no clear cut difference of seasons, the information regarding the variation, if any, in the energy reserve utilisation is also lacking. Similar are situations where informations are lacking on the quantity of reserves available in bivalves of different age groups, and also on the type of reserves (carbohydrate, protein or lipid) from which energy is drawn when these animals are exposed to biotic and abiotic stress.

In order to get more information on these lines, experiments were designed to study the total carbohydrates and glycogen in the haemolymph of S.scripta and V.cyprinoides var. cochinensis of (1) four size-groups, each, (2) under heavy metal stress, and (3) during bacterial challenge. The results of this study are

reported in this chapter.

MATERIALS AND METHODS

Methods of collection of clams, rearing the clams, acclimatisation, selection of size groups, mode of collection of haemolymph, dosing the clams with copper, challenging the clams with bacteria, and the statistical analysis of the data were the same as described in detail in chapter II. As described earlier, concentrations of the copper ions used were different in the two species of clams, but dosage of the bacterial suspension was the same. For S.scripta the salinity used was 30‰ and, for V.cyprinoides var. cochinensis it was 15‰.

The number of clams employed for studying haemolymph glycogen and total carbohydrates in the different size groups are given below. In the case of S.scripta, 12 clams from each size group, and in the case of V.cyprinoides var. cochinensis 13 clams from each size group were employed for the estimation of haemolymph glycogen, and for the estimation of total carbohydrates in the haemolymph 20 clams from each size group of S.scripta, and 13 clams from each size group of V.cyprinoides var. cochinensis were employed.

To study glycogen in the haemolymph of S.scripta of the size group 38-40 mm, dosed with the three concentrations of copper, the total number of clams used is as follows: The experimental group consisted of 180 clams (60 in each of the three experimental batches), and the control group consisted of 60 clams. In the case of V.cyprinoides var. cochinensis also the 38-40 mm size group was used, and the experimental group consisted of 180 clams, and the control group 60 clams. Haemolymph was withdrawn every 24 hrs for 5 days for the estimation of glycogen from 10 specimens each of the experimental batches and 10 from the control group, each day.

To study the total carbohydrate in the haemolymph, the experimental group of each clam species consisted of 180 individuals, and the control group 60 individuals.

For the determination of glycogen and total carbohydrate, the haemolymph samples withdrawn were 0.50 ml and 0.10 ml respectively, from each clam.

For the determination of glycogen in the haemolymph of S.scripta and V.cyprinoides var. cochinensis subsequent to challenge with bacteria at a concentration of 1 x 10^8 cells/0.02 ml, haemolymph samples were collected at 3, 6, 12, 24, 48, 72, 96 and 120 hr-time period post-challenge. For each clam species, for each time period, the untampered control group, 2% saline-injected control group, and the bacteria-injected experimental group consisted of 10 clams each.

For the determination of total carbohydrate in the haemolymph of S.scripta and V.cyprinoides var. cochinensis subsequent to bacterial challenge at a concentration of 1 x 10^8 cells/0.02 ml, haemolymph samples were collected at 3, 6, 12, 24, 48, 72, 96 and 120 hr-time period post-challenge. In the case of S.scripta for each time period, the untampered control group consisted of 20 clams, 2% saline-injected control group consisted of 20 clams, and the bacteria-injected experimental group consisted of 30 clams, whereas in the case of V.cyprinoides var. cochinensis they were 15, 15 and 25, respectively. For the determination of glycogen and total carbohydrate, the samples of haemolymph withdrawn were 0.50 ml and 0.10 ml respectively.

In this experiment, more clams than the required number were reared as experimentals and controls anticipating mortality during the course of the experiment.

ESTIMATION OF GLYCOGEN

Glycogen in the haemolymph was estimated following the method of Montgomery (1957) after deproteinising the sample with 10% TCA. For this, to 1.0 ml of 10% TCA a 0.50 ml sample of haemolymph was pipetted and thoroughly mixed. It was then centrifuged at 2500 rpm for 10 minutes. The supernatant was gently decanted into another test-tube. For the determination of glycogen, 1.0 ml sample of this was taken into another test-tube, and 1.2 ml of 95% ethyl alcohol was added and mixed. It was kept overnight in a refrigerator and then centrifuged at 2500 rpm for 15 minutes. The supernatant was very carefully decanted. To the precipitate, 2.0 ml of distilled water and 0.1 ml of 80% phenol were added. To this, 5.0 ml of concentrated sulphuric acid was added forcefully, to aid mixing. It was then left at room temperature for 30 minutes. After cooling, the optical density was read at 490 nm. The concentrations of glycogen in the samples were found out from the standard graph prepared by employing oyster glycogen (Sigma chemical company, USA.) as the standard.

ESTIMATION OF TOTAL CARBOHYDRATE

Estimation of total carbohydrate was carried out following the method of Dubois et al. (1956). A 0.1 ml sample of the haemolymph was pipetted into a large test-tube containing 0.1 ml of 80% phenol. To this 1.9 ml of distilled water was added bringing the total volume to 2.1 ml. To this, 5.0 ml of concentrated sulphuric acid was added forcefully, allowing thorough mixing. It was left at room temperature for 30 minutes. After cooling, the optical density was determined at 490 nm. From this optical density, the concentration of total carbohydrate in the haemolymph was found out from a standard graph

employing glucose as the standard. The total carbohydrate per ml was then found out and expressed as μg glucose equivalents per ml.

RESULT

GLYCOGEN IN THE HAEMOLYMPH OF THE FOUR SIZE GROUPS OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

Table 5A gives the mean value, standard deviation, and range of haemolymph glycogen in the four size groups of S.scripta and Table 5B shows the same in the four size groups of V.cyprinoides var. cochinensis. In both these cases when the mean value of glycogen, for different size groups were compared, there was no statistically significant difference.

TOTAL CARBOHYDRATE IN THE HAEMOLYMPH OF FOUR SIZE GROUPS OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

Tables 6A and 6B show the mean values, standard deviation, and range of total carbohydrate in haemolymph in the four size groups of S.scripta and V.cyprinoides var. cochinensis, respectively. Comparison of mean values of total carbohydrate within the species showed no statistically significant difference in any of the possible combinations.

GLYCOGEN IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS, DOSED WITH THREE CONCENTRATIONS OF COPPER

Haemolymph glycogen values in S.scripta and V.cyprinoides var. cochinensis when exposed to three sub-lethal concentrations of copper for five days are given in Tables 7 A and 7B, and Figures 3A and 3B, respectively.

In S.scripta dosed with three concentrations of copper, when the mean values of haemolymph glycogen were compared, there was no statistically

Table 5A. Haemolymph Glycogen (µg/ml) in the four size groups of *Sunetta scripta*

Size group	30-32 mm	36-38 mm	38-40 mm	42-44 mm
N	12	12	12	12
Mean value	43.7	46.6	38.4	42.4
± SD	13.55	9.23	13.51	10.90
Range	27.6-71.8	30.8-61.6	23.0-72.0	28.4-64.6

Table 5B. Haemolymph Glycogen (µg/ml) in the four size groups of *Villorita cyprinoides* var. *cochinensis*

Size group	24-26 mm	34-36 mm	38-40 mm	44-46 mm
N	13	13	13	13
Mean value	42.3	39.0	38.4	44.5
± SD	12.57	7.99	11.80	10.89
Range	26.2-68.8	26.2-52.4	23.4-65.0	30.0-67.4

Table 6A. Haemolymph Total Carbohydrate (as µg glucose/ml) in the four size groups of Sunetta scripta

Size group	30-32 mm	36-38 mm	38-40 mm	42-44 mm
N	20	20	20	20
Mean value	196.5	184.0	187.5	174.5
± SD	53.50	63.12	47.84	56.43
Range	110-320	90-310	90-250	100-280

Table 6B. Haemolymph Total Carbohydrate (as µg glucose/ml) in the four size groups of Villorita cyprinoides var. cochinensis

Size group	24-26 mm	34-36 mm	38-40 mm	44-46 mm
N	13	13	13	13
Mean value	157.3	134.6	139.2	163.8
± SD	59.44	33.65	38.52	44.12
Range	90-280	80-200	70-220	80-240

Table 7A. Haemolymph Glycogen (µg/ml) in Sunetta scripta dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	42.59	40.61	37.58	42.20	42.91
	± SD	9.28	13.23	10.87	8.37	9.44
	Range	23.0-57.4	21.8-76.0	25.0-52.4	26.8-55.0	26.8-60.0
1 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	39.20	32.29	36.02	35.03	41.26
	± SD	10.63	7.57	13.08	9.56	11.43
	Range	23.0-57.6	22.0-47.0	22.0-56.2	25.6-53.8	22.2-64.0
3 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	35.55	30.48	28.49	37.43	36.09
	± SD	10.40	9.73	9.26	12.77	6.91
	Range	24.4-61.0	19.0-50.6	22.0-54.0	22.8-57.6	28.4-48.0
5 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	37.57	33.58	28.81	34.44	36.10
	± SD	7.11	6.38	7.26	9.70	6.85
	Range	23.0-50.6	25.0-44.2	21.4-42.2	21.2-54.2	22.0-44.6

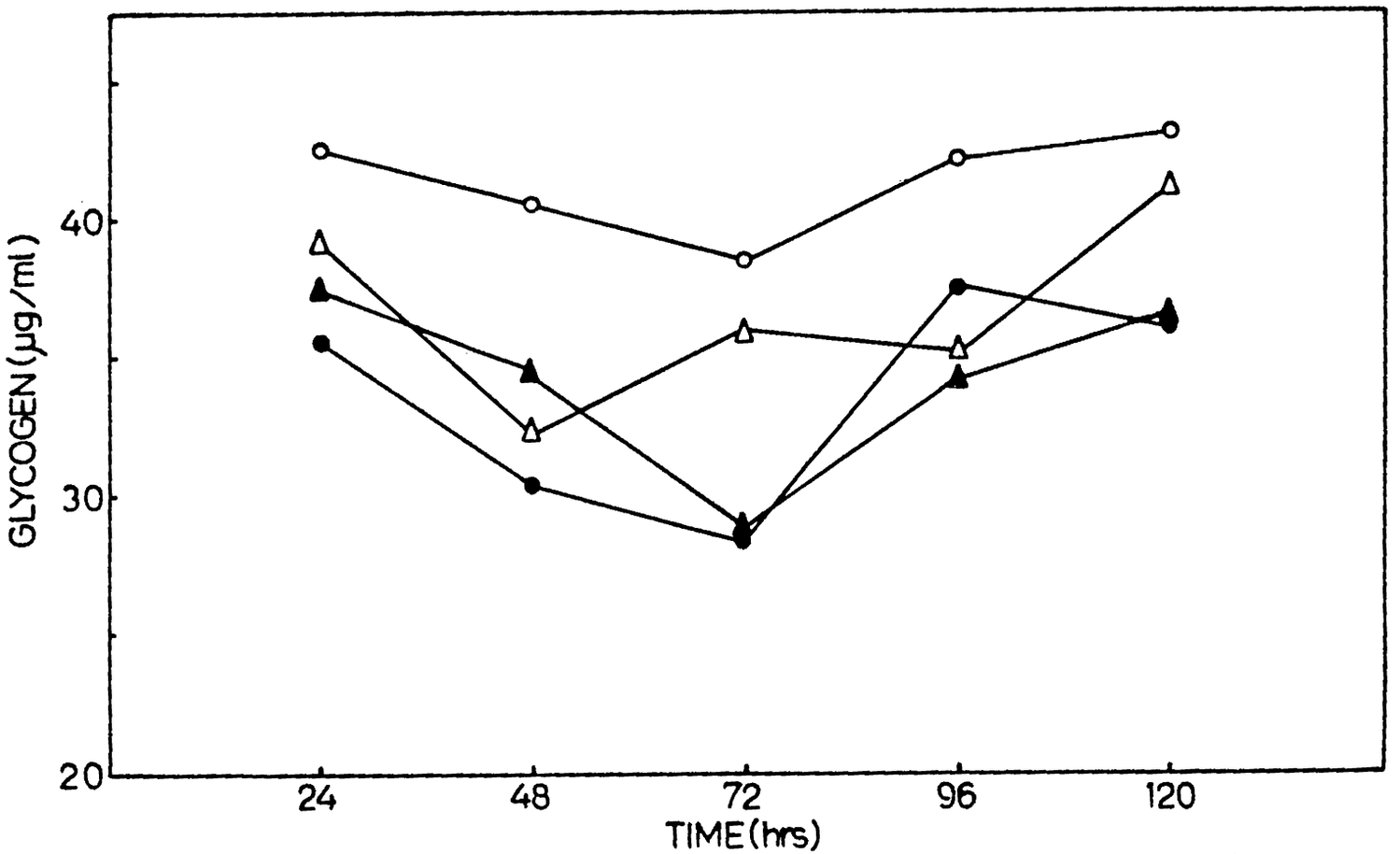

Fig. 3A. Haemolymph Glycogen (µg/ml) in Sunetta scripta dosed with three concentrations of copper. Control (o); 1 ppm copper (Δ); 3 ppm copper (●); 5 ppm copper (▲).

Table 7B. Haemolymph Glycogen (µg/ml) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	31.35	28.02	31.89	35.55	36.20
	± SD	13.47	9.33	8.64	9.21	11.68
	Range	15.0-60.0	15.0-52.5	20.0-47.0	22.0-50.0	15.0-53.0
0.15 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	25.50	18.33	35.30	31.78	36.25
	± SD	6.79	4.94	12.13	5.97	11.36
	Range	15.0-37.5	15.0-30.0	22.0-55.0	22.0-42.3	22.0-60.0
0.30 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	26.65	18.69	28.23	25.30	25.73
	± SD	10.66	5.01	9.91	8.00	6.40
	Range	15.0-53.0	12.0-27.6	22.0-57.0	15.0-38.0	15.0-36.8
0.45 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	31.49	19.88	29.40	22.15	20.80
	± SD	7.12	3.59	7.57	5.44	9.49
	Range	19.0-45.0	15.0-25.0	21.6-44.0	15.0-30.0	12.8-45.0

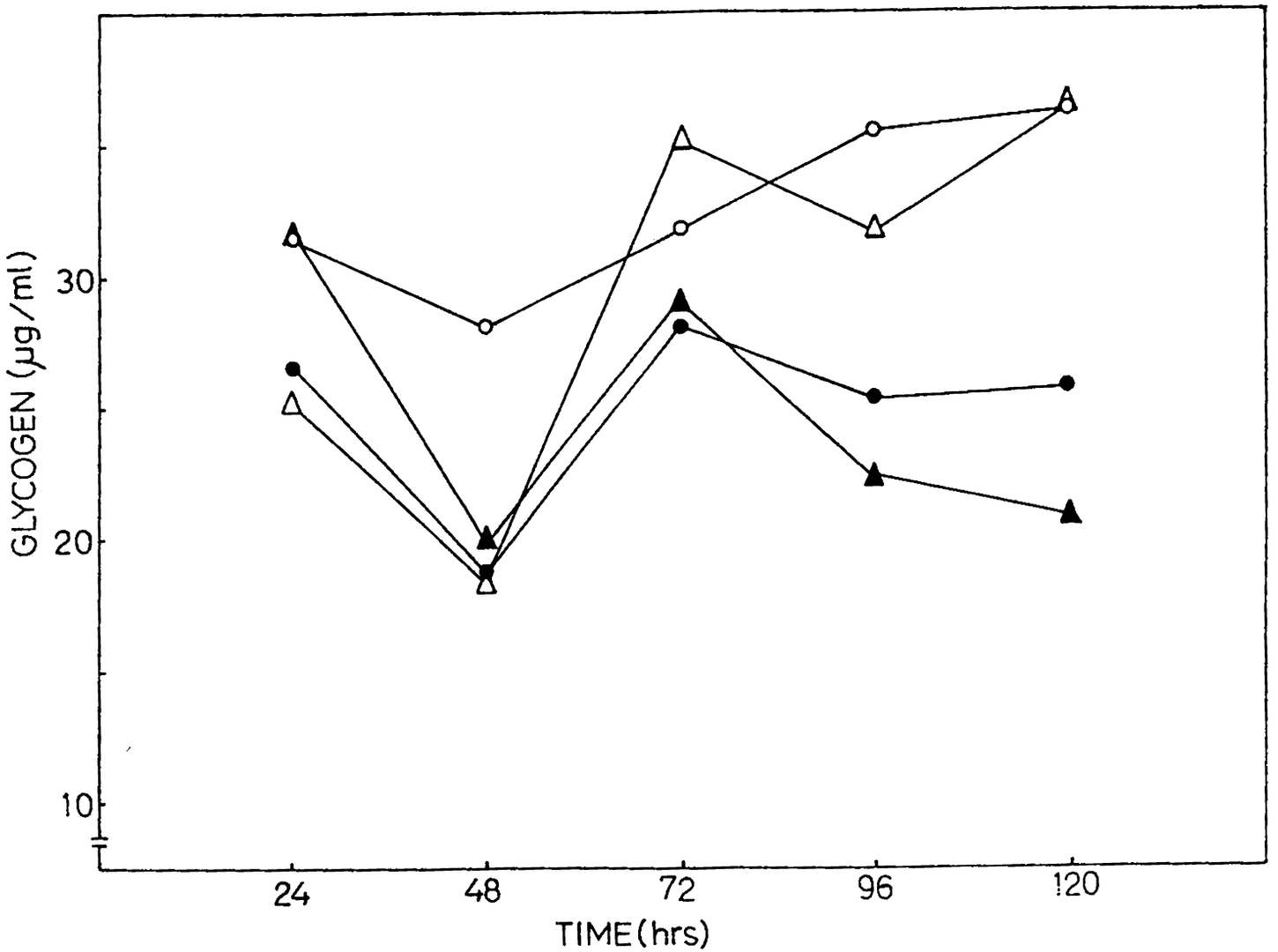

Fig. 3B. Haemolymph Glycogen (µg/ml) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper. Control (o); 0.15 ppm copper (Δ); 0.30 ppm copper (●); 0.45 ppm copper (▲).

significant difference between the control and the experimentals during the exposure period of five days. The same was the case when the mean values of haemolymph glycogen of clams exposed to different concentrations of copper were compared among themselves. In all the above cases the 'P' values were greater than 0.05. In V.cyprinoides var. cochinensis, exposed to copper, the situation was different. When the mean values of 0.15 ppm copper-dosed clams were compared with the mean values for glycogen of the control group, the values of 0.15 ppm copper-dosed clams were significantly lower than the control values at 48 hrs ($P < 0.02$). When the mean values for glycogen of 0.30 ppm copper-dosed clams were compared with the control values, values of the 0.30 ppm copper-dosed clams were significantly lower at 48 hrs ($P < 0.02$), 96 hrs ($P < 0.05$), and at 120 hrs ($P < 0.05$). When the mean values for glycogen of 0.45 ppm copper-dosed clams were compared with the control values, the values of the 0.45 ppm copper-dosed clams were significantly lower at 48 hrs ($P < 0.05$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). When the mean glycogen values of 0.15 ppm copper-dosed clams were compared with those of the 0.30 ppm copper-dosed clams, the values of 0.30 ppm copper-dosed clams were significantly lower at 120 hrs ($P < 0.05$). The values of 0.45 ppm copper-dosed clams were significantly lower at 96 hrs ($P < 0.01$) and 120 hrs ($P < 0.01$), when compared with the values of 0.15 ppm copper-dosed clams. The mean values of glycogen of 0.30 ppm copper-dosed clams and 0.45 ppm copper-dosed clams showed no significant difference when compared.

TOTAL CARBOHYDRATE IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS DOSED WITH THREE CONCENTRATIONS OF COPPER

The total carbohydrate values in the haemolymph of S.scripta and V.cyprinoides var. cochinensis when exposed to the three different concentrations of copper for five days are given in Table 8A and 8B, and Figures 4A and 4B, respectively.

In S.scripta dosed with the three concentrations of copper, the mean values of total carbohydrate of 1 ppm copper-dosed clams when compared with the mean values of the controls, the mean values of 1 ppm copper-dosed clams were significantly higher at 24 hrs ($P < 0.01$), 48 hrs ($P < 0.05$), 72 hrs ($P < 0.01$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). The mean values of total carbohydrate of 3 ppm copper-dosed clams were significantly higher than the control values of 24 hrs ($P < 0.01$), 48 hrs ($P < 0.01$), 72 hrs ($P < 0.01$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). The total carbohydrate values of 5 ppm copper-dosed clams were also significantly higher than the control values at 24 hrs ($P < 0.02$), 48 hrs ($P < 0.01$), 72 hrs ($P < 0.01$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). When the total carbohydrate values of 1 ppm copper-dosed clams were compared with the values of 3 ppm copper-dosed clams, the values of 3 ppm copper-dosed clams were significantly higher at 48 hrs ($P < 0.01$), and 96 hrs ($P < 0.01$). When the values of 1 ppm and 5 ppm copper-dosed clams were compared, the values of 5 ppm copper-dosed clams were significantly higher at 48 hrs ($P < 0.02$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). The total carbohydrate values of 3 ppm and 5 ppm copper-dosed clams did not show any statistically significant difference between themselves.

Table 8A. Haemolymph Total Carbohydrate (as µg glucose/ml) in Sunetta scripta dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	205.0	201.0	220.0	225.0	245.0
	± SD	15.0	18.14	17.32	56.79	84.29
	Range	180-230	170-230	190-250	150-300	150-350
1 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	260.0	226.0	317.0	309.0	380.0
	± SD	34.64	28.36	36.89	43.00	77.72
	Range	220-320	180-270	250-360	250-370	260-500
3 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	243.0	284.0	344.0	383.0	426.0
	± SD	14.18	27.64	76.97	41.49	63.75
	Range	220-270	230-320	220-460	320-440	310-510
5 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	294.0	301.0	293.0	380.0	529.0
	± SD	97.18	81.54	50.41	57.27	133.75
	Range	160-430	210-430	220-390	290-460	310-700

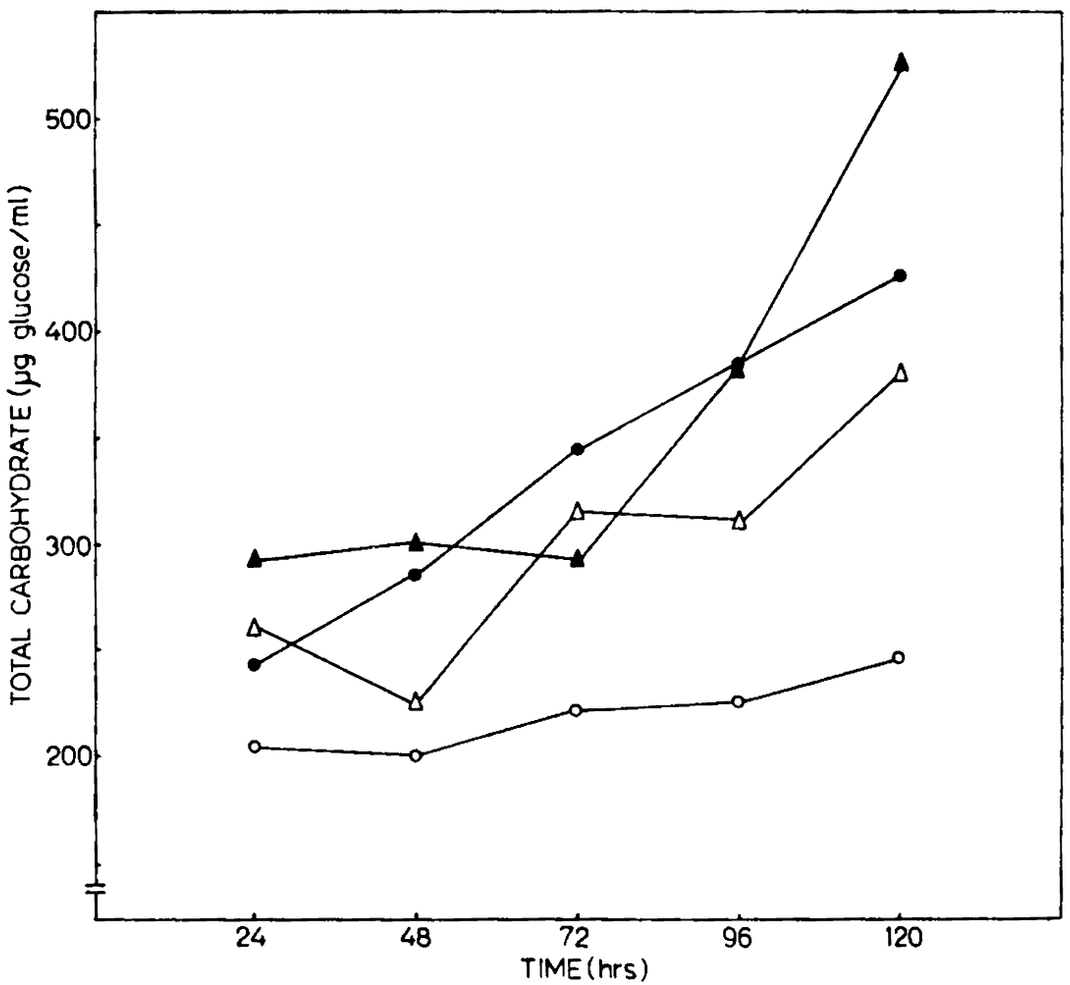

Fig. 4A. Haemolymph Total Carbohydrate (as µg glucose/ml) in Sunetta scripta dosed with three concentrations of copper. Control (○); 1 ppm copper (△); 3 ppm copper (●); 5 ppm copper (▲).

Table 8B. Haemolymph Total Carbohydrate (as μg glucose/ml) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	134.0	138.0	146.0	126.0	130.0
	± SD	25.77	44.45	39.04	38.00	42.43
	Range	90-180	70-240	80-200	80-200	70-200
0.15 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	169.0	161.0	212.0	227.5	187.5
	± SD	60.90	60.07	61.94	66.30	61.94
	Range	100-310	90-270	130-330	150-360	120-310
0.30 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	239.5	280.5	214.5	167.0	225.0
	± SD	73.16	86.73	78.94	39.00	82.61
	Range	130-380	160-460	120-400	100-230	110-330
0.45 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	261.0	195.5	212.5	165.0	195.0
	± SD	46.14	56.14	64-86	27.29	73.93
	Range	170-350	100-310	120-320	130-210	110-280

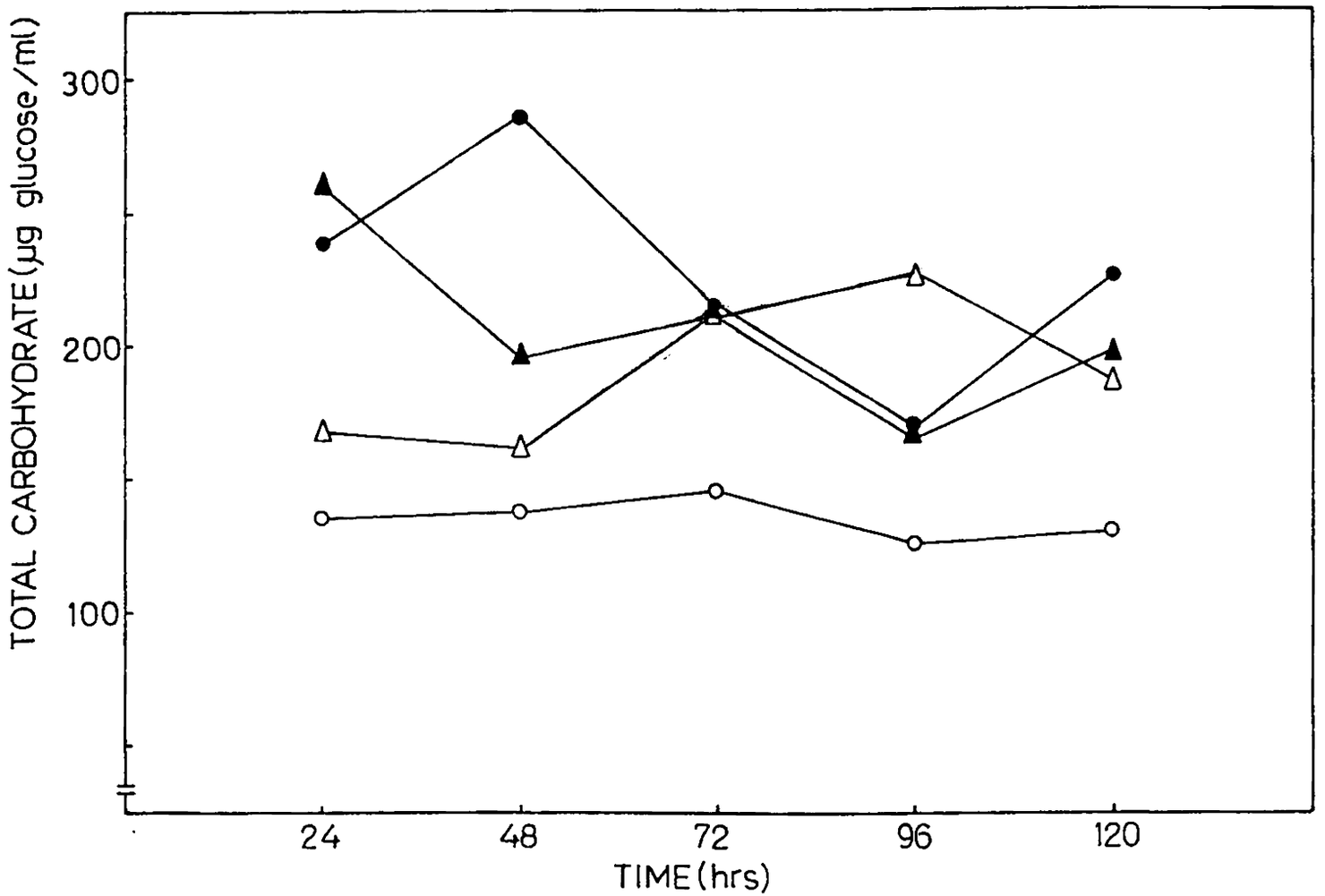

Fig. 4B. Haemolymph Total Carbohydrate (as µg glucose/ml) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper. Control (○); 0.15 ppm copper (△); 0.30 ppm copper (●); 0.45 ppm copper (▲).

In V.cyprinoides var. cochinensis, the total carbohydrate values of 0.15 ppm copper-dosed clams were significantly higher than the control values at 72 hrs ($P<0.02$), 96 hrs ($P<0.01$), and 120 hrs ($P<0.05$). When the total carbohydrate values of 0.30 ppm copper-dosed clams were compared with the values of the controls, the values of 0.30 ppm copper-dosed clams were significantly higher at 24 hrs ($P<0.01$), 48 hrs ($P<0.01$), 72 hrs ($P<0.05$), 96 hrs ($P<0.05$), and 120 hrs ($P<0.01$). The total carbohydrate values of 0.45 ppm copper-dosed clams were also significantly higher than the control values at 24 hrs ($P<0.01$), 48 hrs ($P<0.05$), 72 hrs ($P<0.02$), 96 hrs ($P<0.05$) and 120 hrs ($P<0.05$).

GLYCOGEN IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS WHEN INJECTED WITH VIBRIO SP.

Haemolymph glycogen values in S.scripta, and V.cyprinoides var. cochinensis when challenged with bacteria are given in Tables 9A and 9B, and Figures 5A and 5B, respectively.

In S.scripta when the glycogen values of the saline-injected controls were compared with the values of the untampered controls, the differences in values were found to be insignificant, and when the values of Vibrio-injected clams were compared with those of the untampered controls, the glycogen values of Vibrio-injected clams were significantly higher at 3 hrs ($P<0.01$).

In V.cyprinoides var. cochinensis when the glycogen values of saline-injected controls were compared with the values of untampered controls, there were no significant differences. When values of the Vibrio-injected clams were compared with those of the untampered controls, the glycogen values of the Vibrio-injected clams were significantly higher at 24 hrs ($P<0.02$). The values

Table 9A. Haemolymph Glycogen (µg/ml) in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	10	10	10	10	10	10	10	10
Untampered control	Mean value	29.78	30.93	30.81	24.79	27.90	24.00	23.97	24.20
	± SD	6.59	7.11	8.67	7.67	8.11	12.12	7.43	8.51
	Range	18.0-37.4	20.0-40.2	19.8-44.6	15.0-39.0	17.5-38.0	10.0-48.0	12.0-42.0	12.0-36.0
	N	10	10	10	10	10	10	10	10
2% Saline injected	Mean value	40.59	40.44	28.03	34.90	21.60	29.26	19.48	19.90
	± SD	14.65	14.51	5.58	12.58	5.18	8.28	4.11	4.16
	Range	22.0-58.0	25.0-71.8	22.0-40.5	15.0-50.7	16.0-36.0	20.0-46.0	14.8-28.0	14.0-26.0
	N	10	10	10	10	10	10	10	10
Vibrio sp. injected	Mean value	46.97	37.84	35.46	31.20	21.18	22.42	20.03	24.10
	± SD	13.45	14.72	10.44	11.41	5.76	6.93	4.22	5.12
	Range	28.8-72.0	18.0-69.5	24.0-54.0	15.6-54.0	16.0-36.0	10.0-36.0	15.0-27.0	16.0-35.0

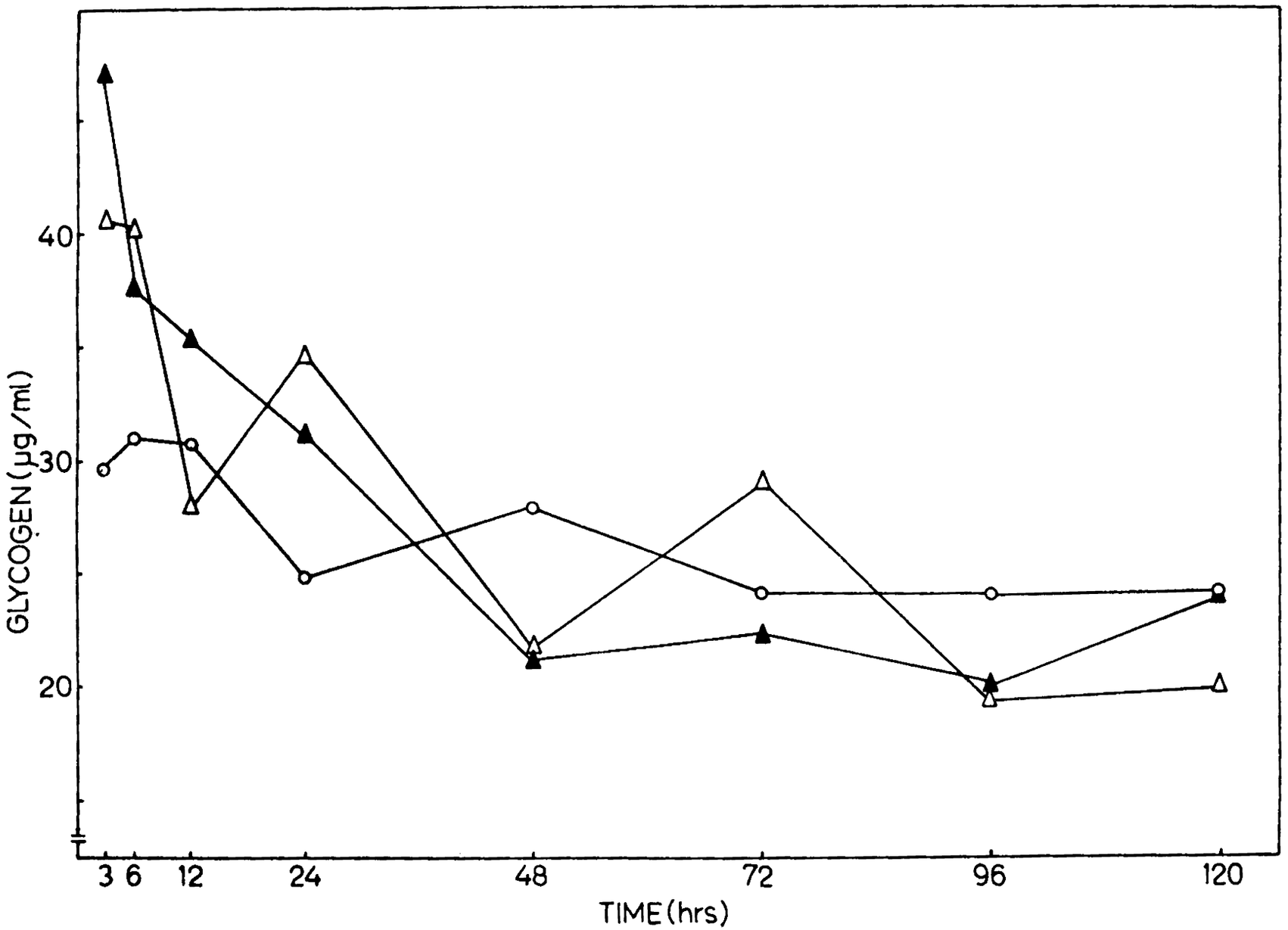

Fig. 5A. Haemolymph Glycogen (µg/ml) in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

Table 9B. Haemolymph Glycogen (µg/ml) in Villorita cyprinoides var. cochinensis injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	10	10	10	10	10	10	10	10
Untampered control	Mean value	30.80	31.22	27.20	23.76	22.68	25.50	23.85	25.20
	± SD	8.48	8.86	6.25	6.90	10.67	8.27	7.48	9.58
	Range	20.4-42.0	20.2-48.0	18.2-36.0	14.4-36.0	12.0-38.4	18.4-40.8	14.6-35.5	12.0-42.0
	N	10	10	10	10	10	10	10	10
2% Saline injected	Mean value	33.60	26.40	32.10	32.40	25.20	22.04	22.70	22.67
	± SD	10.27	6.98	8.88	10.80	13.73	5.32	8.58	8.66
	Range	18.0-54.0	18.0-42.0	18.0-48.0	21.0-50.4	12.0-50.4	12.4-30.2	15.8-43.0	10.0-36.0
	N	10	10	10	10	10	10	10	10
Vibrio sp. injected	Mean value	37.20	37.20	26.67	31.84	35.19	22.99	30.59	26.86
	± SD	13.09	15.15	10.48	5.01	14.54	4.56	8.83	10.39
	Range	24.0-72.0	22.5-66.0	17.0-44.0	26.8-43.8	23.0-63.0	19.0-29.4	13.8-48.3	12.5-48.0

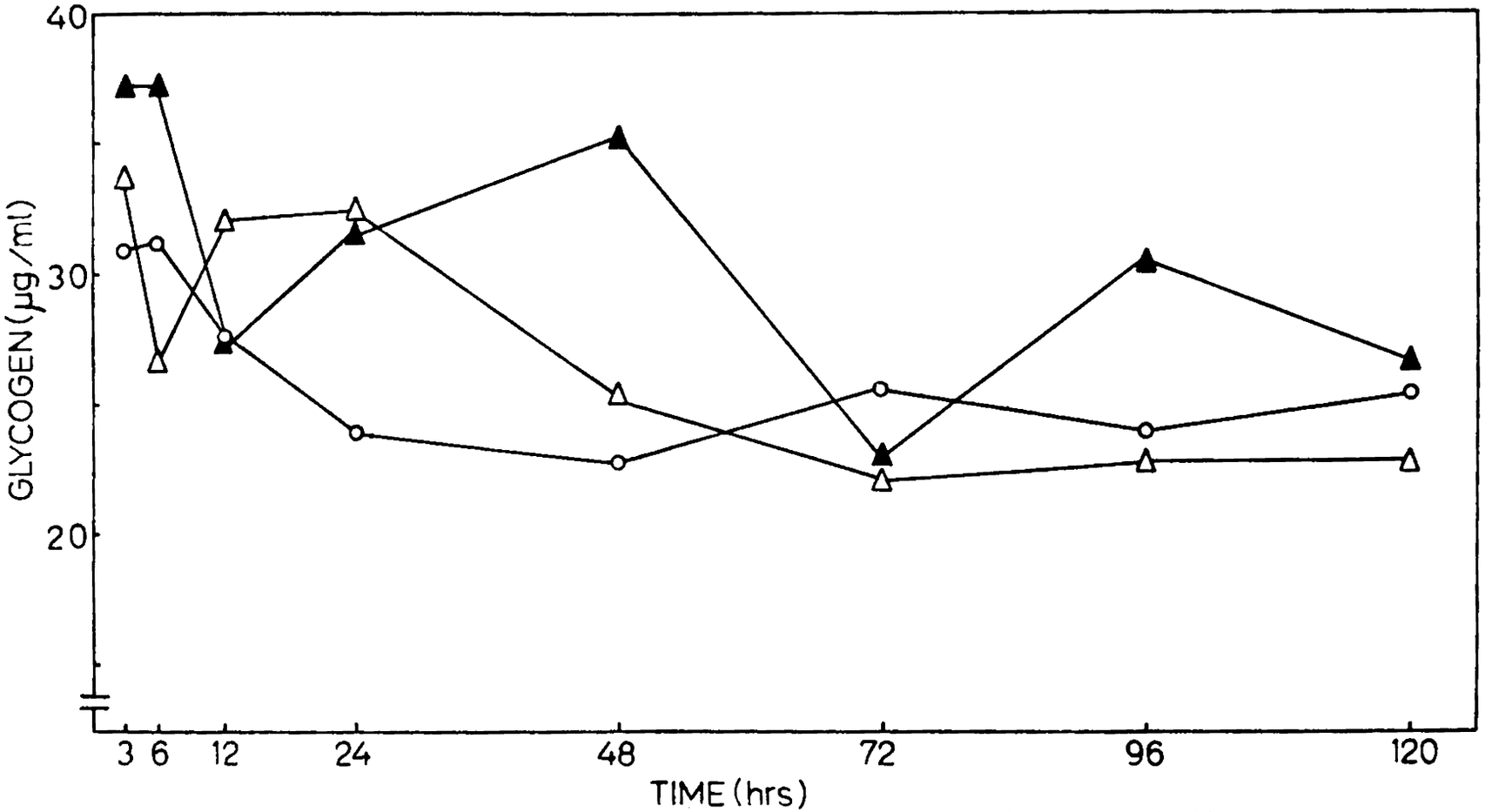

Fig. 5B. Haemolymph Glycogen (µg/ml) in Villorita cyprinoides var. cochinensis injected with $1x10^8$ cells/0.02 ml of Vibrio sp. Untampered control (○); Saline-injected (△); Vibrio-injected (▲).

of saline-injected control clams and Vibrio-injected clams, on comparison, did not show any statistically significant variation.

TOTAL CARBOHYDRATE IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS WHEN INJECTED WITH VIBRIO SP.

Haemolymph total carbohydrate values in S.scripta and V.cyprinoides var. cochinensis when challenged with bacteria are given in Tables 10A and 10B, and Figures 6A and 6B, respectively.

In S.scripta, the mean values of total carbohydrate in saline-injected controls were found to be significantly higher ($P < 0.05$) than the mean value of untampered control at 3 hrs only. When the carbohydrate values of Vibrio-injected clams were compared with the carbohydrate values of untampered controls, Vibrio-injected clams showed significantly higher values at 3 hrs ($P < 0.01$), 6 hrs ($P < 0.01$), and 12 hrs ($P < 0.05$). The carbohydrate values of Vibrio-injected clams and saline-injected clams, when compared, did not show any statistically significant difference during the entire period of the experiment.

In V.cyprinoides var. cochinensis, the total carbohydrate values of the saline-injected controls were significantly higher than the values of the untampered controls at 3 hrs ($P < 0.05$), 6 hrs ($P < 0.02$), and 12 hrs ($P < 0.05$). When the carbohydrate values of the Vibrio-injected clams were compared with those of the untampered controls, Vibrio-injected clams showed significantly higher values at 3 hrs ($P < 0.01$), 6 hrs ($P < 0.01$), 12 hrs ($P < 0.01$), 24 hrs ($P < 0.01$), 48 hrs ($P < 0.02$), 72 hrs ($P < 0.01$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). When the carbohydrate values of the saline-injected controls and

Table 10A. Haemolymph Total Carbohydrate (as µg glucose/ml) in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	20	20	20	20	20	20	20	20
Untampered control	Mean value	212.0	216.0	220.0	200.5	203.8	217.5	226.0	215.5
	± SD	35.44	45.54	29.33	63.52	57.46	54.21	54.26	47.17
	Range	140-250	130-290	180-290	110-320	130-320	130-340	130-310	150-340
	N	20	20	20	20	20	20	20	20
2% Saline injected	Mean value	250.0	236.0	218.5	216.0	195.5	209.8	228.5	243.5
	± SD	59.08	68.73	104.85	63.36	66.89	49.71	81.50	65.67
	Range	160-380	140-380	110-430	110-340	120-330	110-290	130-410	120-350
	N	30	30	30	30	30	30	30	30
Vibrio sp. injected	Mean value	281.3	275.7	254.0	228.0	179.0	230.0	260.7	237.3
	± SD	75.53	80.24	63.80	61.07	58.90	68.94	82.26	74.64
	Range	160-420	150-410	130-390	130-340	90-310	130-400	150-410	130-360

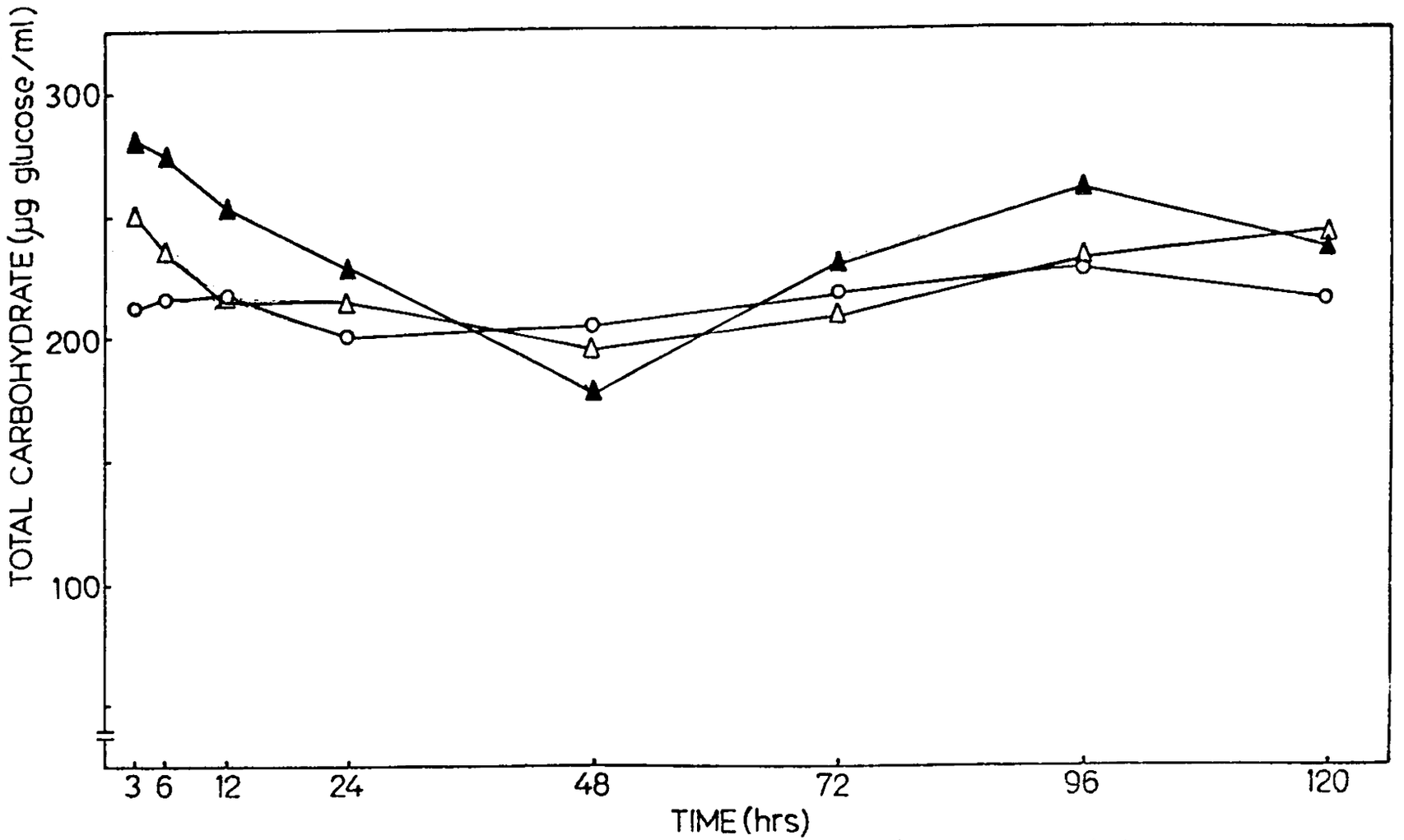

Fig. 6A. Haemolymph Total Carbohydrate (as µg glucose/ml) in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp. Untampered control (○), Saline-injected (△); Vibrio-injected (▲).

Table 10B. Haemolymph Total Carbohydrate (as μg glucose/ml) in Villorita cyprinoides var. cochinensis injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Untampered control	N	15	15	15	15	15	15	15	15
	Mean value	166.0	158.0	162.0	152.0	166.3	143.3	165.3	160.0
	± SD	45.72	43.39	46.78	38.16	35.71	29.81	57.14	29.89
	Range	90-240	90-230	100-240	100-230	110-225	90-190	90-290	100-200
2% Saline injected	N	15	15	15	15	15	15	15	15
	Mean value	211.7	198.0	204.7	181.3	152.7	156.0	170.0	172.0
	± SD	46.75	38.85	45.29	38.62	29.77	42.40	54.77	54.31
	Range	115-290	130-260	140-280	110-250	110-210	110-230	110-290	90-250
Vibrio sp. injected	N	25	25	25	25	25	25	25	25
	Mean value	261.6	266.4	249.0	268.4	223.6	209.8	242.8	247.0
	± SD	62.96	69.91	69.57	83.17	75.47	63.25	71.25	82.00
	Range	140-390	120-390	130-380	140-400	120-400	120-390	130-420	130-420

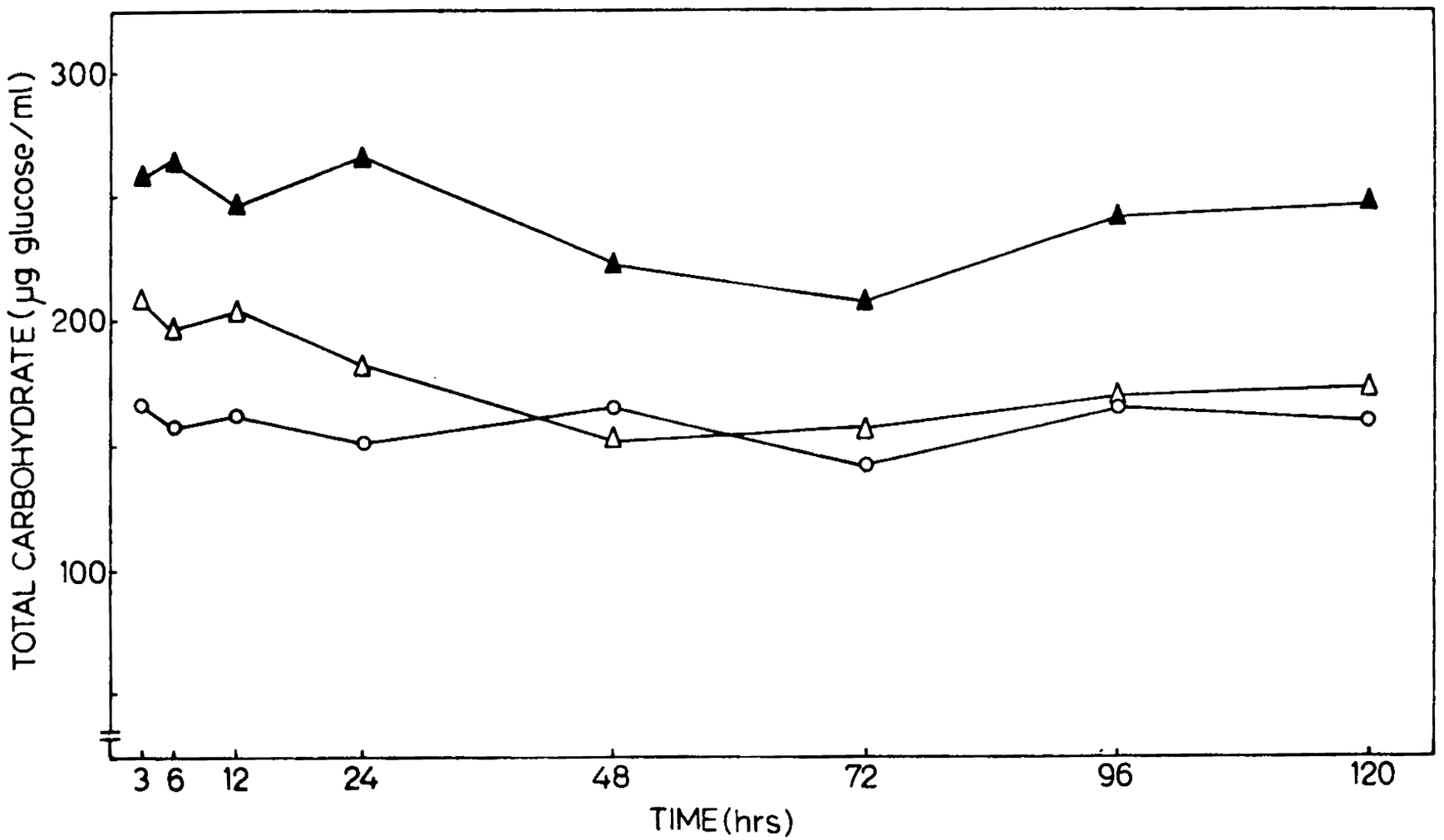

Fig. 6B. Haemolymph Total Carbohydrate (as µg glucose/ml) in Villorita cyprinoides var. cochinensis injected with 1 x 10^8 cells/ 0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

Vibrio-injected ones were compared, the Vibrio-injected clams showed significantly higher values of carbohydrate at 3 hrs ($P < 0.02$), 6 hrs ($P < 0.01$), 12 hrs ($P < 0.05$), 24 hrs ($P < 0.01$), 48 hrs ($P < 0.01$), 72 hrs ($P < 0.01$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$).

DISCUSSION

In the present study, in S.scripta and V.cyprinoides var. cochinensis, no significant differences were found in the mean values of glycogen, and total carbohydrates in the haemolymph among the different size groups of each species studied. Variations in the levels of glycogen and total carbohydrate in tissues can be expected when there is difference in the energy demand generally during growth, reproduction or when they are under stress. Since tissue glycogen and total carbohydrate have not been estimated in the present study, it is difficult to predict the variations of these reserves in the tissues of clams of four size groups. In the present study, since there were no statistical differences in the haemolymph glycogen and total carbohydrate values, it is presumed that in the haemolymph compartment the levels of these reserves are maintained at the upper limit by conversion into tissue glycogen, as indicated by Barry and Munday (1959).

In copper-stress experiments, in S.scripta, the haemolymph glycogen values did not show any significant variation from the control values. In V.cyprinoides var. cochinensis, however, the glycogen level was lower in the experimentals than in the controls, especially towards the end of the experimental period. At 96 and 120 hrs, a gradation in the glycogen content was clearly evident, ie., the higher the concentration of the metal ions, the lower the glycogen

values in the haemolymph.

Interestingly, in S.scripta, the carbohydrate values were significantly higher in all the copper-dosed clams than in the controls, and values of the 3 ppm, and 5 ppm copper-dosed clams were significantly higher than those of the 1 ppm copper-dosed clams, particularly towards the end of the experimental period. In V.cyprinoides var. cochinensis also the carbohydrate values were significantly higher in the copper-dosed clams than in the controls. In this species, however, the values in the experimentals did not show a definite gradation with respect to concentration as was evident in the case of S.scripta.

The minor deviation in the trend seen in the values of haemolymph glycogen and total carbohydrate in S.scripta and V.cyprinoides var. cochinensis is attributed to the species difference. Moreover, the concentrations of copper ions employed in each species were also different. In S.scripta, the concentrations employed were far below the LC_{50} limits, while in the case of V.cyprinoides var. cochinensis, the concentrations employed were near to its LC_{50} limit. This aspect has also contributed to the difference shown by the two species.

Interestingly, in both the clam species, during copper stress, there is a strong tendency for increased carbohydrate concentration in the haemolymph compartment. The increase was, to a certain extent, dependent on the concentration of metal ion used, and in general, increasing with increasing concentration. Similarly, though there was a strong tendency for a reduction in the glycogen content in the haemolymph compartment in V.cyprinoides var. cochinensis after 72 hrs, it was only apparent in S.scripta.

A survey of literature indicates very little work on carbohydrate and glycogen contents in the haemolymph compartment of bivalves under normal conditions, and also while under stress. Jeffries (1972) recognised a stress syndrome in response to environmental pollution, and infection of the shells by the polychaete, Polydora, and indicated that the levels of carbohydrate and free amino acid fell and the molar ratio of free taurine to glycine increased in the tissues.

In normal cases, in marine molluscs the level of blood sugar is controlled at the upper limit by conversion into tissue glycogen (Barry and Munday, 1959), and the loss of a true hepatic function in molluscs has led to the proliferation of Leydig tissue for the local storage of glycogen (Eble, 1969). This removes the requirement for a high blood glucose level because the body tissues have their own supply of carbohydrate (Gabbott, 1976).

The observed increase in the total carbohydratae values in the haemolymph compartment points out to the fact that the carbohydrate might have come from other sources. In the later period of the experiments, the observed reduction in haemolymph glycogen in V.cyprinoides var. cochinensis may be due to the conversion of glycogen to glucose for reasons mentioned below. The absence of a reduction in haemolymph glycogen in S.scripta may be because in this species the breakdown of glycogen in the haemolymph compartment may be taking place only at a later stage or the conversion of glycogen to glucose is less. The observed increase in total carbohydrates in haemolymph may partly be due to haemolymph glycogen, breakdown product (glucose), and also due to glucose transported through haemolymph from metabolically less active tissues to metabolically more active tissues

to meet the stress situation. From the present data, it can be rightly assumed that glycogen contributes only around 1/5th towards the total carbohydrate values in the haemolymph compartment, and the major share is obviously contributed by other simple sugars. In their experiments, Thompson et al. (1978) have also indicated that glycogen accounted only approximately 22% of the carbohydrate present in the haemolymph of M.californianus.

During copper-stress experiments, the behavioural responses of the clams used in the present study, in response to copper ions are, valve closure and secretion of mucus. Studies by Latha Thampuran (per. comm.) have revealed that there is a reduction in the uptake of oxygen from the environment, and filteration rate. Valve closure by the clams and the respiration 'depressing' activity of the Cu^{2+} ions (Shapiro, 1964; Brown and Newell, 1972; Scott and Major, 1972; Prabhudeva and Menon, 1986) lead to anaerobiosis and result in a drop in pH. The valve closure response may be a signal mechanism for 'turning down' the activity of phosphofructokinase resulting in a major reduction in overall metabolic rate (de Zwaan, 1983). This is highly essential because if the metabolic rate is not reduced, the energy demand will be high, and to meet this anaerobically the animal has to draw its energy reserves heavily. The reduction in pH leads to a pronounced fall in the affinity of phosphofructokinase for fructose-6-phosphate, and general inhibition of catalytic rate at all substrate levels (Ebberink, 1980). The degree of the pH drop, and hence the fall in affinity of phosphofructokinase, varies with the tissues as there can be differences in the degree of anaerobiosis by the different tissues. This contention is strengthened by a number of evidences in favour of the existence of specific tissue differences in anaerobic maintenance metabolism as

reflected in enzyme spectra (de Zwaan, 1977; Collicutt, 1975; Addink and Veenhof, 1975; Collicutt and Hochachka, 1977; Holwerda and de Zwaan, 1980).

In all the tissues for the basal metabolism, glycolysis will proceed upto the point of phosphoenolpyruvate (PEP). In carbohydrate metabolism in bivalves, the PEP is a branch point and the flow of carbon through phosphoenolpyruvate branch point is determined by the degree of tissue hypoxia and both pathways (under aerobic condition, PEP is converted into pyruvate, and under anaerobic condition (phosphoenolpyruvate carboxykinase activity is favoured by the inhibition of pyruvate kinase by alanine and low pH) PEP is converted into oxaloacetate and ultimately into succinate) operate together (Livingstone and Bayne, 1974; de Zwaan and de Bont, 1975). The extent to which one pathway will predominate over the other will depend on the particular conditions within the body tissues, where the level of hypoxia is reduced, succinate will be removed by oxidative metabolism or if hypoxia persists, it may be converted to propionate. This is supported by the fact that during anaerobic fermentation of carbohydrate, volatile fatty acids can be one of the end products in some instances (Kluytmans et al., 1977; 1978; Zurburg and Kluytmans, 1980). The ability to respire anaerobically also varies in bivalve tissues, depending on the easy accessibility of oxygen from the environment or circulatory system. For example, deeply located tissues have a greater tendency to respire anaerobically than superficially located tissues as a result of relative oxygen availability (de Zwaan, 1983). It has been suggested that in bivalve molluscs, some tissues may be adapted to function anaerobically while others which are near to the sites of gas exchange may be primarily aerobic (Booth and Mangum, 1978). Chaplin and Loxton (1976) have reported that the gill is able to obtain oxygen

during aerial exposure, and will follow a predominantly aerobic fuel degradation.

From the foregoing account, it seems that during stress, different tissues draw energy by predominantly aerobic or predominantly anaerobic processes. For example, to combat the stress, the vital tissues will have to be provided with the required energy, and for this, the energy requirement of some other tissues (eg. gonad) may have to be greatly reduced. In such circumstances, there is a differential flux of carbon between the tissues. Carbohydrate from metabolically less active tissues may be transferred to the metabolically more active tissues through haemolymph. The clams may be trying to adjust to the inhibition of phosphofructokinase activity resulting from the copper dosage by providing the substrate, fructose-6-phosphate, in a greater concentration within the cells to maintain the carbon flux to meet the maintenance metabolism. This can be effected only by maintaining a higher concentration of glucose in the haemolymph so as to retain a concentration gradient.

The other observed behaviour, ie., secretion of more mucus in response to copper, is the function of the epithelial layer lining the mantle cavity. For the production of mucus, glucose is essential and may be transported from metabolically less active tissues. Glucose-6-phosphate (formed from the breakdown of glycogen or from the gluconeogenic pathways) can be dephosphorylated to glucose and transported around the body in the haemolymph (Gabbott, 1976).

Reddy and Rao (1983), based on the fact that copper readily forms copper soaps with long fatty acids, have tentatively hypothesised that the observed increased levels of free fatty acids in copper exposed freshwater gastropod, as a mechanism for reducing copper toxicity or mechanisms for

copper storage. They have also indicated that during copper exposure the immediate demand for energy might be met from lipids. It may be noted that in molluscs, conversion of glycogen into fatty acids or triglycerides also takes place (Gabbott, 1976). For this, glycogen may be mobilised from other tissues, where it can be spared, through the haemolymph. When such mobilisation takes place from tissues, through haemolymph, there will be elevation of total carbohydrate levels in the haemolymph concomitant with depletion of this fuel reserve in the tissues. The observed elevated total carbohydrate values in the haemolymph may be due to this mechanism also. Depletion of tissue glycogen and total carbohydrates in copper-exposed bivalves (Lakshmanan and Nambisan, 1977, 1985), and of carbohydrates in copper-exposed gastropod (Ramesh Babu, 1980., quoted from Reddy and Rao, 1983) is already documented.

Regarding other stress factors, widdows and Bayne (1971) have shown that there is no increase in blood sugar during the first seven days of acclimation to high temperature. Thompson et al. (1978) have reported that in M.californianus subjected to nutritional, thermal, and exposure stress, there were no changes in plasma carbohydrates, lipid or protein attributable to exposure, ration or increased temperature.

In bacteria-challenged S.scripta, the haemolymph glycogen values were found to be significantly higher in the Vibrio-injected clams, than in the untampered controls at 3 hrs. In V.cyprinoides var. cochinensis, the values of Vibrio-injected ones showed significant increase than those of the untampered controls at 24 hrs. This increase in the glycogen content at 3 hrs in S.scripta, and 24 hrs in V.cyprinoides var. cochinensis is attributed to the glycogen synthesised through degradation of bacteria by the phagocytes. The time

difference shown by the two clam species is attributed to the species difference.

It may be added that cytochemical studies conducted on the haemocytes of S.scripta and V.cyprinoides var. cochinensis, at 2 hr post challenge with bacterium revealed high glycogen content in these cells. That there is species difference in the process of degradation is evident from the works of Cheng (1975), Cheng and Rudo (1976b) and Rodrick and Ulrich (1984). Cheng (1975) has reported a significant rise in haemolymph glycogen in C.virginica at 24 hr post-challenge with Escherichia coli in vivo, and Rodrick and Ulrich (1984) have obtained similar results in M.campechiensis, C.virginica and A.ovalis at 1 hr post-challenge with E.coli and Vibrio anguillarum, in vivo. By employing ^{14}C-labelled B.megaterium as the challenging agent, Cheng and Rudo (1976b), and Cheng (1977b) also have demonstrated that the degradation of phagocytosed bacteria in C.virginica haemocytes leads to the synthesis of glycogen from sugar of bacterial orign and its eventual release from phagocytes. Cheng and Rudo (1976b) reported that ^{14}C activity was detected in the glycogen extracted from the sera at 24 hrs post-injection and detected in haemolymph cells at 16 hr post-injection. All these clearly indicate that the time taken for the degradation of bacteria differs from species to species. Electron-microscopic studies on the granulocytes of C.virginica (Cheng and Cali, 1974), and M.mercenaria (Mohandas, 1985) have revealed that subsequent to bacterial exposure, the bacteria are degraded in the phagosomes, by lysosomal enzymes, and the glycogen thus formed is finally released into serum.

In S.scripta subsequent to bacterial challenge, the total haemolymph

carbohydrate of the Vibrio-injected clams showed significantly higher values at 3, 6 and 12 hrs than the untampered controls, and the Vibrio-injected V.cyprinoides var. cochinensis showed higher values at 3, 6, 12, 24, 48, 72, 96, and 120 hrs.

Removal mechanisms for the bacteria introduced into the body include the phagocytic activities of cells in the shellfish haemolymph (Bang, 1967). These phagocytic cells are continuously lost to the exterior through epithelial borders (Cheng et al., 1969), a process which provides an efficient clearance mechanism for phagocytosed bacteria and other materials (Malek and Cheng, 1974). The exteriorised phagocytes are carried away in mucus or faeces by the water stream set up by natural pumping action in the body of the shellfish (Houser, 1964; Malek and Cheng, 1974). These mechanisms are believed to be operating in Vibrio-injected S.scripta and V.cyprinoides var. cochinensis also. The above mentioned pumping action is affected by ciliary action. The epithelial tissues bearing cilia consume energy for setting up the water current. A steady supply of glucose is provided to this area of the clam body by the mobilisation of stored glycogen from other tissues. The transfer of glucose is through haemolymph, and hence the high total carbohydrate content in the haemolymph of Vibrio-injected S.scripta and V.cyprinoides var. cochinensis when compared with the respective controls. That addition of glucose to water increased the filtration rate of M.edulis has been demonstrated by Theede (1963), and Thompson and Bayne (1972). Manohar and Rao (1975) have reported a hyperglycemic factor in Lymnaea luteola in response to parasitism. Perhaps, such a hyperglycemic factor may be operating in these clam species also in response to the presence of Vibrio in the body.

In the saline-injected clams an increase in the total carbohydrate values when compared with the control values was observed in the initial period of the experiment. This is interpreted as a short-term response to the injection.

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CHAPTER IV

PROTEIN IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

In vertebrates, the serum proteins play an important role in maintaining the osmolality, transport of ions, immune mechanisms, and homoeostasis. In lower forms, including bivalves, proteins do the same function. The main difference, however lies in the actual mechanism. In bivalves, the haemolymph proteins have been implicated to perform a variety of functions, such as transport of ions, detoxification of metal ions (Simkiss and Mason, 1983), recognition of self and non-self materials (Cheng, 1986), etc.

In many animals the circulating fluids contain specific proteins that transport metal ions around the body, and may even deliver them specifically to particular cell types. In vertebrates such functions have been shown for nickel transport by nickel plasmin, and for iron transport by transferrin, whereas albumin carries copper and zinc ions (Harrison and Hoare, 1980). Information on the way by which metals are transported in molluscs is often sparse, although it has been shown that the main plasma proteins, which are often the respiratory pigments, can function in this way (Simkiss and Mason, 1983). The retention of metal ions in the blood may be a problem for molluscs, in general. It appears, therefore, that haemocytes in the blood, like mucus on the epidermis, may be a particularly interesting molluscan adaptation of great relevance to metal metabolism. Simkiss and Mason (1984) have shown that many heavy metals are retained within the tissues by reactive ligands. These may be inducible, metabolised and excreted, and it is the 'turnover' and availability of these ligands which may ultimately determine the final concentration of the metal in the animal. They have also indicated

that ligands are very specific for particular metals or are non-specific in nature and capable of binding a wide variety of metals, and the ligands may contribute to a general system of detoxification while the former appear to be integrated into precise metabolic pathways. They have added that specific cells, such as basophils, appear to contain high capacity ligands that will bind a variety of metals.

Many molluscs have a remarkably permeable epidermis from which it is possible to obtain simple diffusion pontentials for metal ions and from which a large number of blood proteins can also escape (Simkiss and Wilbur, 1977). The gills, foot, mantle and the alimentary tract have all been implicated as sites of uptake (Chipman, 1966; Shuster and Pringle, 1969; Harrison, 1969; Schulz-Baldes, 1974; Fowler and Benayoun, 1974; George and Pirie, 1980; Roesijadi, 1980; Simkiss and Taylor, 1981). During the uptake of metals across molluscan epithelia, endocytosis has been described. The metals are transported across the epithelial cells and exocytosed into the blood on the basal side of the cells, from which they are circulated around the body in amoebocytes and retained or excreted. There is extensive evidence that copper in L.stagnalis (Spronk et al., 1973) and in B.glabrata (Cheng and Sullivan, 1974), mercury and iron in M.mercenaria (Fowler et al., 1975), cadmium in M.edulis (George and Coombs, 1977) and in Protothaca staminea (Roesijadi, 1980), are all taken up by molluscs at a rate that is roughly proportional to the concentrations in the water. The main blood proteins of molluscs are the respiratory pigments.

It is generally agreed that the metals are protein bound as soon as they enter the vascular system so that there is probably a concentration gradient

inward (Simkiss and Mason, 1983). Evidence in favour of this interpretation has been provided by George and Coombs (1977), who have shown that ligand binding of cadmium, iron or lead ions increases the rate at which they enter M.edulis. It is possible that mucus binding may provide a ligand source for increasing metal ion uptake although some workers have suggested intracellular sources of metallothioneins, copper-chelation or low molecular weight compounds as alternative mediating molecules (Roesijadi, 1980). George and Pirie (1980) have identified a zinc-binding protein in M.edulis. There is also evidence that, in tissue homogenates, a number of low molecular weight compounds, such as taurine, lysine and homarine bind metals such as zinc, and copper in O.edulis (Coombs, 1974), although it is uncertain whether the complexed metals occur in the blood or are confined within the tissues. Besides the metal-binding proteins, the other cellular storage systems include metalloproteins (ferritin); metalloenzymes (iron, copper and zinc containing enzymes; George and Coombs, 1975), and metal containing cells (Pore cells and connective tissue calcium cells). As with the storage systems, the detoxification system can be separated into the formation of inorganic precipitates (Bouquegneau and Martoja, 1982), compartmentation process within membrane limited vesicles of granulocytes (George et al., 1978; Simkiss and Mason, 1983), binding to specific proteins (Noel-Lambot, 1976; Talbot and Magee, 1978; George et al., 1979; Frankenne et al., 1980; Roesijadi, 1980; 1980-81; Viarengo et al., 1980b; 1981b; 1984a; 1985), and to accumulation in lysosomes and residual bodies (Viarengo et al., 1981a).

In the present investigation an attempt is made to find out the haemolymph protein values in clams of different size groups, and also to find out whether

there are differences in the values in clams exposed to copper, and challenged with bacteria.

MATERIALS AND METHODS

Methods of collection of clams, rearing the clams, acclimatisation, selection of size groups, mode of collection of haemolymph, dosing the clams with copper, challenging the clams with bacteria, and the statistical analysis of the data were the same as described in detail in chapter II. As mentioned earlier, concentrations of the copper ions used were different in the two species of clams, but dosage of the bacterial suspension was the same. For S.scripta the salinity used was 30‰ and for V.cyprinoides var. cochinensis, it was 15‰.

In the case of S.scripta, 20 clams from each size group, and in the case of V.cyprinoides var. cochinensis, 13 clams from each size group were employed for the estimation of haemolymph protein in different size groups of clams.

To study haemolymph protein in S.scripta, dosed with three concentrations of copper, 330 clams were used for the experimental group (110 clams in each of the three experimental batches), and the control group consisted of 85 clams. In the case of V.cyprinoides var. cochinensis the experimental group consisted of 180 clams, and the control group 60 clams. The size group selected for both the clams species was 38-40 mm. Haemolymph was withdrawn every 24 hrs for 5 days for the estimation of haemolymph protein from 20 specimens each of the experimental batches, and 15 from the control group in the case of S.scripta, and in the case of V.cyprinoides var. cochinensis, from 10 specimens each of the experimental batches and 10 from the control group, each day.

To study haemolymph protein in S.scripta and V.cyprinoides var.cochinensis

subsequent to bacterial challenge at a concentration of 1 x 10^8 cells/0.02 ml, haemolymph samples were collected at 3, 6, 12, 24, 48, 72, 96 and 120-hr time period post-challenge. In the case of S.scripta, for each time period, the untampered control group and 2% saline-injected control group consisted of 20 clams each, and the bacteria-injected experimental group consisted of 30 clams, whereas in the case of V.cyprinoides var. cochinensis, they were 15, 15 and 25 respectively.

More clams than the required number were reared as experimentals and controls anticipating mortality during the course of the experiment.

ESTIMATION OF PROTEIN

Estimation of protein was done by the method of Lowry et al. (1951). Haemolymph was withdrawn and expelled into a clean test-tube. A 0.30 ml sample of the expelled haemolymph was then pipetted into a centrifuge tube containing one ml of 10% TCA. It was shaken well and then centrifuged at 2500 rpm for 15 minutes. The supernatant was slowly poured out. The precipitate was dissolved in one ml of 0.1N NaOH. From this a 0.5 ml sample was pipetted into a large test-tube, made upto one ml with distilled water. Five ml of alkaline copper reagent was then added and shaken well. After 10 minutes, 0.5 ml of Folin's Phenol reagent was added and shaken well. After 45 minutes, the optical density was read at 500 nm. From this optical density, the corresponding concentrations were found out from the standard graph employing bovine serum albumin as the standard.

RESULT

PROTEIN IN THE HAEMOLYMPH OF THE FOUR SIZE GROUPS OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

The mean value, standard deviation and range of haemolymph protein in the four size groups of S.scripta are given in the Table 11A and those of V.cyprinoides var. cochinensis in Table 11B. In both cases, when the mean value of haemolymph protein in different size groups were compared, there was no statistically significant difference.

PROTEIN IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS DOSED WITH THREE CONCENTRATIONS OF COPPER

Haemolymph protein values in S.scripta and V.cyprinoides var. cochinensis when exposed to three sub-lethal concentrations of copper for five days are given in Tables 12A and 12B, and Figures 7A and 7B respectively.

In S.scripta, when the mean values of haemolymph protein of 1 ppm copper-dosed clam were compared with the mean values of the controls, those of 1 ppm copper-dosed clams were significantly lower at 48 hrs ($P < 0.02$), 72 hrs ($P < 0.05$), 96 hrs ($P < 0.02$), and 120 hrs ($P < 0.01$). The mean values of protein of 3 ppm copper-dosed clams were significantly lower than the control values at 120 hrs ($P < 0.01$). The mean values of control and 5 ppm copper-dosed clams, on comparison, did not show any significant difference. When the mean protein values of 1 ppm and 3 ppm copper-dosed clams were compared, the protein values of 1 ppm copper-dosed clams were significantly lower at 72 hrs ($P < 0.05$), 96 hrs ($P < 0.01$) and 120 hrs ($P < 0.01$). When the values of 1 ppm and 5 ppm copper-dosed clams were compared, the protein values of 1 ppm copper-dosed clams were significantly lower at 48 hrs

Table 11A. Haemolymph Protein (µg/ml) in the four size groups of Sunetta scripta

Size-group	30-32 mm	36-38 mm	38-40 mm	42-44 mm
N	20	20	20	20
Mean value	816.0	757.0	735.0	738.5
± SD	169.22	221.90	159.83	212.54
Range	550-1000	540-1180	410-990	530-1250

Table 11B. Haemolymph Protein (µg/ml) in the four size groups of Villorita cyprinoides var. cochinensis

Size-group	24-26 mm	34-36 mm	38-40 mm	44-46 mm
N	13	13	13	13
Mean value	1036.2	956.2	997.7	1077.7
± SD	183.83	243.90	214.66	176.77
Range	710-1380	630-1450	740-1400	770-1410

Table 12A. Haemolymph Protein (µg/ml) in Sunetta scripta dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	15	15	15	15	15
	Mean value	839.3	829.3	694.0	700.0	793.3
	± SD	192.82	221.22	226.09	209.00	137.24
	Range	570-1130	440-1210	450-1300	450-1300	480-960
1 ppm of Cu^{2+} dosed	N	20	20	20	20	20
	Mean value	895.5	615.0	543.0	557.5	493.5
	± SD	183.04	234.40	125.86	110.45	94.83
	Range	500-1200	340-1120	400-940	400-880	290-620
3 ppm of Cu^{2+} dosed	N	20	20	20	20	20
	Mean value	932.5	696.0	671.0	711.5	636.0
	± SD	173.81	208.43	228.84	192.02	136.54
	Range	560-1160	480-1230	390-1150	480-1140	400-920
5 ppm of Cu^{2+} dosed	N	20	20	20	20	20
	Mean value	981.5	869.5	691.0	753.5	760.5
	± SD	215.92	249.99	277.94	226.81	119.94
	Range	610-1300	490-1300	340-1280	440-1290	500-960

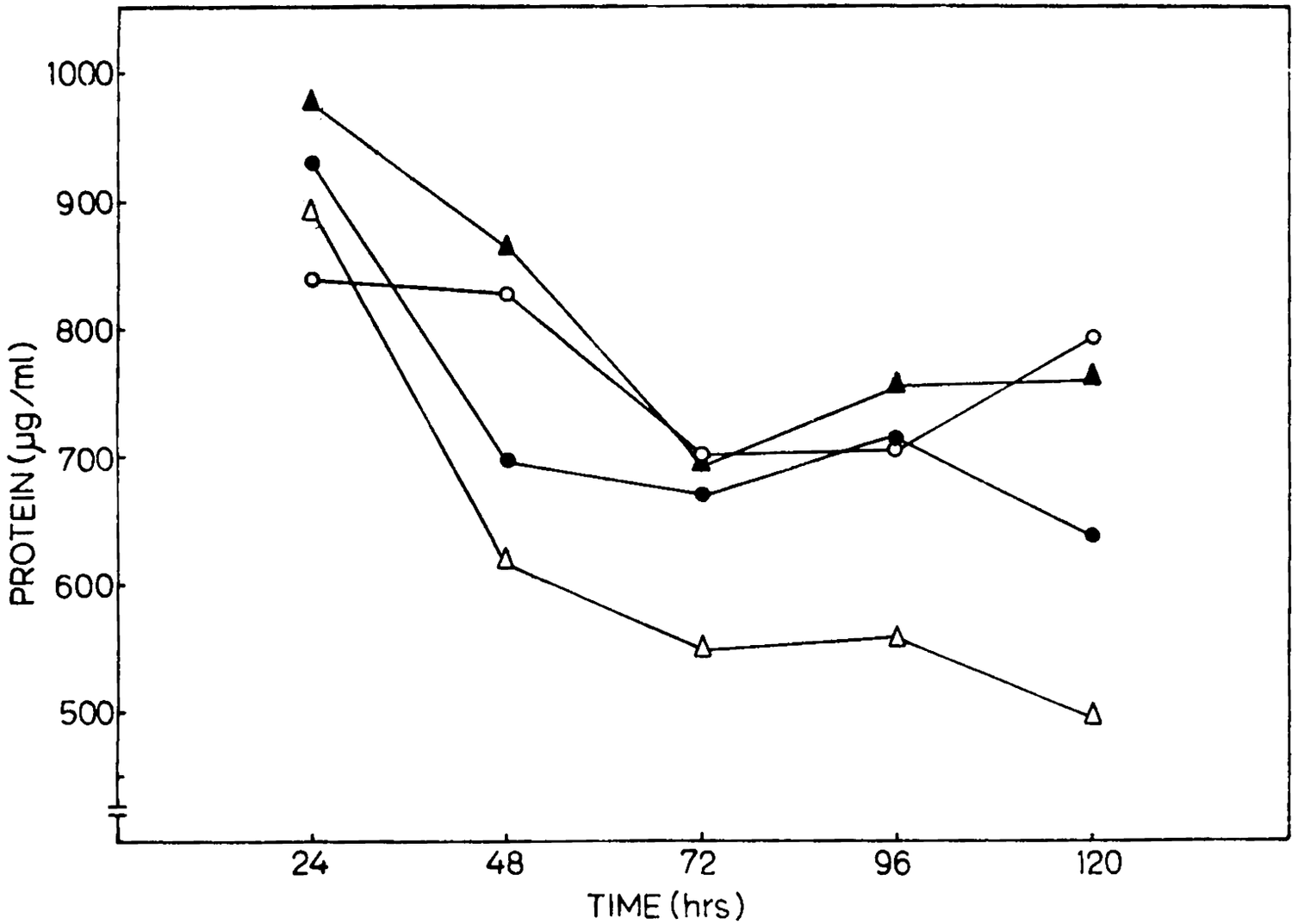

Fig. 7A. Haemolymph Protein (µg/ml) in Sunetta scripta dosed with three concentrations of copper. Control (o); 1 ppm copper (Δ); 3 ppm copper (●); 5 ppm copper (▲).

Table 12B. Haemolymph Protein (µg/ml) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	1226.0	1326.0	1302.0	1137.0	1053.0
	± SD	200.11	269.79	269.07	159.25	327.35
	Range	860-1450	800-1800	790-1790	830-1450	690-1770
0.15 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	849.0	871.0	939.0	907.0	722.0
	± SD	219.88	230.28	324.82	196.57	166.18
	Range	480-1180	520-1300	610-1700	510-1180	500-1020
0.30 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	1013.0	843.0	884.0	812.0	940.0
	± SD	296.68	199.05	209.53	228.77	150.86
	Range	600-1520	600-1360	530-1180	540-1300	710-1080
0.45 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	1058.0	982.0	879.0	846.0	965.0
	± SD	382.80	389.35	224.39	334.88	121.26
	Range	620-1980	560-1920	570-1270	580-1500	700-1080

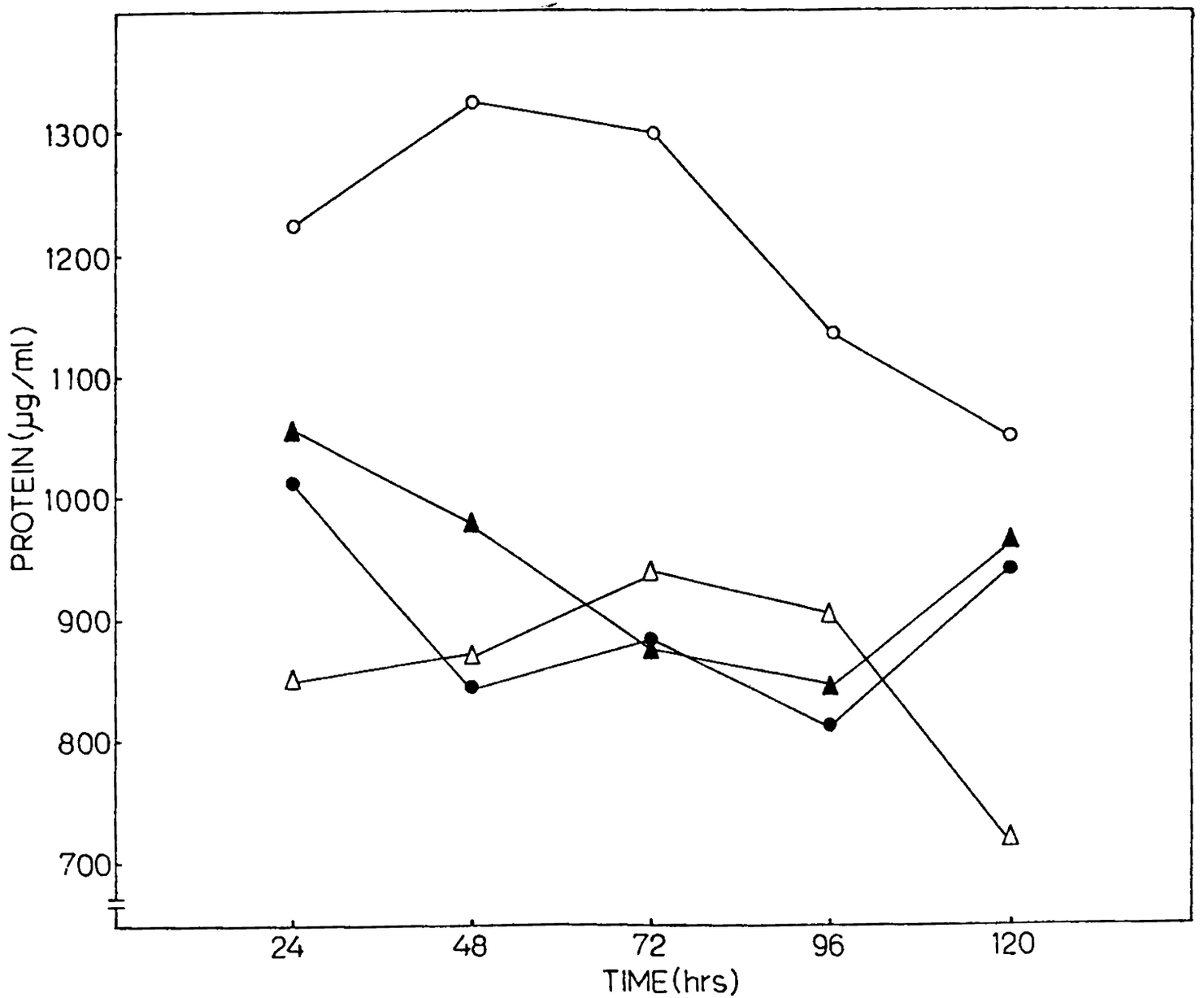

Fig. 7B. Haemolymph Protein (µg/ml) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper. Control (o);

($P < 0.01$), 72 hrs ($P < 0.05$), 96 hrs ($P < 0.01$) and 120 hrs ($P < 0.01$). The values of 3 ppm copper-dosed clams were significantly lower than the 5 ppm copper-dosed clams at 48 hrs ($P < 0.05$) and 120 hrs ($P < 0.01$).

In V.cyprinoides var. cochinensis, haemolymph protein values in 0.15 ppm copper-dosed clams were significantly lower than the control values at 24 hrs ($P < 0.01$), 48 hrs ($P < 0.01$), 72 hrs ($P < 0.02$), 96 hrs ($P < 0.02$), and 120 hrs ($P < 0.02$). When the mean protein values of 0.30 ppm copper-dosed clams were compared with the values of the controls, the values of 0.30 ppm copper-dosed clams were significantly lower at 48 hrs ($P < 0.01$), 72 hrs ($P < 0.01$), and 96 hrs ($P < 0.01$). On comparing the mean protein values of 0.45 ppm copper-dosed clams with the values of the controls, it was found that the protein values of 0.45 ppm copper-dosed clams were significantly lower at 48 hrs ($P < 0.05$), 72 hrs ($P < 0.01$), and 96 hrs ($P < 0.05$). When the protein values of 0.15 ppm and 0.30 ppm copper-dosed clams were compared, it was found that the protein values of 0.15 ppm copper-dosed clams were significantly lower ($P < 0.01$) at 120 hrs. When the values of 0.15 ppm and 0.45 ppm copper-dosed clams were compared, the protein values of 0.15 ppm copper-dosed clams was significantly lower than those of 0.45 ppm copper-dosed clams at 120 hrs. On comparison, the values of 0.30 ppm and 0.45 ppm copper-dosed clams did not show any significant difference.

PROTEIN IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS INJECTED WITH VIBRIO SP.

Haemolymph protein values in S.scripta and V.cyprinoides var.cochinensis at different time periods subsequent to Vibrio-injection are given in Tables 13A and 13B, and Figures 8A and 8B, respectively.

Table 13A. Haemolymph Protein (µg/ml) in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	20	20	20	20	20	20	20	20
Untampered control	Mean value	759.0	777.0	776.0	702.0	691.5	769.0	724.0	712.0
	± SD	109.54	109.87	102.24	158.95	163.99	206.88	210.25	115.65
	Range	540-960	650-970	550-870	500-1020	400-990	400-1120	430-1200	460-880
	N	20	20	20	20	20	20	20	20
2% Saline injected	Mean value	813.5	672.5	777.0	717.5	596.0	634.5	623.0	716.5
	± SD	209.82	222.44	212.35	213.82	146.40	206.38	109.27	108.87
	Range	420-1230	360-1050	440-1200	440-1190	410-870	340-1070	460-870	530-830
	N	30	30	30	30	30	30	30	30
Vibrio sp. injected	Mean value	858.3	879.3	805.3	758.7	666.0	707.3	616.0	624.7
	± SD	197.50	241.69	231.69	182.77	185.72	215.84	173.31	188.92
	Range	460-1300	550-1580	410-1250	490-1180	400-1020	410-1020	390-920	340-1020

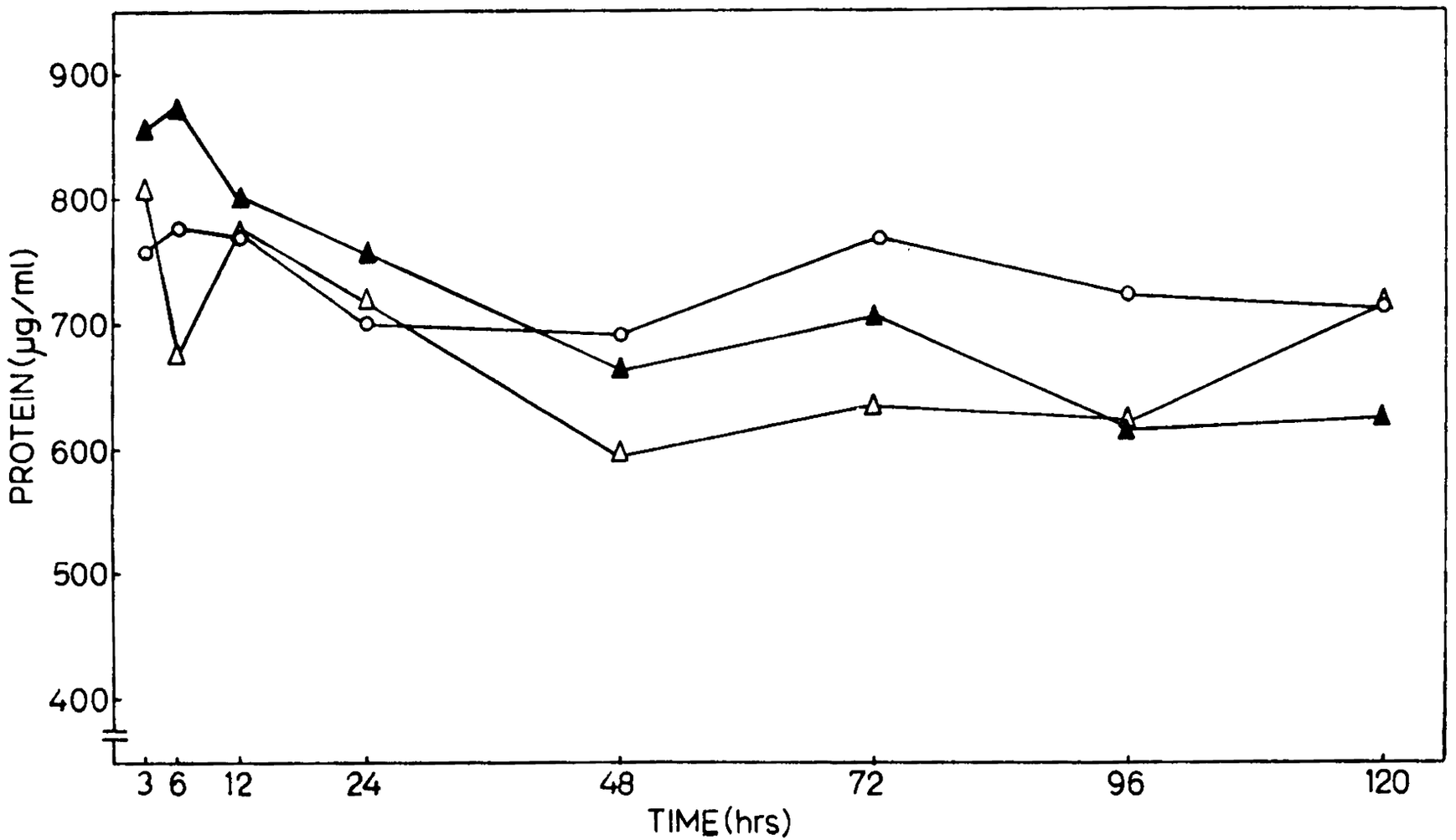

Fig 8A. Haemolymph Protein (µg/ml) in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

Table 13B. Haemolymph Protein (µg/ml) in Villorita cyprinoides var. cochinensis injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	15	15	15	15	15	15	15	15
Untampered control	Mean value	1020.0	1086.0	1096.0	1022.0	986.0	1200.0	1227.3	1084.0
	± SD	255.03	241.30	248.40	211.54	310.52	307.42	356.62	310.63
	Range	830-1590	740-1330	630-1320	730-1330	510-1390	820-1780	800-1920	660-1790
	N	15	15	15	15	15	15	15	15
2% Saline injected	Mean value	1101.3	1021.3	1207.3	975.3	999.3	1212.0	1042.0	1088.0
	± SD	242.15	338.48	316.57	335.10	168.22	339.95	207.53	282.26
	Range	790-1720	710-1710	720-1630	560-1760	750-1360	680-1960	680-1370	660-1670
	N	25	25	25	25	25	25	25	25
Vibrio sp. injected	Mean value	1171.2	1100.0	1107.6	1070.0	1088.8	1068.4	1036.8	976.8
	± SD	194.72	377.38	207.97	213.39	207.60	233.27	304.80	226.32
	Range	880-1590	580-2010	730-1570	680-1430	630-1460	610-1660	610-1800	650-1540

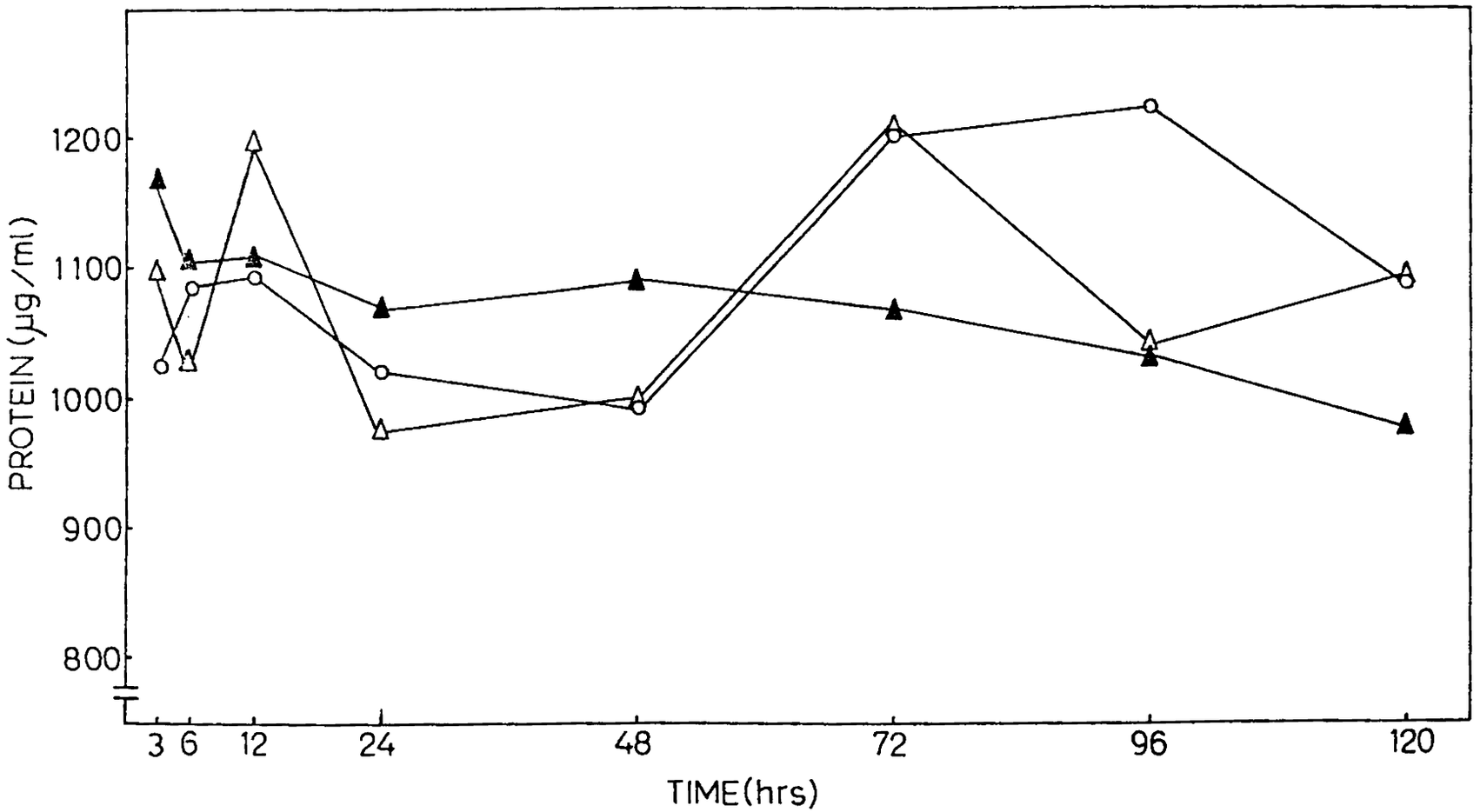

Fig. 8B. Haemolymph Protein (µg/ml) in Villorita cyprinoides var. cochinensis injected with $1x10^8$ cells/0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

In S.scripta, the mean values of protein of the saline-injected and Vibrio-injected clams did not show any significant difference from the values of the controls. When the values of saline-injected and Vibrio-injected clams were compared, there was no significant difference.

In V.cyprinoides var. cochinensis, the mean protein value of saline-injected controls showed no significant difference with the values of untampered controls at any time period. The mean value of Vibrio-injected clams also, did not show any significant difference with the value of untampered control at any time period. When the values of the saline-injected and Vibrio-injected clams were compared, there was no significant difference.

DISCUSSION

The mean haemolymph protein values in the four size groups of each species, S.scripta and V.cyprinoides var. cochinensis, showed no significant difference. In the haemolymph compartment protein/ml remained the same irrespective of the differences in the size groups in both the species studied.

In the copper-dosing experiments with S.scripta, the pattern in protein values shown by the copper-dosed clams was different depending on the concentration of copper employed. One ppm copper-dosed clams showed significantly lower values than the control from 48 hrs onwards upto the end of the experimental period (120 hrs), whereas 3 ppm copper-dosed clams showed significantly lower values than the controls only at 120 hrs. Five ppm copper-dosed clams showed no significant difference in values from the controls. One ppm copper-dosed clams showed significantly lower protein values than those of the 3 ppm copper-dosed clams from 72 hrs onwards,

whereas 1 ppm copper-dosed clams showed significantly lower protein values than those of the 5 ppm copper-dosed clams from 48 hrs onwards. Three ppm copper-dosed clams showed significantly lower values than those of the 5 ppm copper-dosed clams at 48 and 120 hrs.

From the results briefly described above, it is evident that the protein value in the haemolymph compartment in copper stressed condition is a matter of balance between entry and exit of protein into and out of the haemolymph compartment. This view is strengthened by the fact that wherever there is significant difference, within the copper-dosed clams, the clams dosed with higher concentration of copper showed greater amount of haemolymph protein. When compared with the controls, the lowest concentration employed showed significantly lower protein values.

Similar trend in haemolymph protein values is shown by copper-dosed V.cyprinoides var. cochinensis also as briefly described below. 0.15 ppm copper-dosed clams showed significantly lower levels than the controls from 24 hrs onwards upto the end of the experimental period (120 hrs), whereas 0.30 ppm and 0.45 ppm copper-dosed clams showed significantly lower levels than the contrlos from 48 hrs onwards upto 96 hrs only. The absence of any significance for the value at 120 hrs is explained as the establishment of the balance between the entry and exit of protein into and from the haemolymph compartment. As in copper-dosed S.scripta, here also, the clams dosed with higher concentration of copper showed significantly higher values, but only towards the end of the experimental period.

Difference in trend in V.cyprinoides var. cochinensis (ie., lower protein values in all the copper-dosed clams than the control values at 48, 72 and

96 hrs) from S.scripta, is attributed to the species difference, and also to differences in the concentrations of the metal ion used. It may be noted that George et al. (1984) have reported interspecies differences in the heavy metal detoxication in oysters.

Many molluscs have a remarkably permeable epidermis so that metal ions can reach the haemolymph by simple diffusion (Simkiss and Wilbur, 1977). Though mechanisms for the transport of metals in the blood of molluscs are not clearly understood, there are, however, numerous examples that show that metals can rapidly reach the tissues by this route. Various systems for losing or immobilising metals have been recognised in bivalve molluscs, apart from the possibility of diffusion. These include the immobilisation of heavy metals in membrane-bound vesicles prior to their excretion from the kidney (Bryan, 1973; George and Pirie, 1980; Carmichael et al., 1980; Viarengo et al., 1981a; Simkiss et al., 1982; Calabrese et al., 1984), and by binding to wandering leucocytes, polysaccharides, amino acids and proteins, eg., metallothioneins (George et al., 1979; Bryan, 1979; Roesijadi, 1980; Viarengo et al., 1980a,b).

In 1 ppm copper-dosed S.scripta and in 0.15 ppm copper-dosed V.cyprinoides var. cochinensis, the metal ions entering the haemolymph compartment (because of the permeability of the epidermis) are removed preventing the accumulation of copper ions to toxic levels. This is achieved by binding it with haemolymph protein and the denatured protein is removed from the haemolymph through the mediation of haemocytes. This is the reason for the significant drop in the concentration of the haemolymph protein in 1 ppm copper-dosed S.scripta, and in 0.15 ppm dosed V.cyprinoides var. cochinensis. In these clams, exposed to low concentration of copper, alteration in cell

permeability may be less or nil, resulting in less or no seepage of cytosol into the haemolymph compartment. Earlier it has been pointed out that in 0.15 ppm copper-dosed V.cyprinoides var. cochinensis, there is migration of metal laden haemocytes to bring down the Cu^{2+} ions in the haemolymph compartment, which was manifested as a drop in the number of circulating haemocytes. In 1 ppm copper-dosed S.scripta, on the other hand, though the removal of ions may be taking place with the involvement of protein (resulting in a drop in protein values), there was no significant difference in the number of circulating haemocytes. This is interpreted to mean that in S.scripta, the loss of haemocytes from the haemolymph compartment may be balanced by the mobilisation of haemocytes into the haemolymph.

The role of wandering leucocytes (circulating haemocytes) in bringing down the metal ions in the haemolymph compartment has been recorded (George et al., 1976; Coombs, 1977). Amoebocytes (haemocytes) have been observed leaving the body by diapedesis after traversing the epithelial layers of the heart and kidney (Brown and Brown, 1965). The metal, either in a particulate form or in association with denatured proteins in the blood, are phagocytosed by amoebocytes in an indiscriminate way (Simkiss and Mason, 1983).

In 3 ppm copper-dosed S.scripta and 0.30 ppm copper-dosed V.cyprinoides var. cochinensis also, removal of ions from the haemolymph compartment by haemocytes is taking place. In V.cyprinoides var. cochinensis, the significant drop in the number of circulating haemocytes points out the above fact. In S.scripta at this concentration also, there was no significant difference in the number of circulating haemocytes, because, even this concentration may not

be sufficient to alter the balance of haemocyte's exit and entry into the haemolymph compartment. However, since the concentration of ions is greater at these levels, the removal of ions by the above-mentioned process alone may not be sufficient to decrease the cytotoxic effect of copper ions. In such circumstances, the permeability of the cell membrane is altered and seepage of protein occurs from within the tissue cells into the haemolymph. The protein added in this way into the haemolymph may be balancing the loss of protein caused by removal of denatured protein through the mediation of haemocytes. The no significance in the haemolymph protein values when compared with the controls in the case of 3 ppm copper-dosed S.scripta upto 96 hrs, and in the case of 0.30 ppm copper-dosed V.cyprinoides var. cochinensis upto 24 hrs may be due to the entry of protein due to seepage. When the permeability of the cell mambrane is thus altered by Cu^{2+} ions in higher concentration, it leads to seepage of protein into haemolymph compartment but also results in an alteration of osmolality, and maintenance of osmotic balance becomes essential to combat the stress. In 3 ppm copper-dosed S.scripta, this correction of altered permeability sets in between 96 hrs and 120 hrs, whereas in 0.30 ppm copper-dosed V.cyprinoides var. cochinensis, it sets in much earlier ie., between 24 hrs and 48 hrs. This correction may be achieved by the use of a compatible solute system (Brown and Simpson, 1972) involving amino acids along with other solutes. The contribution of protein degradation to the free amino acid pool may be substantial under certain conditions (Bishop et al., 1981). It is possible that while some denatured proteins are removed by the haemocytes, some are degraded further into amino acids and these amino acids may also be contributing, at least in part,

to the amino acid pool. Pickwell and Steinert (1984) have reported that relatively high initial concentrations of cupric ion seemed to initiate early increases in haemolymph proteolytic activity above control values. As mentioned earlier, in 3 ppm copper-dosed S.scripta it sets in between 96 hrs and 120 hrs, and in 0.30 ppm copper-dosed V.cyprinoides var. cochinensis, between 24 and 48 hrs. When such a correction mechanism is operating, the protein loss from the haemolymph compartment by the earlier mentioned metal removing process involving haemocytes (Which were operating all the time periods) will be more pronounced as there is no entry of protein into the haemolymph. In this situation, there will be a reduction in the total haemolymph protein values which is shown by S.scripta at 120 hrs and by V.cyprinoides var. cochinensis at 48, 72 and 96 hrs. The loss of protein also cannot be borne indefinitely, and in V.cyprinoides var. cochinensis, after 96 hrs the correction mechanism stops for a while, and more protein may be coming to the haemolymph compartment again to make good the loss of protein in the metal detoxication process and this may be a cyclic process. As a result in V.cyprinoides var. cochinensis, at 120 hrs, no significant difference in protein value was observed. In S.scripta, this may occur at a later stage, ie., after 120 hrs.

In 5 ppm copper-dosed S.scripta and 0.45 ppm copper-dosed V.cyprinoides var. cochinensis, since the concentrations of metal ions are the greatest, here also the membrane permeability is altered and the seepage of protein occurs. In S.scripta it persists during the entire period of the experiment. In 0.45 ppm copper-dosed V.cyprinoides var. cochinensis, the seepage is checked by 24 hrs as in 0.30 ppm copper-dosed clams but starts again after

96 hrs. This is the reason for the observed no significant difference in protein values when compared with the controls during the entire period of the experiment in S.scripta, and upto 24 hrs in V.cyprinoides var. cochinensis. In V.cyprinoides var. cochinensis once the correction mechanism sets in by about 24 hrs, the protein loss from the haemolymph compartment by the already mentioned metal removing process involving haemocytes (which were operating all the time) will be more pronounced as there is no entry of protein into the haemolymph. In this situation also as mentioned earlier, there will be a reduction in the haemolymph protein values, which is shown at 48, 72 and 96 hrs. After 96 hrs more protein may be seeping into the haemolymph compartment because of the temporary suspension of correction mechanism, to make good the loss of protein in the metal detoxication process, and this may be a cyclic process. Again, in S.scripta, this may be occurring at a later stage.

In copper-dosed V.cyprinoides var. cochinensis, at 120 hrs, and in S.scripta at all time periods, there is a tendency of protein increase with increasing metal ion concentration. The neo-synthesised metallothionein-like protein in response to metal ions may be contributing to this trend in protein increase. The wide spread occurrence of a low molecular weight, sulphydryl-rich, heat-stable and soluble metal binding protein in vertebrates and invertebrates is now well established (Roesijadi, 1980-81). This protein has a high affinity for various metals, including copper, mercury, silver and zinc (Cherian and Goyer, 1978), and is highly adapted to form metal complexes. The large number of cysteinyl residues are actively involved in metal binding and form mercaptide bonds. They are also characterised by the absence of aromatic

amino acids and histidine (Vallee, 1979). The induction of metallothionein synthesis is controlled at the transcriptional level (Roesijadi, 1980-81). Although Vallee and Ulmer (1972) and Weser and Hubner (1970) have observed that cadmium can stimulate the activity of RNA polymerase, Viarengo et al. (1982) have reported reduced synthesis of RNA polymerase I and II in the digestive gland cells of copper exposed mussels, and have indicated that this may depend not upon a direct effect of copper ions on the transcriptional process, as believed earlier, but rather, on the cytotoxic effect the metal has on the cytoplasm. Recent studies on molluscs have demonstrated that Cd^{2+}, Cu^{2+} Hg^{2+}, etc. are able to induce the synthesis of metallothionein-like proteins (Casterline and Yip, 1975; Noel-Lambot, 1976; Noel-Lambot et al., 1978; George et al., 1979; Marshall and Talbot, 1979; Carmichael et al., 1980; Viarengo et al., 1980b; 1981a,b; 1982; 1984a; 1985; Roesijadi and Hall, 1981; among others). The molluscan metal binding protein, however, exhibit certain properties, not characteristic of metallothioneins (Ridlington and Fowler, 1979; George et al., 1979; Marshall and Talbot, 1979), and are, therefore, often referred to as metallothionein-like proteins. In general, however, the similarities between the molluscan proteins and metallothionein appear to be strong when one considers their metal binding properties.

The general perception of metallothionein as primarily a detoxifying protein, is altered with the reported occurrence of these proteins in unexposed animals also. Because of this it is suggested that this protein plays a regulatory role in the metabolism of metals, such as, Cd, Zn, and Cu (Roesijadi, 1980-81; Engel, 1987). Further, Cousins (1985) has indicated that it is also involved in stress reactions, and Udom and Brady (1980) and Li et

al. (1980) have suggested that, besides regulatory, it has a transport function also in normal physiology.

It may be noted that Viarengo et al. (1980a,b; 1982) have demonstrated that in the cells of the gill, digestive gland and mantle from mussels exposed to sub-lethal concentration of Cu^{2+}, amino acid uptake, overall protein synthesis, ATP content and the synthesis of RNA polymerase I and II are significantly reduced. Metal ions are known to inactivate protein molecules through non-specific binding or cross-linking of essential side chains and by promoting irreversible denaturation (Ulmer, 1970).

In Vibrio-injected experiments, the haemolymph protein did not show any significant variation between the experimentals and the controls during the entire experimental period in both the clam species used for the study. Because bacteria are not totally composed of carbohydrates, naturally the question arises as to what happens to the non-carbohydrate constituents. Since there is no difference in the protein values in the experimentals, it is possible, as suggested by Cheng (1975), that they are expelled from phagosomes to the exterior. Or the protein may be degraded further into amino acids.

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CHAPTER V

ACID PHOSPHATASE ACTIVITY IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

The impact of pollutants on an organism is realised as disturbances at different levels of functional complexity. It includes responses to toxic environmental chemicals at the sub-cellular and cellular levels of organisation. In many cases, the earliest detectable changes are associated with a particular type of sub-cellular organelle, such as lysosomes, endoplasmic reticulum and mitochondria (Fowler et al., 1975; Sternlieb and Goldfischer, 1976; Moore and Stebbing, 1976; Moore, 1977, 1980; Zaba and Harris, 1978; George, 1983a,b; Pickwell and Steinert, 1984; Moore et al., 1984; Akberali et al., 1984).

There are many ways in which the structure and/or function of organelles and cells can be disturbed by toxic contaminants (Slater, 1978). Toxicological investigations in mammals have shown that much of the damaging action of environmental xenobiotics is produced by highly reactive metabolites (Slater, 1978). It is these activated chemical forms that are responsible for the initiation of primary intracellular disturbances. These may rapidly spread into a complex network of associated secondary and higher order disturbances which become progressively more difficult for the cell to reverse or modify.

In mammalian toxicological studies, study of enzyme activity is a routine practice, since this provides a method for the determination of subtle, sub-lethal alterations. When it comes to bivalves, it can give a greater insight on the nature of mechanisms where there is a greater bioaccumulation of trace metals which is unparallel. Even to draw on the resources of mammalian toxicology is of limited value, as what little is known of mechanisms in lower organisms indicates that they are often markedly different from those of higher

organisms (Parke, 1981).

Study of cellular responses to pollutant-induced cell injury may make it possible to observe alteration in the structural-functional organisation in individual target cells or groups of cells at an early stage of a reaction to cell injury, before an integrated cellular response would manifest at the level of whole animal physiological processes, and long before there were any changes at the population level (Moore, 1980).

As circumstantially linked to pollutant effects, a number of abnormal cellular conditions have been reported in mussels and bivalves. These include the presence of proliferative neoplastic cells in M.edulis, possibly linked to exposure to polynuclear aromatic hydrocarbons (Lowe and Moore, 1978; Mix et al., 1979), abnormal occlusion of haemolymph sinuses by large numbers of haemocytes, termed granulocytomas (Lowe and Moore, 1979a) which was linked especially with the presence of pollutant-related effects at a number of environmental sites where the mussels were known to be stressed.

More concrete evidence of contaminant-related pathological changes in cell structure has been obtained from investigations of the hepatopancreas of marine mussels. This has involved pollutant-induced alterations in the epithelial cells of the digestive tubules, often leading to epithelial thinning or apparent atrophy (Couch, 1984). These morphological disturbances have been quantitatively assessed in M.edulis in response to water accomodated fraction of North Sea crude oil (Lowe et al., 1981), and in the clam M.mercenaria in response to benzo(a)pyrene, hexa chlorobenzene and pentachlorophenol (Tripp et al., 1984). Lysosomal disruption in mussels in response to copper and phenanthrene (Pickwell and Steinert, 1984; Moore et al., 1984), extracellular

release of lysosomal enzymes (Harrison and Berger, 1982), and inhibition of cellular immunity in molluscs (Cheng and Sullivan, 1984) have also been reported. Mussels and periwinkles have a proven sensitivity to the sub-lethal chemical perturbations that may be expressed at the cellular and sub-cellular levels of organisation (Bayne et al., 1981; 1985; Dixon, 1982).

Lysosomes are an ideal starting point for investigations of generalised cellular injury in marine molluscs as many cell types in these animals are particularly rich in lysosomes (Summer, 1969), and lysosomes are noted for their compartmentalization and accumulation of a wide variety of organic chemicals and metals (Allison, 1969; Moore, 1980; George, 1983a,b). And many of these xenobiotics induce alterations in the bounding membrane of the lysosomes leading to destabilisation (Viarengo et al., 1981a; Moore and Lowe, 1985). Investigations of digestive cell atrophy or thinning have revealed that there is significant increase in lysosomal volume in mussels (M.edulis). This increase in lysosomal volume involved the formation of pathologically enlarged or giant lysosomes and this alteration is associated with membrane destabilisation or increased permeability resulting in reduced latency of lysosomal enzymes (Moore and Clarke, 1982; Moore et al., 1985). Release of degradative hydrolytic enzymes from the lysosomal compartment into the cytosol may result from destabilisation of the lysosomal membrane (Moore, 1976; Baccino, 1978), and such destabilisation may also involve increased lysosomal fusion with other intracellular vacuoles leading to the formation of pathologically enlarged lysosomes.

The lysosomal membrane is often a target of injury by xenobiotics. Injury resulting in destabilisation of the lysosomal membrane bears a

quantitative relationship to the magnitude of the stress response (Bayne et al., 1979a; 1982), and this presumably contributes to the intensity of catabolic or degradative effects, as well as to the level of pathological change that results. The destabilised membrane allows the release of lysosomal hydrolytic enzymes into the cytoplasm and extracellular environment (Bayne et al., 1976; Moore and Stebbing, 1976; Pickwell and Steinert, 1984).

Lysosome may be important as a detoxication system particularly for metals (Viarengo et al., 1981a; 1984b; George, 1983a,b). As with other detoxication systems, this process is effective until the storage capacity of the lysosomes is overloaded, or the lysosomes are damaged directly by the accumulated contaminant (Moore, et al., 1985). The consequences of these lysosomal changes would be increased autolysis activity leading to atrophy.

Cytochemical techniques such as the measurement of lysosomal membrane stability (hydrolase latency) and study of the activity of marker enzymes, acid phosphatase and alkaline phosphatase, can provide insight into the mechanisms of xenobiotic-induced cell injury. Caution is required in the interpretation of lysosomal damage as a primary injury, when it may, in some instances, be a secondary or higher order effect. This type of approach provides information on mechanisms involving molecular alterations in the lysosomal membranes, which undoubtedly contribute to disturbances of the integration of cellular function (Slater, 1978).

Also biochemical interpretations of effects in many molluscan (and mammalian) tissues are often plagued by problems incurred from the fact that many cell types are present, whereas only a small number of these may be susceptible to initial damage. Also, the same cell type in various physiological/

structural states will probably respond quite differently, so it is important to identify cyclical activity in cells of interest (Lowe et al., 1981; 1982).

Apart from this, there is emerging evidence of modification of cellular reactions in mixtures of xenobiotics. Chandy and Patel (1985) have reported that the tropical blood clam exposed to mercury, selenium and glutathione individually and in combination showed different effects in lysosomal enzyme activity in vitro and in vivo. These types of modifications can be subtle rather than direct is clear from recent work on mussels, where some of the interactive modifications affected cellular recovery from xenobiotic insult by copper and phenanthrene, rather than the immediate reactions to cell injury (Moore et al., 1984).

Lysosomal systems has been shown to be very sensitive to changes in the intra and extracellular environment and subsequently to be involved directly or indirectly in regulating many physiological and pathological processes. In mussels and clams, the lysosomal destabilisation has been demonstrated also in response to hypothermia, and thermal death, starvation, changes in salinity, exposure to air, experimentally induced spawning, steroid hormone etc. (Moore et al., 1979; Patel and Patel, 1985). Autolytic responses, involving lysosomes, have also been induced in molluscs by parasitic and nutritional stresses (Thompson et al., 1974; Yoshino, 1976; Moore and Halton, 1977). Besides, challenging with bacteria also causes destabilisation of lysosomes and subsequent release of acid hydrolases (Cheng, 1983a).

Evaluation of the possible role of lysosomes, in the deposition or degradation of pollutants including heavy metals, and in the degradation of micro-organisms like bacteria, could be utilised to quantify the changes in

lysosomal latency which in turn could be monitored as an index for pollution caused by biotic and abiotic factors. This can be achieved through a knowledge of the concentrations of the important lysosomal marker enzymes. The present study was, therefore, designed to evaluate the activity pattern of one of the lysosomal marker enzymes, acid phosphatase, in the haemolymph compartment of two clam species exposed to different concentrations of copper and challenged with bacteria.

MATERIALS AND METHODS

Methods of collection of clams, rearing the clams, acclimatisation, selection of size groups, mode of collection of haemolymph, dosing the clams with copper, challenging the clams with bacteria and the statistical analysis of the data were the same as described in detail in chapter II. The concentration of the copper ions, the dosage of bacterial suspension used, and the salinity used for the two clam species were also the same as described before.

In the case of S.scripta, 12 clams from each size group, and in the case of V.cyprinoides var. cochinensis, 13 clams from each size group were employed for studying the haemolymph acid phosphatase activity in the different size groups selected.

For studying the haemolymph acid phosphatase activity during stress induced by copper ions, the following setup was used. For S.scripta, the experimental group consisted of 180 clams (60 in each of the three experimental batches), and the control group consisted of 60 clams. In the case of V. cyprinoides var. cochinensis also, the experimental group consisted of 180 clams and the control group 60 clams. Haemolymph was withdrawn every

24 hrs for 5 days for the estimation of acid phosphatase activity from 10 specimens each of the experimental batches and 10 from the control group, each day.

For the determination of acid phosphatase activity in the haemolymph of S.scripta and V.cyprinoides var. cochinensis, subsequent to challenge with bacteria at a concentration of 1x10 cells/0.02 ml, haemolymph samples were collected at 3, 6, 12, 24, 48, 72, 96 and 120 hr-time period post challenge. For each clam species, for each time period, the untampered control group, 2% saline-injected control group, and the bacteria-injected experimental group consisted of 10 clams, each.

In this experiment also, as described earlier, more clams than the required number were reared as experimentals and controls anticipating mortality during the course of the experiment.

ESTIMATION OF ACID PHOSPHATASE ACTIVITY

Acid phosphatase activity was determined following the methodology of Sigma technical bulletin (No: 104) with some modifications. To study the enzyme activity, 0.1M citrate buffer of pH 3.6 was used. The incubation temperature was 37±0.1° C.

To 1 ml of the frozen buffer containing 100 mM of NaCl, 0.1 ml haemolymph was added using a 0.1 ml pipette, and immediately frozen till analysed. At the time of analysis, the buffer-enzyme mixture was kept in a water-bath at 37° C. When the temperature of buffer-enzyme mixture reached 37° C, 0.1 ml of substrate (2 mg of p-nitrophenyl phosphate sodium salt (Merck) in 0.1 ml) was added using a 0.1 ml pipette to start the reaction. After incubating for one hour at 37° C, the reaction was stopped by adding

2 ml of 0.25N NaOH. The yellow colour of p-nitrophenol in the alkaline medium was read at 410 nm. The concentration of p-nitrophenol formed was found out from a standard graph of p-nitrophenol. Simultaneously, the protein content of 0.1 ml haemolymph was found as described before. From this μmoles of p-nitrophenol liberated per mg protein per hour was calculated.

RESULT

ACID PHOSPHATASE ACTIVITY IN THE HAEMOLYMPH OF THE FOUR SIZE GROUPS OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

Table 14A gives the mean value, standard deviation and range of haemolymph acid phosphatase activity in the four size groups of S.scripta, and Table 14B shows the same in the four size groups of V.cyprinoides var. cochinensis. In both these cases when the mean value of acid phosphatase activity for different size groups were compared, there was no statistically significant difference.

ACID PHOSPHATASE ACTIVITY IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS DOSED WITH THREE CONCENTRATIONS OF COPPER

Haemolymph acid phosphatase activity values in S.scripta and V.cyprinoides var. cochinensis when exposed to three sub-lethal concentrations of copper for five days are given in Tables 15A and 15B, and Figures 9A and 9B, respectively.

In S.scripta, when the values of acid phosphatase activity of 1 ppm copper-dosed clams were compared with the values of the controls, the values

Table 14A. Haemolymph Acid Phosphatase activity (μmol/mg protein/hr) in the four size groups of Sunetta scripta

Size group	30-32 mm	36-38 mm	38-40 mm	42-44 mm
N	12	12	12	12
Mean value	0.2068	0.2316	0.2583	0.2210
± SD	0.0427	0.0704	0.0829	0.0609
Range	0.1584-0.3100	0.1300-0.3750	0.1442-0.4715	0.1283-0.3402

Table 14B. Haemolymph Acid Phosphatase activity (μmol/mg protein/hr) in the four size groups of Villorita cyprinoides var. cochinensis

Size group	24-26 mm	34-36 mm	38-40 mm	44-46 mm
N	13	13	13	13
Mean value	0.7080	0.5513	0.6321	0.6829
± SD	0.2819	0.1394	0.2294	0.2202
Range	0.3162-1.1232	0.3617-0.7563	0.1965-1.0187	0.2990-0.9495

Table 15A. Haemolymph Acid Phosphatase Activity (μmol/mg protein/hr) in Sunetta scripta dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	0.2724	0.2913	0.2814	0.2683	0.2690
	± SD	0.0645	0.0546	0.0607	0.0775	0.0703
	Range	0.1731-0.3586	0.2051-0.3659	0.1595-0.3516	0.1742-0.3686	0.1905-0.4563
1 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.3679	0.4361	0.2675	0.2057	0.2212
	± SD	0.0624	0.1222	0.1040	0.0609	0.0675
	Range	0.2941-0.4733	0.2333-0.6500	0.1375-0.4579	0.1300-0.3019	0.1446-0.3433
3 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.3311	0.3549	0.2687	0.2077	0.2000
	± SD	0.0886	0.1317	0.0675	0.0528	0.0475
	Range	0.2129-0.5034	0.1856-0.5615	0.2101-0.4423	0.1181-0.2827	0.1198-0.2973
5 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.2208	0.4154	0.2652	0.2026	0.1973
	± SD	0.0796	0.0905	0.0712	0.0410	0.0416
	Range	0.1267-0.3516	0.2417-0.5604	0.1667-0.3925	0.1663-0.3095	0.1382-0.2692

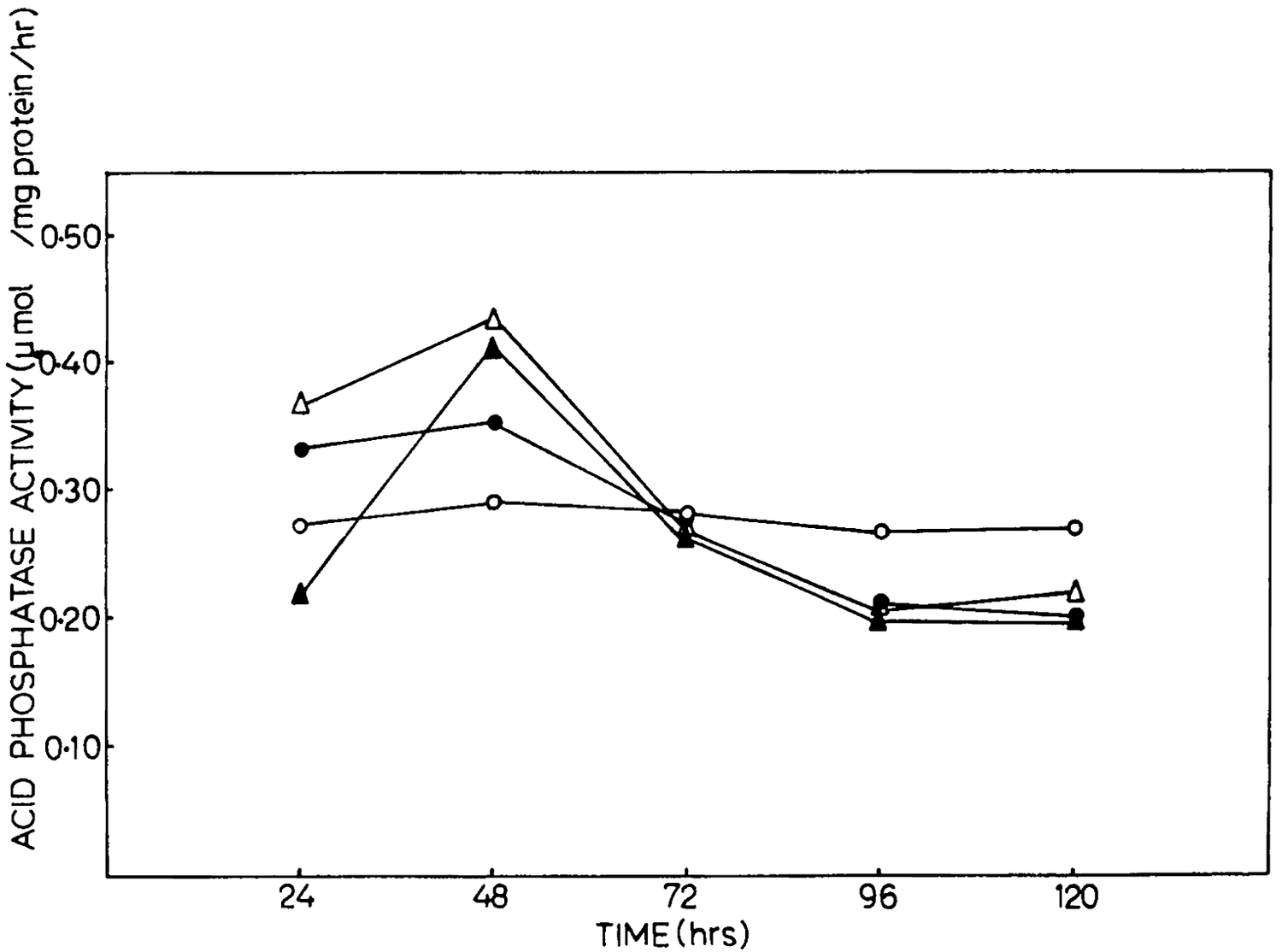

Fig. 9A. Haemolymph Acid Phosphatase activity (µmol/mg protein/hr) in Sunetta scripta dosed with three concentrations of copper. Control (o); 1 ppm copper (Δ); 3 ppm copper (●); 5 ppm copper (▲).

Table 15B. Haemolymph Acid Phosphatase Activity (µmol/mg protein/hr) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	0.5913	0.4764	0.5862	0.5983	0.6180
	± SD	0.1712	0.1515	0.2009	0.2268	0.1920
	Range	0.3984-1.0152	0.2484-0.6909	0.3750-1.1007	0.3491-1.0187	0.3250-0.9206
0.15ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.8191	0.6428	0.4353	1.0634	0.7067
	± SD	0.2040	0.1603	0.1621	0.3036	0.2352
	Range	0.4854-1.2317	0.3585-0.9859	0.2295-0.6957	0.6273-1.6129	0.4200-1.0595
0.30ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.6894	0.5933	0.7072	0.6958	0.3350
	± SD	0.1721	0.2222	0.2929	0.1926	0.0972
	Range	0.4419-1.1233	0.2941-1.0147	0.3947-1.2195	0.4091-1.0319	0.2037-0.4507
0.45ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.7684	0.3153	0.1600	0.5510	0.1558
	+ SD	0.2224	0.0845	0.0477	0.2145	0.0144
	Range	0.3694-1.1151	0.1744-0.4324	0.0866-0.2468	0.2568-0.8974	0.1362-0.1754

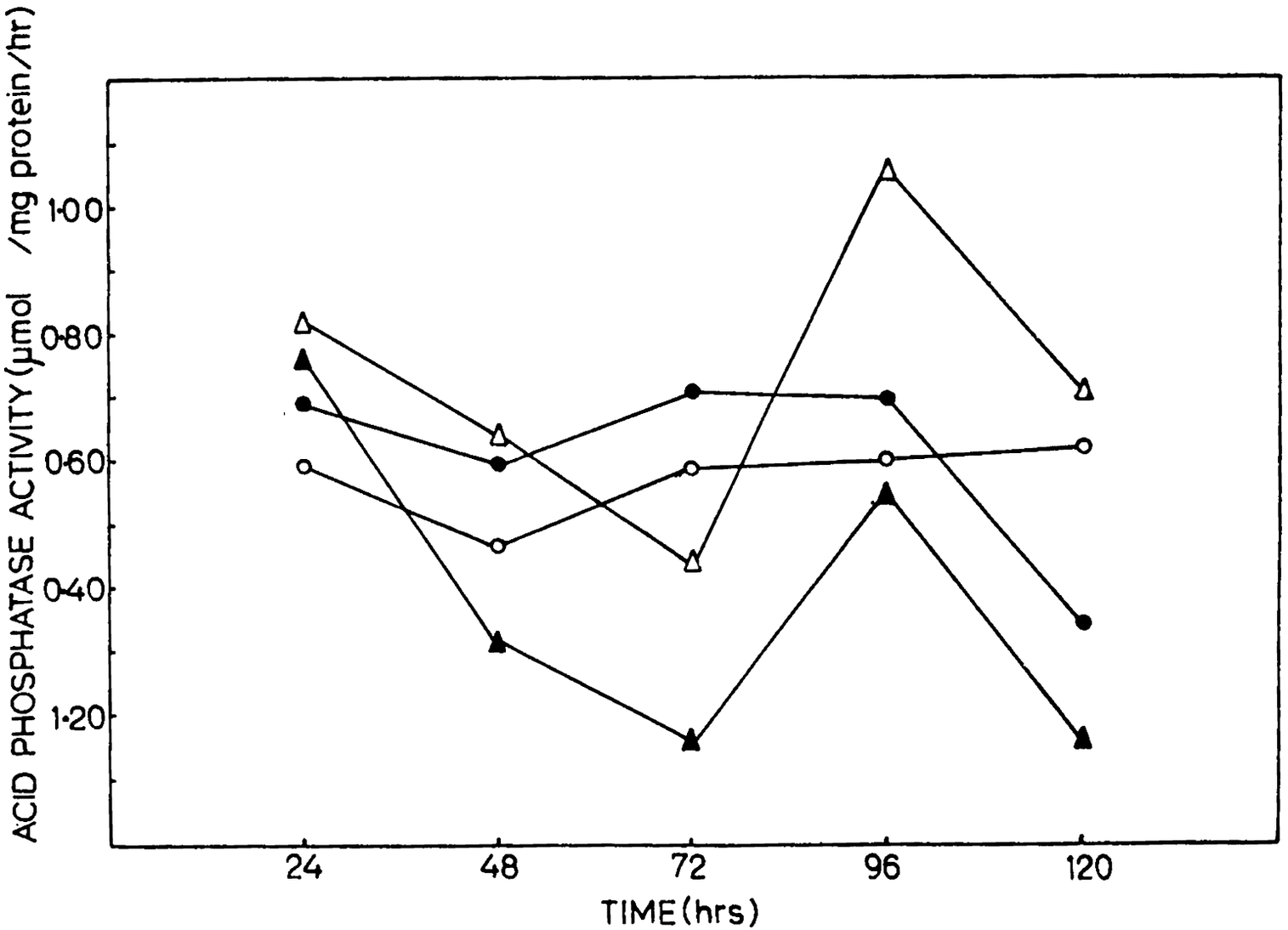

Fig. 9B. Haemolymph Acid Phosphatase activity (µmol/mg protein/hr) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper. Control (○); 0.15 ppm copper (△); 0.30 ppm copper (●); 0.45 ppm copper (▲).

of 1 ppm copper-dosed clams were found to be significantly higher at 24 hrs ($P < 0.01$) and 48 hrs ($P < 0.01$). Acid phosphatase activity of 3 ppm copper-dosed clams were found to be significantly lower than that of the controls at 120 hrs ($P < 0.05$). Five ppm copper-dosed clams showed significantly higher values than the controls at 48 hrs ($P < 0.01$) but significantly lower values at 96 hrs ($P < 0.05$), and 120 hrs ($P < 0.02$). The values of 1 ppm copper-dosed clams on comparison with the values of 3 ppm copper-dosed clams, did not show any significant difference. The values of 1 ppm copper-dosed clams were significantly higher than the values of 5 ppm copper-dosed clams only at 24 hrs ($P < 0.01$). When the values of 3 ppm and 5 ppm copper-dosed clams were compared, the values of 3 ppm copper-dosed clams were significantly higher at 24 hrs ($P < 0.02$).

In V.cyprinoides var. cochinensis, the values of acid phosphatase activity of 0.15 ppm copper-dosed clams were found to be significantly higher at 24 hrs ($P < 0.05$), 48 hrs ($P < 0.05$), and 96 hrs ($P < 0.01$) than the values of the controls in the respective days. The mean value of 0.30 ppm copper-dosed clams were significantly lower than the values of the control at 120 hrs ($P < 0.01$). The mean values of 0.45 ppm copper-dosed clams were significantly lower than the mean value of the control at 48 hrs ($P < 0.02$), 72 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). When the mean values of 0.15 ppm and 0.30 ppm copper-dosed clams were compared, the values of 0.15 ppm copper-dosed clams were significantly lower at 72 hrs ($P < 0.05$), but significantly higher at 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). The mean values of 0.15 ppm copper-dosed clams were significantly higher than the mean values of 0.45 ppm coper-dosed clams at 48 hrs ($P < 0.01$), 72 hrs ($P < 0.01$), 96 hrs ($P < 0.01$), and 120 hrs

($P < 0.01$). Similarly the mean values of 0.30 ppm copper-dosed clams were significantly higher than the mean values of 0.45 ppm copper-dosed clams at 48 hrs ($P<0.01$), 72 hrs ($P<0.01$), and 120 hrs ($P<0.01$).

ACID PHOSPHATASE ACTIVITY IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS WHEN INJECTED WITH VIBRIO SP.

Haemolymph acid phosphatase activity in S.scripta and V.cyprinoides var. cochinensis when challenged with bacteria are given in Tables 16A and 16B, and Figures 10A and 10B, respectively.

In S.scripta, 2% saline-injected controls showed significantly higher values than the untampered controls at 3 hrs ($P<0.01$). The mean values of Vibrio-injected clams were significantly higher than the mean values of the untampered controls at 3 hrs ($P< 0.01$), 6 hrs ($P< 0.02$), 12 hrs ($P< 0.05$), and 24 hrs ($P< 0.05$). When the mean values of the 2% saline-injected controls and Vibrio-injected clams were compared, the mean values of Vibrio-injected clams were significantly higher at 12 hrs ($P<0.02$), and 72 hrs ($P<0.02$).

In V.cyprinoides var. cochinensis, the mean values of 2% saline-injected controls were significantly higher at 3 hrs ($P< 0.02$). Vibrio-injected clams showed significantly higher mean values than the controls at 3 hrs ($P< 0.01$), 6 hrs ($P< 0.01$), and 12 hrs ($P< 0.01$). The mean value of 2% saline-injected clams was significantly lower than the mean value of Vibrio-injected clams at 6 hrs ($P<0.01$).

Table 16A. Haemolymph Acid Phosphatase Activity (μmol /mg protein/hr) in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hour	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	10	10	10	10	10	10	10	10
Untampered control	Mean value	0.2684	0.2624	0.2701	0.2829	0.2672	0.2799	0.2288	0.2435
	± SD	0.0799	0.0530	0.1032	0.0851	0.1159	0.0757	0.0759	0.0531
	Range	0.1472-0.3735	0.1621-0.3614	0.1629-0.4615	0.1653-0.4698	0.1078-0.4843	0.1650-0.3636	0.1605-0.3824	0.1481-0.3277
	N	10	10	10	10	10	10	10	10
2% Saline injected	Mean value	0.4266	0.3263	0.2801	0.3732	0.3183	0.2174	0.2172	0.2428
	± SD	0.0857	0.0772	0.0429	0.0994	0.1170	0.0759	0.0589	0.0665
	Range	0.2946-0.5652	0.2417-0.4862	0.2093-0.3362	0.2150-0.5614	0.0869-0.5001	0.1100-0.3333	0.1212-0.2766	0.1494-0.3789
	N	10	10	10	10	10	10	10	10
Vibrio sp injected	Mean value	0.4252	0.3415	0.3671	0.3659	0.3257	0.3184	0.2428	0.2444
	± SD	0.1240	0.0655	0.0868	0.0684	0.1816	0.0836	0.0629	0.0376
	Range	0.2667-0.7119	0.2523-0.4417	0.2378-0.5345	0.2521-0-4677	0.1100-0.7722	0.1569-0.4403	0.1563-0.3544	0.2066-0.3200

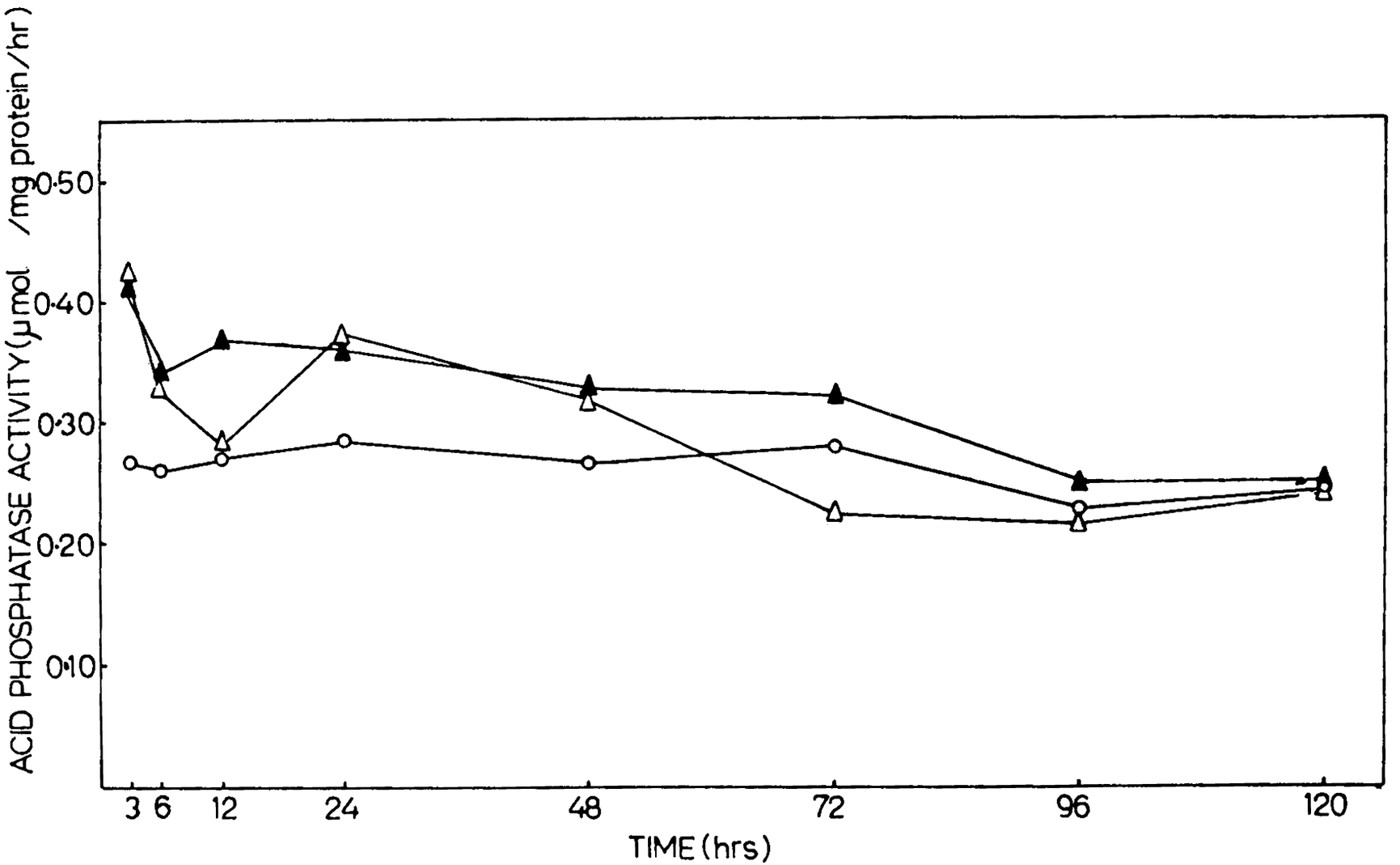

Fig. 10A. Haemolymph Acid Phosphatase activity (µmol/mg protein/hr) in Sunetta scripta injected with 1 x 10^8 cells/0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

Table 16B. Haemolymph Acid Phosphatase Activity (μmol /mg protein/hr) in Villorita cyprinoides var. cochinensis injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	10	10	10	10	10	10	10	10
Untampered control	Mean value	0.6062	0.5556	0.6047	0.5287	0.6304	0.6307	0.6251	0.6215
	± SD	0.1482	0.1334	0.1234	0.2172	0.2825	0.3524	0.2147	0.2557
	Range	0.4372-0.8262	0.4042-0.7521	0.4624-0.7832	0.2273-1.0162	0.1500-1.1021	0.1965-1.5300	0.2762-0.9284	0.1701-0.8760
	N	10	10	10	10	10	10	10	10
2% Saline injected	Mean value	1.0666	0.6667	0.6219	0.6581	0.5650	0.5919	0.5024	0.7828
	± SD	0.4630	0.2131	0.4398	0.2353	0.2472	0.3375	0.2413	0.3662
	Range	0.4818-2.0317	0.3582-1.0625	0.2010-1.8109	0.3214-1.1298	0.2083-0.8676	0.1912-1.2220	0.2744-1.1170	0.2366-1.6491
	N	10	10	10	10	10	10	10	10
Vibrio sp injected	Mean value	0.9879	1.0775	0.9687	0.6717	0.5228	0.5955	0.7010	0.7908
	± SD	0.2372	0.2748	0.3397	0.2990	0.2732	0.3025	0.4258	0.4230
	Range	0.4903-1.3664	0.8722-1.5220	0.5310-1.6264	0.3289-1.2409	0.1408-0.9085	0.1728-1.1415	0.2636-1.4920	0.3231-1.6689

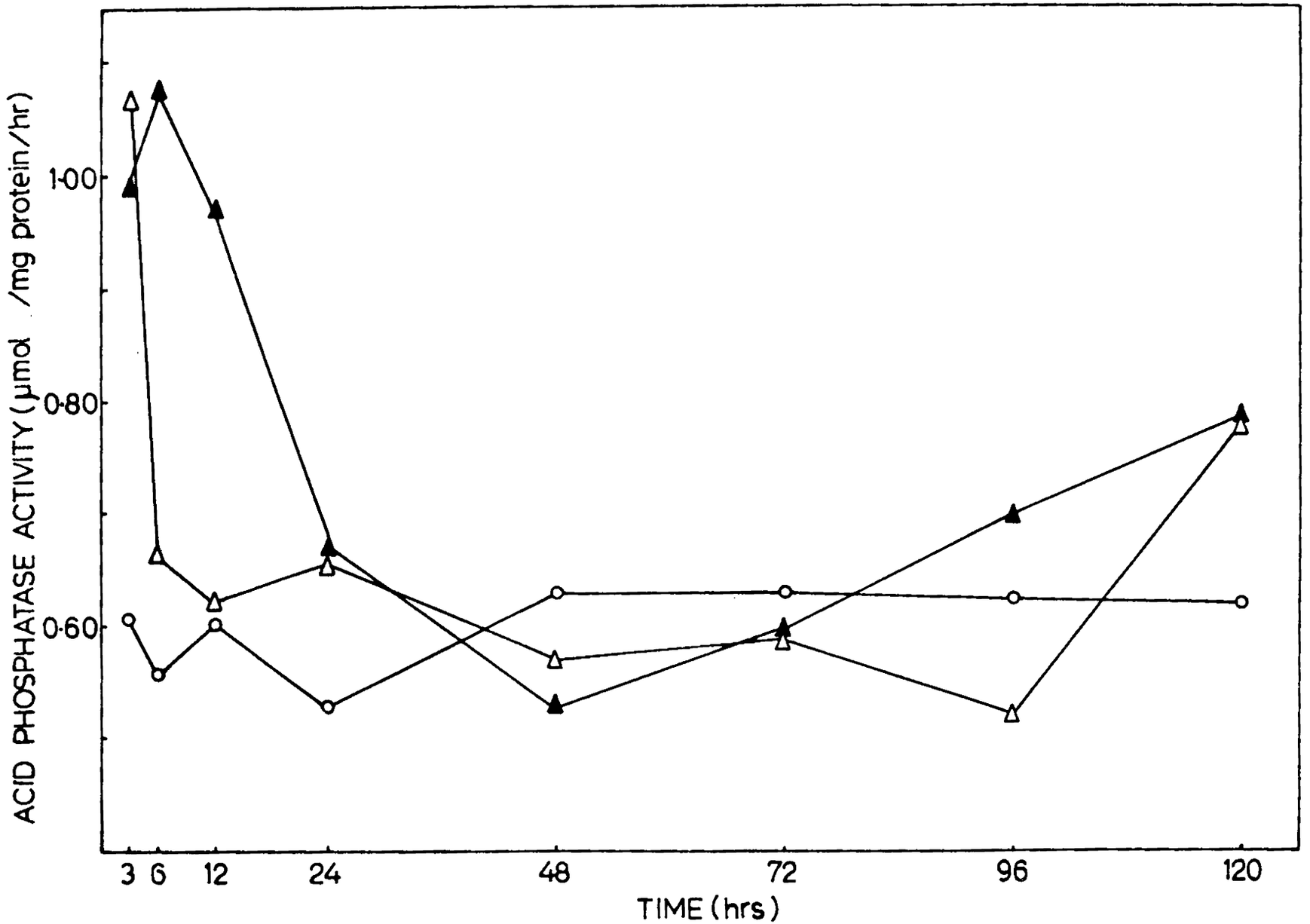

Fig. 10B. Haemolymph Acid Phosphatase activity (µmol/mg protein/hr) in Villorita cyprinoides var. cochinensis injected with $1x10^8$ cells/ 0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ);

DISCUSSION

In the present study, the mean values of haemolymph acid phosphatase activity did not show any significant difference among the four size groups of the clam species studied. This shows that under normal conditions, the acid phosphatase activity will not vary significantly among the size groups.

In copper-stress experiments with S.scripta and V.cyprinoides var. cochinensis, the pattern of peak activity clearly shows that in the haemolymph the acid phosphatase activity is determined by its synthesis, its release into the haemolymph compartment and its final loss. In the two clam species studied, the differences in the time-periods, showing the maximum and minimum mean activities, are attributed to species difference, and to the concentrations of the metal ions used. Apart from these, the onset of destabilisation, and reduction of latency of enzyme also influence the activity pattern of the enzyme.

There are reports that many of the xenobiotics induce alterations in the bounding membrane of the lysosomes leading to destabilisation (see review, Moore and Lowe, 1985). This destabilisation causes release of hydrolytic enzymes from the lysosomal compartment into the cytosol (Moore, 1976; Baccino, 1978, and others), and such destabilisation may also involve increased lysosomal fusion with other intracellular vacuoles leading to the formation of pathologically enlarged lysosomes. In the light of these reports, the observed increase in haemolymph acid phosphatase activity in S.scripta dosed with 1 ppm copper and in V.cyprinoides var. cochinensis dosed with 0.15 ppm copper in the early time periods is interpreted as the release of the enzyme into the haemolymph compartment from lysosomes as a result of destabilisation of

lysosomal membrane. This may have also resulted from the cytotoxicity of copper ions through increased turn over of organelles, and hence, increased lysosomal activity resulting in increased release of lysosomal enzymes. Such an explanation was also given by Koenig (1963), Norseth (1968), and Roesijadi (1980) for increased lysosomal enzyme activities in organisms exposed to sub-lethal concentrations of metal ions. It is also possible that, the lowest copper ion concentration used in the present study was readily detoxified before significant lysosomal damage took place. In this context it is worth mentioning the presumption of Chandy and Patel (1985) that at lower concentrations the metal ions that have entered the system are engulfed into lysosomes and subsequently transformed into biologically inactive forms. There is reason to believe that the presence of metal ions causes increased production and release of acid hydrolases into the haemolymph, and subsequently the metal ions, in non-toxic forms, are taken into the lysosomes of haemocytes and eventually removed. This transformation and inactivation of metal ions, are probably carried out by the acid hydrolases released from lysosomes. In comparatively low concentrations of copper, the transformation and inactivation of the metal ion are carried out by the lysosomal enzymes normally present in the haemolymph although the presence of metal ions may lead to a hypersynthesis of lysosomal enzymes. The hypersynthesised enzyme is not immediately made use of, and this is reflected as increased enzyme activity in the early time periods. But in the later time periods, the hypersynthesised enzyme is also made use of for the inactivation and transformation of the metal ion as the clams are still under exposure to copper, and hence, no significant difference in the values are noticed in the later time

periods. Since destabilisation has already taken place in the early time periods, it is also possible that the enzyme is not released in significant amount into the haemolymph and hence no significant difference in the activity values at later time periods. The hypersynthesis of acid hydrolases, in situations like this, has been well explained by Cheng (1983a,b), and Cheng and Mohandas (1985) with the involvement of recognition sites on molluscan haemocytes.

In S.scripta dosed with 3 ppm copper and in V.cyprinoides var. cochinensis dosed with 0.30 ppm copper, the absence of increased enzyme activity in the early time periods may be due to two reasons: (1) because of the higher metal ion concentration, the peak enzyme activity (because of destabilisation of lysosomes) might have taken place much earlier, ie., before 24 hrs, or (2) because of the concentration of the metal ion was higher, the hypersynthesised enzyme was also made use of from the very first day itself for modification and inactivation of metal ions, and hence no significant difference need be expected in the values in the initial time periods. However, as the clams continue to be under metal-exposure condition, the higher concentration of copper has affected the synthesis of the enzyme also, and this is reflected as a significant drop in the enzyme activity by 120 hrs.

The above mentioned two reasonings are applicable in the case of S.scripta dosed with 5 ppm copper, and V.cyprinoides var. cochinensis dosed with 0.45 ppm copper. The pattern of activity, however, is different in the sense that since the concentrations used were the highest, the adverse effect of metal ions on the synthesis of the enzyme had started much earlier. With the result, the significant drop in haemolymph enzyme activity in S.scripta started by 96 hrs, and in V.cyprinoides var. cochinensis by 48 hrs.

When we look at the data, it is evident that at certain time period between two time periods, in the experimentals, the enzyme activity value has shown significant or no significant difference from the controls at the respective time periods. In 5 ppm copper-dosed S.scripta, the value at 72 hrs does not show significant difference, but at 48 and 96 hr-time periods they do. The value at 48 hrs is significantly higher but those at 24 and 72 hrs are not. Similarly in v.cyprinoides var. cochinensis in 0.15 ppm copper-dosed clams the value at 72 hrs is not significantly higher or lower but the values at 48 and 96 hr-time periods show significant variations. The same is the case with the value of 0.45 ppm copper-dosed clams at 96 hr-time period when no significant difference was noticed. This difference in the pattern of activity is explained in the following way. It is noted that lysosomes even in single cell types are quite variable in their enzymatic constitution (Dean, 1977); and the heterogeneity in size and shape reflects the divergent functional activities of lysosomes in different cell types (Schellens et al., 1977). Moreover, the cytoplasmic granules in the granulocytes of several species of molluscs have been considered to be true lysosomes (see Mohandas et al., 1985). The finding of Yoshino and Cheng (1976a) that not all of the cytoplasmic granules within the granulocytes of M.mercenaria include acid phosphatase activity prompted them to suggest that these vesicles represent a chemically heterogenous population or there is a non-synchronised chemical cycle which occures within the granules (lysosomes). In the present study, the observed differences in the pattern of haemolymph acid phosphatase activity is attributed to the non-synchronised chemical cycle occurring in the lysosomes.

It has been reported that integrity of lysosomal membrane is adversely

affected by Hg^{2+} ions, resulting in subsequent release of acid hydrolytic contents into the cytoplasm (Verity and Reith, 1967; Lauwerys and Buchet, 1972). A similar association between lysosomal system and heavy metals has also been reported in marine lamellibranchs, especially in M.edulis (Moore, 1977; Lowe and Moore, 1979b; George et al., 1982). Roesijadi (1980) observed increased activity of acid phosphatase in the gills of the clam P.staminea at sub-lethal exposure to copper. In lower concentrations of copper, Pickwell and Steinert (1984) found that haemolymph lysozyme levels in M.edulis did not differ significantly from the control values. In their experiments with A.granosa, Chandy and Patel (1985) observed that Hg, Se and glutathione, either alone or in combination had no significant effect on acid phosphatase activity, in vivo, but in vitro inhibition of arylsulphatase and acid phosphatase depending upon the concentration of mercury used and inhibition of acid phosphatse activity by selenium were also observed. They concluded that clams exposed to these, individually or in combination showed different effects on lysosomal enzyme activity in vivo and in vitro. It is also known that heavy metals inhibit the activities of several enzymes (Dixon and Webb, 1967). Yoshino et al. (1966) have shown that Hg^{2+} inhibits enzymes of Kreb's cycle. Have (1969), Sporn et al. (1970), Vallee and Wacker (1970) have found that cadmium influences the rate of action of certain enzymes by activation, inactivation, uncoupling reactions or mechanisms yet to be defined. Webb (1966) and Jakim et al. (1970) reported that heavy metals usually inhibit enzymatic and metabolic process in vitro. Jackim (1974) who studied the influence of cadmium, copper, lead and silver on the activities of xanthine oxidase, acid and alkaline phosphatases, catalase, Na^+ and K^+ ATPase, and

Mg^{2+} ATPase in vivo and in vitro showed that there are no consistent relationships between in vitro effects of the metal on enzyme (which are usually inhibitory) and the effect of exposing the whole animal to the same metal. This report clearly shows that the effect of heavy metal ions is not a direct effect alone. It acts on the membrane system of the cell also, and the observed effect of enzyme activity when the whole animal is exposed to a metal is the result of a combination of the above two or more reactions of heavy metal ions with the various cytosystems. In M.edulis, concentration dependent labilisation of lysosomes was observed on exposure to copper (Harrison and Berger, 1982); no significant reduction in lysosomal integrity when copper concentration was low, but significant reduction in lysosomal latency when the concentration was the highest.

In general, the higher the heavy metal ions, the lesser will be the synthesis, storage, and release of the enzymes, and alteration in membrane permeability and/or membrane destabilisation caused by metal ions in higher concentrations will result in lesser enzyme turn over-ie., lesser synthesis, storage, and release which will reflect as lesser enzyme activity in the haemolymph.

In S.scripta injected with Vibrio sp. the acid phosphatase activity was significantly higher from 3 hrs upto 24 hrs whereas in V.cyprinoides var. cochinensis it was from 3 hrs upto 12 hrs. This is interpreted as the release of this hydrolytic enzyme from the lysosomes in response to a biotic challenge. Studies by Cheng (1975), Cheng et al. (1975) and Foley and Cheng (1977) on M.mercenaria; Cheng and Rodrick (1974) and Cheng and Yoshino (1976a) on Mya arenaria; and Cheng and Yoshino (1976b), Cheng et al. (1977; 1978a,b)

Cheng and Butler (1979) and Cheng and Mohandas (1985) on B.glabrata have demonstrated that challenge with heat-killed and/or live B.megaterium results in the hypersynthesis of lysosomal enzymes within haemocytes and subsequent release of these hydrolases into serum. It may be noted that intense staining for acid phosphatase activity was observed in the haemocytes of S.scripta and V.cyprinoides var. cochinensis, 2 hr post-challenge with Vibrio sp., in vitro. That the lysosomal enzymes released from the granulocytes into the serum act on bacterial cell walls, causing their degradation is evident from the work of Cheng (1975; 1977a,b) and Mohandas et al. (1985). From these contributions, it appears that the partial degradation of bacterial cell wall, at least of certain species, may be a pre-requisite for endocytosis of bacteria by granulocytes. It may also be noted that the release of lysosomal enzymes, into serum subsequent to bacterial challenge is through degranulation (Cheng, 1975; Cheng et al., 1975; Foley and Cheng, 1977; Mohandas et al., 1985) and is not due to the disruption of cell membrane (Cheng et al., 1975; Mohandas et al., 1985; Mohandas and Cheng, 1985b). It may also be noted that the degranulation is not restricted to actively phagocytosing cells, but in enhanced during phagocytosis (Foley, 1974; Cheng, 1975; Foley and Cheng, 1977).

This investigation, as well as that of Jyodhirmyi and Rao (1987), reveal that just as in vertebrates, changes in the haemolymph enzyme activity profiles as a result of exposure to pollutants, parasitism or challenge with bacteria, can be of significant diagnostic value.

The observed increase in enzyme activity at 3 hrs in the 2% saline-injected calms of both the species, may be due to the tissue trauma. Cheng et al. (1977) in B.glabrata found an elevated lysozyme-like activity both in water and bacteria injected clams.

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CHAPTER VI

ALKALINE PHOSPHATASE ACTIVITY IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

As described in chapter V, the lysosomal membrane is often the target of injury by xenobiotics. Injury resulting in destabilisation of the lysosomal membrane bears a quantitative relationship to the magnitude of the stress response (Bayne et al., 1979a; 1982), and this presumably contributes to the intensity of catabolic or degradative effects, as well as to the level of pathological change that results. Literature concerning this has been briefly reviewed in chapter V.

Apart from the lysosomal detoxication systems, many organic xenobiotics are metabolised by the microsomal (smooth endoplasmic reticulum) cytochrome P-450 dependent monooxygenases. Some of these toxic chemicals can stimulate the proliferation of the smooth endoplasmic reticulum and/or its associated monooxygenases, and cytochrome P-450 reductase (Conney and Burns, 1972).

Though no detoxication system has been implicated for the plasma membrane, its behaviour towards a xenobiotic insult is of interest. Plasma membrane which forms the bounding membrane will be the first membrane to confront a xenobiotic insult. Since it has been reported that destabilisation of the lysosomal membrane as a result of injury by xenobiotics, bears a quantitative relationship to the magnitude of the stress response (Bayne et al., 1979a; 1982; and others), it is of interest to study whether the same destabilisation occurs in the plasma membrane. For this alkaline phosphatase, which is considered as a marker enzyme for the plasma membrane (Bogitsh, 1974), is selected, and its activity followed during the stress imposed by abiotic (copper) and biotic (bacteria) agents for a period of 120 hrs. A large number

of metalloenzymes, and metal ion-activated enzymes are now known. A variety of metals, including copper and zinc, are involved structurally in the formation of metalloenzymes in molluscs; and alkaline phosphatase is one among these enzymes (George and Coombs, 1975).

Alkaline phosphatase also catalyses the hydrolytic cleavage of phosphoric acid esters with pH optimum in the alkaline range. In bivalves, Narain (1973) states that there are indications that the phosphatase activity of the amoebocytes is somehow involved during the process of transfer and deposit of shell material.

MATERIALS AND METHODS

Methods of collection of clams, rearing the clams, acclimatisation, dosing the clams with copper, challenging the clams with bacteria and the statistical analysis of the data were the same as described in detail in chapter II. The concentration of the copper ions, the dosage of the bacterial suspension used, and the salinity used for the two clam species were also the same as described before.

In the case of S.scripta, 12 clams from each size group, and in the case of V.cyprinoides var. cochinensis, 13 clams from each size group were employed for studying the haemolymph alkaline phosphatase activity in the different size groups selected.

For studying the haemolymph alkaline phosphatase activity in the two clam species during stress induced by copper ions, the experimental setup was the same as described in chapter V for acid phosphatase.

For studying the alkaline phosphatase activity in the haemolymph of S.scripta and V.cyprinoides var. cochinensis, subsequent to challenge with

bacteria, the same experimental setup as described in chapter V was used.

In this experiment also, as described earlier, more clams than the required number were reared as experimentals and controls anticipating mortality during the course of the experiment.

ESTIMATION OF ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase activity in the haemolymph was determined following the methodology of Sigma technical bulletin (No: 104) with some modifications. To study the enzyme activity, 0.05M glycine-NaOH buffer of pH 9.6 was used. The incubation temperature was 37 $\pm$0.1° C.

To 1 ml of the frozen buffer containing 100 mM of NaCl and 0.1 mg of $MgCl_2$, 0.2 ml of haemolymph was added using a 0.2 ml pipette, and immediately frozen till analysed. At the time of analysis, the buffer-enzyme mixture was kept in a water-bath at 37° C. When the temperature of the buffer-enzyme mixture came to 37° C, 0.1 ml substrate (2 mg of p-nitrophenyl phosphate sodium salt (Merck) in 0.1 ml) was added using a 0.1 ml pipette to start the reaction. After incubation for one hour, the reaction was stopped by adding 10 ml of 0.05N NaOH. The yellow colour was read at 410 nm. The concentration of p-nitrophenol was found out from a standard graph of p-nitrophenol. Protein content of 0.2 ml haemolymph was found out simultaneously and activity was expressed as µmoles of p-nitrophenol liberated/ mg protein/hour.

RESULT

ALKALINE PHOSPHATASE ACTIVITY IN THE HAEMOLYMPH OF THE FOUR SIZE GROUPS OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS

Table 17A gives the mean value, standard deviation, and range of haemolymph alkaline phosphatase activity in the four size groups of S.scripta, and Table 17B shows the same in the four size groups of V.cyprinoides var. cochinensis. In both these cases when the mean values of alkaline phosphatase activity for different size groups were compared, there was no statistically significant difference.

ALKALINE PHOSPHATASE ACTIVITY IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS, DOSED WITH THREE CONCENTRATIONS OF COPPER

Haemolymph alkaline phosphatase activity values in S.scripta and V.cyprinoides var. cochinensis when exposed to three sub-lethal concentrations of copper for five days are given in Tables 18A and 18B, and Figures 11A and 11B, respectively.

In S.scripta, when the values of alkaline phosphatase activity of 1 ppm copper-dosed clams were compared with the mean values of the controls, the mean values of 1 ppm copper-dosed clams were found to be significantly higher at 24 hrs ($P < 0.02$), 72 hrs ($P < 0.01$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). The 3 ppm copper-dosed clams showed significantly higher values than the controls at 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$); and 5 ppm copper-dosed clams showed significantly higher values than the controls at 24 hrs ($P < 0.01$), 72 hrs ($P < 0.05$), 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). When the mean

Table 18A. Haemolymph Alkaline Phosphatase Activity (μmol/mg protein/hr) in Sunetta scripta dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	0.0814	0.0918	0.0947	0.0719	0.0901
	± SD	0.0136	0.0269	0.0291	0.0206	0.0287
	Range	0.0612-0.1168	0.0603-0.1355	0.0472-0.1384	0.0466-0.1125	0.0640-0.1243
1 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.1178	0.1137	0.1536	0.1203	0.1810
	± SD	0.0381	0.0261	0.0526	0.0201	0.0618
	Range	0.0633-0.1962	0.0714-0.1589	0.0909-0.2619	0.0848-0.1477	0.1182-0.3225
3 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.1014	0.1193	0.1125	0.1203	0.1439
	± SD	0.0432	0.0474	0.0234	0.0212	0.0343
	Range	0.0527-0.1788	0.0669-0.2346	0.0750-0.1584	0.0864-0.1607	0.1083-0.2273
5 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.1462	0.1000	0.1386	0.1203	0.1696
	+ SD	0.0284	0.0357	0.0444	0.0244	0.0406
	Range	0.1139-0.1865	0.0563-0.1792	0.0719-0.2350	0.0759-0.1685	0.1160-0.2435

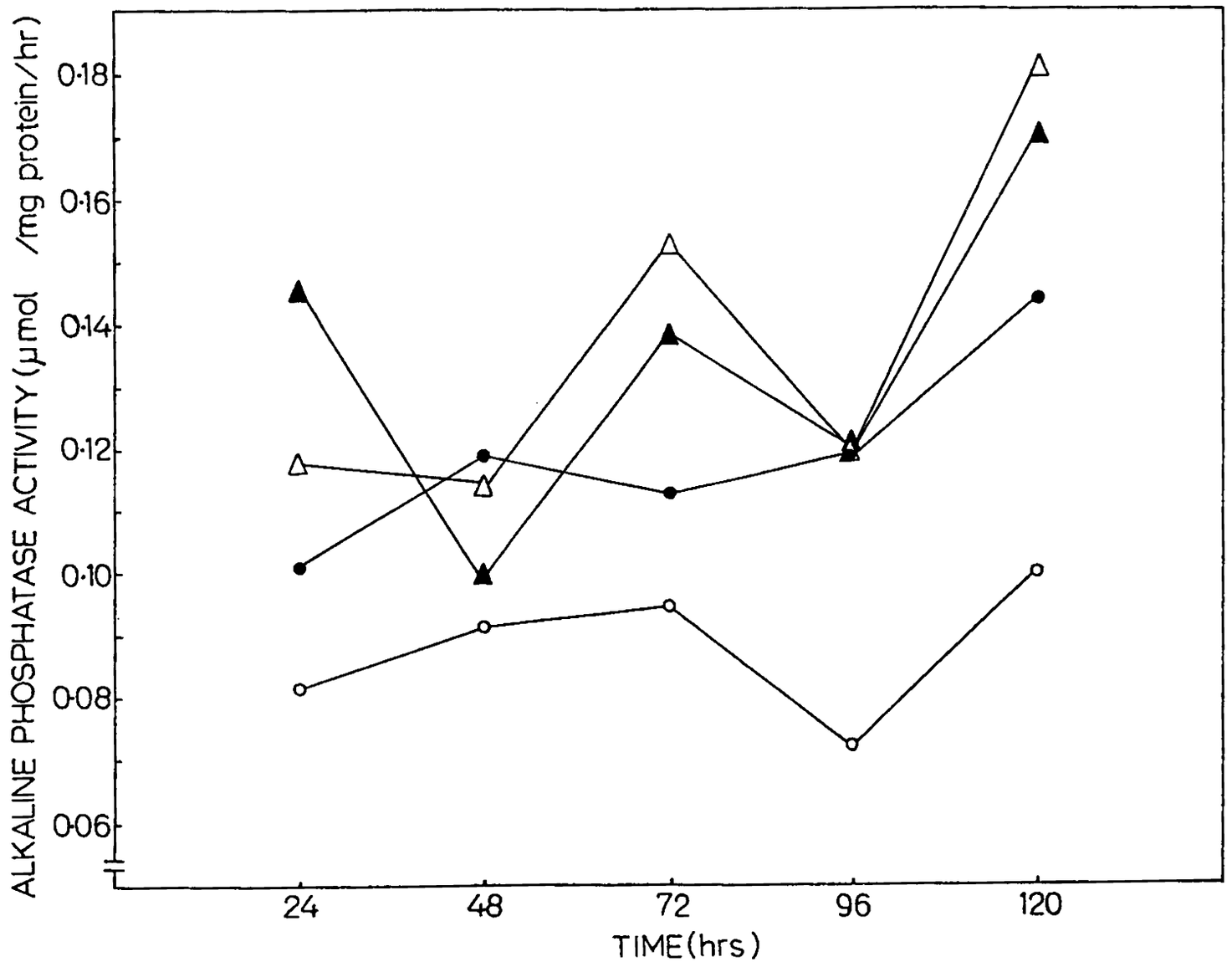

Fig. 11A. Haemolymph Alkaline Phosphatase activity (µmol/mg protein/hr) in Sunetta scripta dosed with three concentrations of copper. Control (o); 1 ppm copper (Δ); 3 ppm copper (•);

Table 18B. Haemolymph Alkaline Phosphatase Activity (µmol/mg protein/hr) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper

	Hours	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	0.1276	0.1067	0.1024	0.1246	0.0989
	± SD	0.0490	0.0217	0.0253	0.0221	0.0317
	Range	0.0643-0.2424	0.0782-0.1631	0.0738-0.1434	0.0802-0.1582	0.0579-0.1625
0.15ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.1571	0.1354	0.1216	0.1115	0.1357
	± SD	0.0482	0.0447	0.0340	0.0253	0.0431
	Range	0.0944-0.2396	0.0787-0.2024	0.0667-0.1842	0.0728-0.1526	0.0714-0.2180
0.30ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.1370	0.1313	0.1126	0.1461	0.1019
	± SD	0.0434	0.0376	0.0411	0.0272	0.0208
	Range	0.0758-0.2013	0.0679-0.1750	0.0694-0.1905	0.1064-0.2095	0.0667-0.1172
0.45ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	0.1618	0.1149	0.0908	0.1182	0.0931
	± SD	0.0305	0.0421	0.0155	0.0250	0.0081
	Range	0.1267-0.2258	0.0651-0.1910	0.0651-0.1127	0.0878-0.1502	0.0798-0.1022

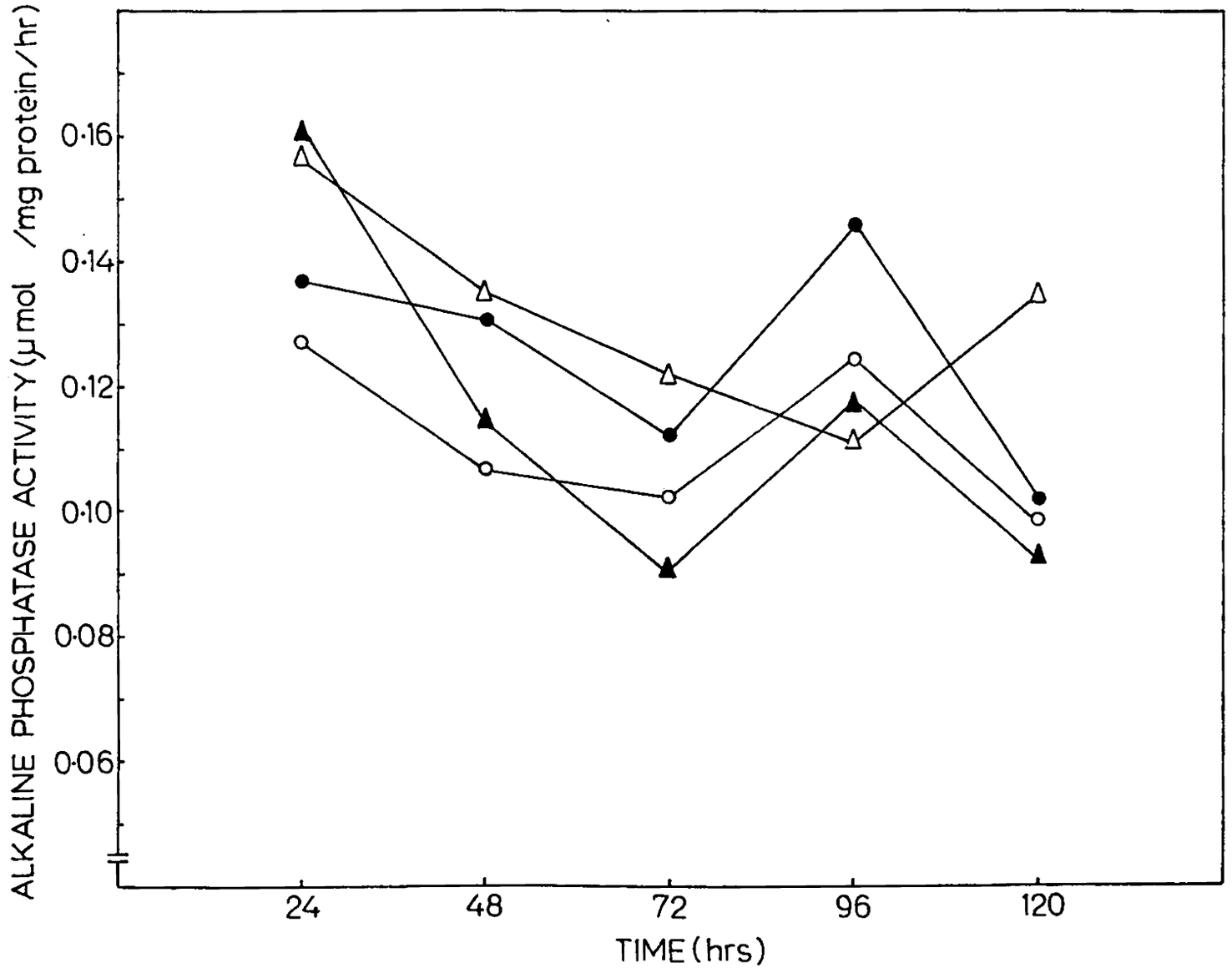

Fig. 11B. Haemolymph Alkaline Phosphatase activity (µmol/mg protein/hr) in Villorita cyprinoides var. cochinensis dosed with three concentrations of copper. Control (o); 0.15 ppm copper (Δ); 0.30 ppm

values of 1 ppm and 3 ppm copper-dosed clams were compared, 1 ppm copper-dosed clams showed significantly higher values at 72 hrs ($P < 0.05$), and 120 hrs ($P < 0.01$). The mean values of 1 ppm copper-dosed clams did not show any significant difference when compared with the values of 5 ppm copper-dosed clams. When the mean values of 3 ppm copper-dosed clams were compared with the values of 5 ppm copper-dosed clams, 5 ppm copper-dosed clams showed significantly higher values at 24 hrs ($P < 0.02$), and 120 hrs ($P < 0.02$).

In V.cyprinoides var. cochinensis, the mean values of 0.15 ppm, 0.30 ppm and 0.45 ppm copper-dosed clams showed no significant difference from the mean values of the controls, during the entire experimental period. The mean values of 0.15 ppm copper-dosed clams were significantly lower at 96 hrs ($P < 0.02$) but significantly higher at 120 hrs ($P < 0.05$) when compared with the mean values of 0.30 ppm copper-dosed clams. The mean values of 0.45 ppm copper-dosed clams were significantly lower than the values of 0.15 ppm copper-dosed clams at 72 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$). Similarly, the mean values of 0.45 ppm copper-dosed clams were significantly lower than the values of 0.30 ppm copper-dosed at 96 hrs ($P < 0.05$).

ALKALINE PHOSPHATASE ACTIVITY IN THE HAEMOLYMPH OF SUNETTA SCRIPTA AND VILLORITA CYPRINOIDES VAR. COCHINENSIS, WHEN INJECTED WITH VIBRIO SP.

Haemolymph alkaline phosphatase activity values in S.scripta, and V.cyprinoides var. cochinensis when challenged with bacteria are given in Tables 19A and 19B, and Figures 12A and 12B respectively.

In S.scripta, the mean values of the 2% saline-injected clams were

Table 19A. Haemolymph Alkaline Phosphatase Activity (µmol/mg protein/hr) in Sunetta scripta injected with 1 x 10^8 cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	10	10	10	10	10	10	10	10
Untampered control	Mean value	0.0596	0.0546	0.0551	0.0568	0.0532	0.0470	0.0475	0.0554
	± SD	0.0143	0.0142	0.0202	0.0211	0.0184	0.0124	0.0164	0.0201
	Range	0.0312-0.0700	0.0332-0.0762	0.0278-0.0851	0.0347-0.0963	0.0277-0.0951	0.0297-0.0769	0.0292-0.0801	0.0342-0.0950
	N	10	10	10	10	10	10	10	10
2% Saline injected	Mean value	0.0703	0.0603	0.0474	0.0617	0.1048	0.0435	0.0537	0.0543
	± SD	0.0263	0.0260	0.0152	0.0200	0.0295	0.0120	0.0200	0.0158
	Range	0.0388-0.1288	0.0333-0.1280	0.0336-0.0902	0.0393-0.0952	0.0663-0.1603	0.0276-0.0690	0.0285-0.0878	0.0347-0.0776
	N	10	10	10	10	10	10	10	10
Vibrio sp injected	Mean value	0.0682	0.0668	0.0408	0.0685	0.0708	0.0933	0.0687	0.0660
	± SD	0.0169	0.0497	0.0122	0.0191	0.0216	0.0270	0.0216	0.0225
	Range	0.0421-0.1095	0.0261-0.2083	0.0250-0.0622	0.0378-0.1048	0.0408-0.1138	0.0503-0.1381	0.0466-0.1149	0.0408-0.1027

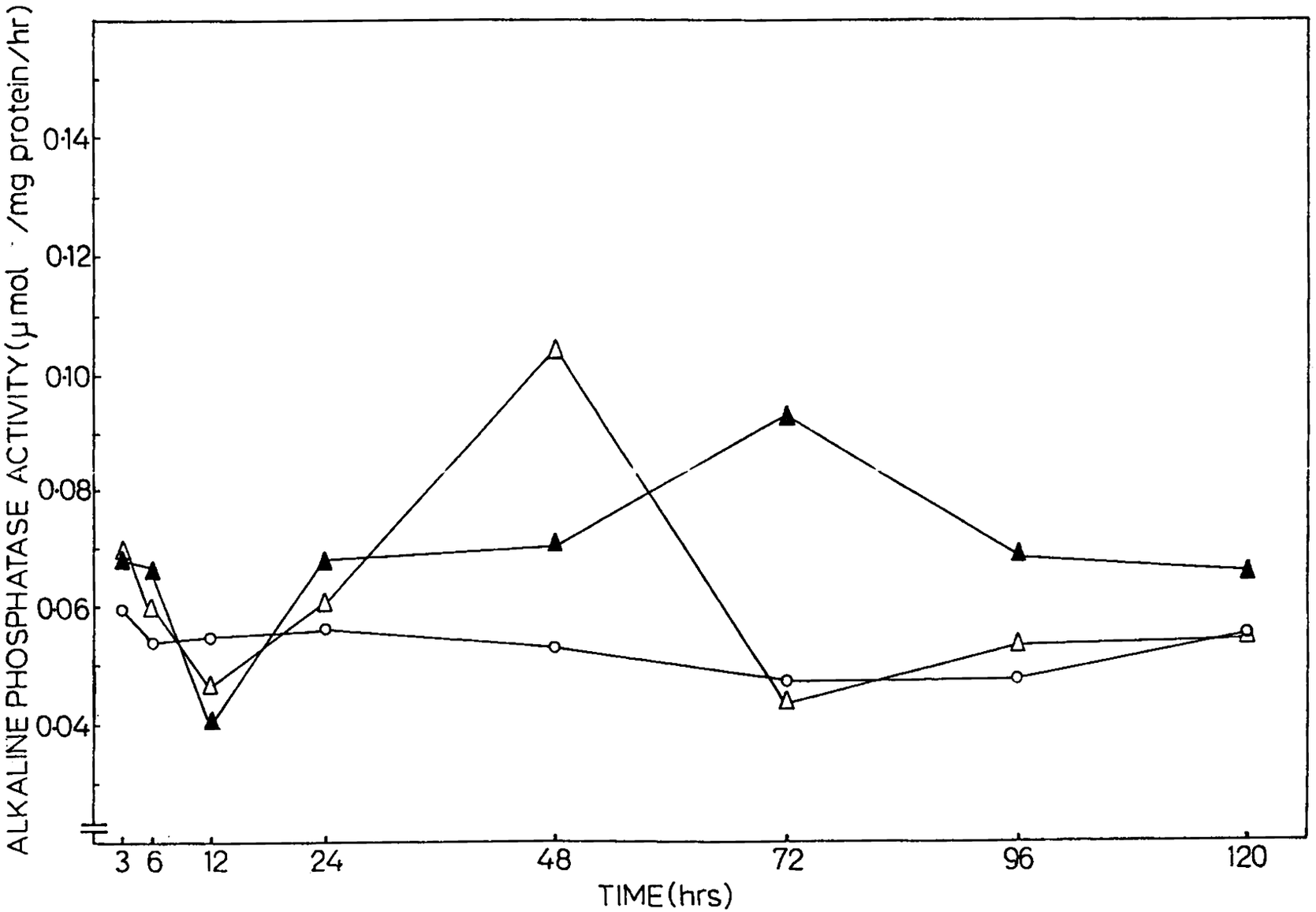

Fig 12A. Haemolymph Alkaline Phosphatase activity (µmol/mg protein/hr) in Sunetta scripta injected with $1x10^8$ cells/0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

Table 19B. Haemolymph Alkaline Phosphatase Activity (μmol/mg protein/hr) in Villorita cyprinoides var. cochinensis injected with $1x10^8$ cells/0.02 ml of Vibrio sp.

	Hours	3 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
	N	10	10	10	10	10	10	10	10
Untampered control	Mean value	0.0714	0.0724	0.0800	0.0741	0.0677	0.0528	0.0631	0.0546
	± SD	0.0204	0.0226	0.0379	0.0296	0.0299	0.0198	0.0128	0.0187
	Range	0.0505-0.1089	0.0500-0.1150	0.0519-0.1537	0.0496-0.1226	0.0214-0.1154	0.0348-0.1074	0.0385-0.0813	0.0367-0.0894
	N	10	10	10	10	10	10	10	10
2% Saline injected	Mean value	0.0951	0.0879	0.1015	0.0652	0.0759	0.0729	0.0524	0.0538
	± SD	0.0300	0.0465	0.0291	0.0324	0.0314	0.0165	0.0119	0.0145
	Range	0.0640-0.1577	0.0318-0.1648	0.0542-0.1449	0.0312-0.1339	0.0357-0.1500	0.0535-0.1143	0.0354-0.0777	0.0345-0.0868
	N	10	10	10	10	10	10	10	10
Vibrio sp injected	Mean value	0.1015	0.0864	0.0868	0.0538	0.0672	0.0748	0.0784	0.0769
	± SD	0.0379	0.0315	0.0234	0.0334	0.0332	0.0238	0.0147	0.0175
	Range	0.0511-0.1950	0.0406-0.1373	0.0539-0.1361	0.0132-0.1375	0.0340-0.1524	0.0492-0.1361	0.0581-0.1038	0.0464-0.1064

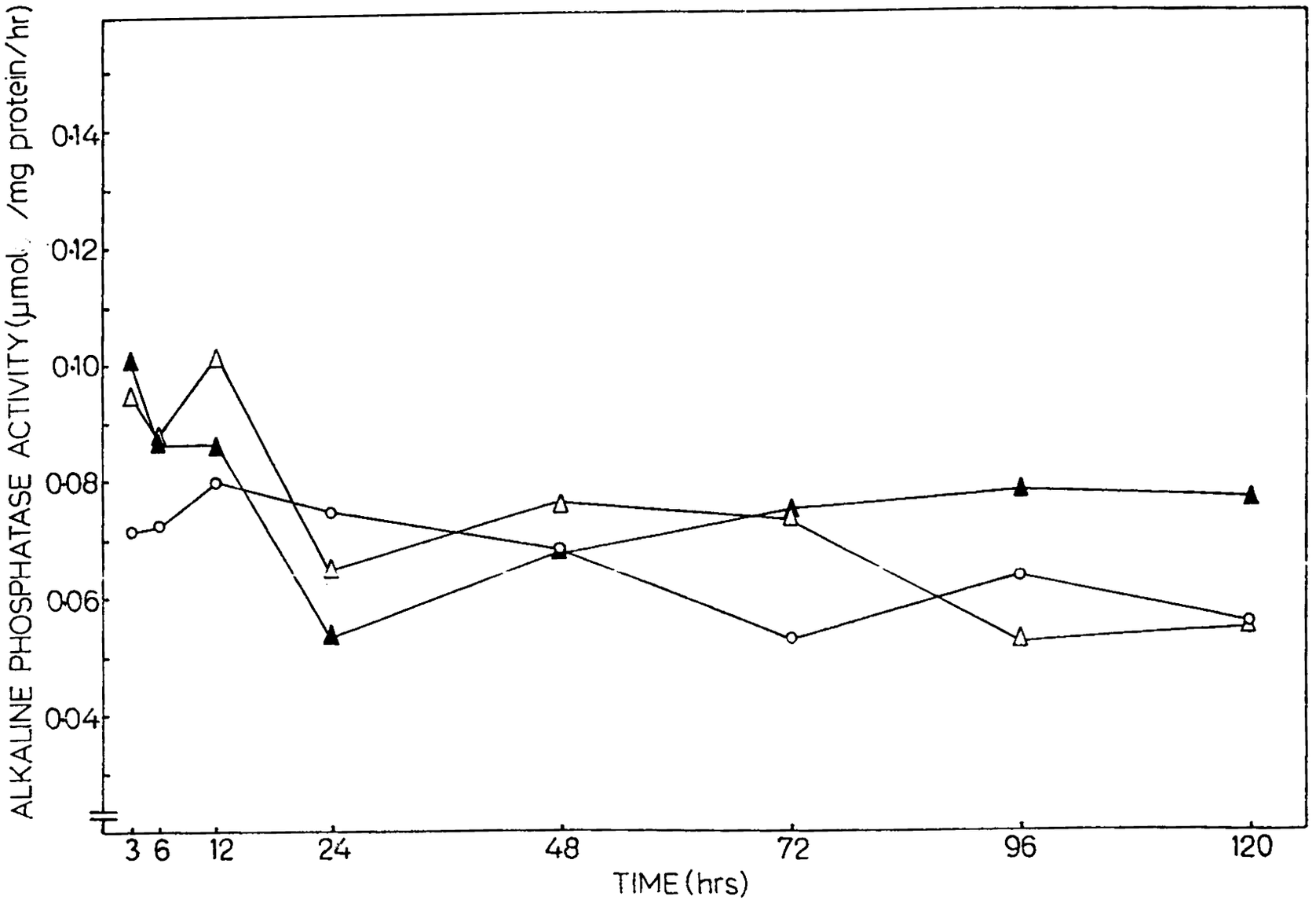

Fig. 12B. Haemolymph Alkaline Phosphatase activity (µmol/mg protein/hr) in Villorita cyprinoides var. cochinensis injected with 1x10 cells/0.02 ml of Vibrio sp. Untampered control (o); Saline-injected (Δ); Vibrio-injected (▲).

significantly higher at 48 hrs ($P < 0.01$) than the values of the untampered controls. The values of Vibrio-injected clams were significantly higher than the control values at 72 hrs ($P < 0.01$), and 96 hrs ($P < 0.05$). When the mean values of the Vibrio-injected clams were compared with the values of 2% saline-injected clams, the Vibrio-injected clams showed significantly lower values at 48 hrs ($P < 0.05$) but significantly higher values at 72 hrs ($P < 0.02$).

In V.cyprinoides var. cochinensis, the mean value of 2% saline-injected clams showed significantly higher values than the controls at 72 hrs ($P < 0.05$), whereas the Vibrio-injected clams showed significantly higher values at 72 hrs ($P < 0.05$), 96 hrs ($P < 0.05$), and 120 hrs ($P < 0.02$). When the mean values of 2% saline--injected and Vibrio-injected clams were conpared, the Vibrio-injected clams showed significantly higher values at 96 hrs ($P < 0.01$), and 120 hrs ($P < 0.01$).

DISCUSSION

In this study also, the mean values of haemolymph alkaline phosphatase activity in the two clam species did not show any significant difference among the different size groups. This shows that normally, the alkaline phosphatase activity will not vary significantly among the size groups.

In copper-dosed experiments, in S.scripta, 1 ppm copper-dosed clams showed significantly higher alkaline phosphatase activity at 24, 72, 96 and 120 hrs, whereas acid phosphatase activity (in the previous experiment) showed significant increase only at 24 and 48 hrs, than the controls. Clams dosed with 3 ppm copper showed significantly higher alkaline phosphatase activity at 96 hrs and 120 hrs, whereas acid phosphatase activity was significantly lower at 120 hrs

on comparing with the controls. When the values of the 5 ppm copper-dosed clams were compared with the control values, alkaline phosphatase activity was significantly higher at 24, 72, 96 and 120 hrs, whereas acid phosphatase activity was significantly higher at 48 hrs but significantly lower at 96 and 120 hrs.

In V.cyprinoides var. cochinensis, dosed with copper, 0.15 ppm copper-dosed clams showed no significant difference in the alkaline phosphatase activity during the entire experimental period when compared with the controls, whereas significantly higher acid phosphatase activity values were shown by the 0.15 ppm copper-dosed clams at 24, 48, and 96 hrs. Clams dosed with 0.30 ppm copper also did not show any significant difference in alkaline phosphatase activity whereas acid phosphatase showed a significant decrease in activity at 120 hrs. When values of the 0.45 ppm copper-dosed clams were compared with the control values, alkaline phosphatase activity showed no significant variation, whereas acid phosphatase activity showed significantly lower values at 48, 72 and 120 hrs.

From a comparison of alkaline and acid phosphatase activity patterns in the same time periods in S.scripta and V.cyprinoides var. cochinensis, it is clear that the two enzymes need not be released simultaneously into the haemolymph compartment. This study gives an insight into the efficient mechanism of compartmentalization of enzymes in different membrane systems.

Since, in clams dosed with different concentrations of copper, the values of alkaline phosphatase activity showed either non-significant variation (as in V.cyprinoides var. cochinensis), or were significantly higher (as in S.scripta), it is apparent that only destabilisation of the plasma membrane has happened,

resulting in the release of the enzyme but synthesis was not affected. But in the case of acid phosphatase activity, the destabilisation of lysosomal membrane had taken place in the early time-periods, and in later time-periods, and also in higher concentrations of copper, the synthesis of the enzyme was also affected. Obvious from the data are the information that (1) the metal ion has different effects on different enzymes, and (2) the release pattern of the enzymes need not coincide. Although Cheng and Rodrick (1975), and Huffman and Tripp (1982) have treated alkaline phosphatase as one of the lysosomal enzymes, the data from the present study do not give enough supporting evidence to share this view mainly because lysosomal membrane destabilisation has not resulted in simultaneous release of both the enzymes. However, considering the fact that (1) lysosomes even in a single cell type are quite variable in their enzymatic constitution (Dean, 1977), (2) there are different sub-populations of haemocytes- molecularly and antigenically distinct- with some degree of functional compartmentalization (Yoshino and Granath, 1985), and (3) different sub-populations may show different lysosomal enzyme activities, and the particular challenging agent may evoke the same or different stimulus in the different sub-populations resulting in the release of one or several types of lysosomal enzymes (Mohandas, 1985), it is possible that the observed difference in the release pattern of the two enzymes, may be due to the non-synchronised chemical cycle within the cell, as suggested by Yoshino and Cheng (1976a).

In S.scripta, the Vibrio-injected clams showed significant higher activity than the controls at 72 and 96 hrs, whereas significantly higher acid phosphatase activity was seen at 3, 6, 12 and 24 hrs. The saline-injected clams showed

significantly higher activity at 48 hrs than the controls, but the acid phosphatase activity of saline-injected clams was significantly higher at 3 hrs.

In V.cyprinoides var. cochinensis, the Vibrio-injected clams showed significantly higher activity than the controls at 72, 96, and 120 hrs, whereas significantly higher acid phosphatase activity was shown at 3, 6 and 12 hrs. The saline-injected clams showed significantly higher alkaline phosphatase activity than the controls at 72 hrs whereas the acid phosphatase activity showed significantly higher values in saline-injected clams at 3 hrs.

In S.scripta and V.cyprinoides var. cochinensis, challenged with bacteria, the increase in alkaline phosphatase activity does not coincide with the increase in acid phosphatase activity. Acid phosphatase is a lysosomal hydrolysing enzyme, and discharge of lysosomal hydrolases has been implicated in internal defence (Cheng, 1975), whereas alkaline phosphatase activity has been implicated in the deposit of shell material (Narain, 1973). The function they perform in the body is different, and hence the observed non-coincidence in their activity.

The role of haemocyte plasma membrane in the uptake of bacteria is well documented (Mohandas, 1985). It is also known that the bacteria that are endocytosed have altered body structure caused by the action of lysosomal enzymes, including acid phosphatases (Cheng, 1977b, 1978; Mohandas, 1985). Since the uptake of bacteria takes place subsequent to the alteration of bacterial cell wall, the higher activity of acid phosphatase in the early time periods to effect alteration of bacterial cell wall; and the higher activity of alkaline phosphatase in the later time periods, probably right at the time of uptake of bacteria involving haemocyte plasma membrane, may be correlated.

During saline and bacterial injections, when the shell is opened for inserting the needle some damage to the shell occurs in both the clam species. Since V.cyprinoides var. cochinensis has a thin shell, the damage is more in this species. It was observed that, by about 72 hrs onwards, the shell margin at the site of damage was slowly being covered by a leathery membrane. The significant increase in alkaline phosphatase activity also starts at that time period. So, the increase in alkaline phosphatase activity is considered also as a response towards shell repair. Alkaline phosphatase activity has been implicated in the deposit of shell meterial (Narain, 1973). In the shell regeneration process only the contribution of alkaline phosphatase is probable because shell deposition is possible only in the alkaline pH. This view is supported by the fact that calcium from the shell is taken to buffer the lowering of pH during anoxia or hypoxia (Crenshaw and Neff, 1969; Richardson et al., 1981; Wilbur and Saleuddin, 1983). The fact that saline-injected clams of the two species showed significantly higher alkaline phosphatase activity at 48 and 72 hrs, and the Vibrio-injected ones from 72 hrs onwards indicates that both the explanations may be true and valid.

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CHAPTER VII

LACTIC ACID IN THE HAEMOLYMPH OF SUNETTA SCRIPTA

It is well known that heavy metal ions when present above threshold concentrations in marine ecosystems act as pollutants to organisms. In pollution bioassay studies, estimation of oxygen consumption is a routine practice to assess the metabolic rates of the test animals. But the lowest estimated rate based on oxygen uptake does not necessarily apply to situations in which metabolism is partly or totally anaerobic (de Zwaan, 1983). Bivalves are known for their ability to engage in anaerobic metabolism during stress. Therefore, estimation of metabolic rates in these invertebrates during total or partial anaerobiosis must also involve the biochemical analysis of end-products together with the determination of oxygen uptake. By identifying the end-products accumulated during glycolytic fermentation, the ATP equivalent can be derived for each identified end-product from the reaction sequences of glycolytic fermentation. By adding to the catabolic ATP production the net utilisation of energy reserves from the phosphagen and ATP pools, the total ATP consumption rate can be obtained (de Zwaan, 1983). This indirect method has been applied to assess the metabolic rate in a variety of bivalves (de Zwaan and Wijsman, 1976; Ebberink et al., 1979; de Zwaan et al., 1980; Gäde, 1980; Meinardus and Gäde, 1981; Livingstone, 1982; and others).

Switching over to anaerobic metabolism under anoxic and hypoxic conditions does not necessarily mean that aerobic metabolism does not occur at reduced oxygen tension. Livingstone and Bayne (1974, 1977), de Zwaan and de Bont (1975), Mangum and Burnett (1975), among others, have recorded the operation of both the pathways in bivalves at reduced oxygen tensions. In all animals where anoxia leads to an activation of the phosphoenolpyruvate carboxykinase

pathway resulting in the production of succinate or a derivative, other routes, employed either to maintain redox balance or to gain ATP by substrate level phosphorylations, may lead to the accumulation of a variety of other intermediates (Kluytmans et al., 1980).

Investigations on anaerobic metabolism in molluscs have revealed increasing complexity and flexibility regarding the number of end-products formed. In bivalves, besides the classical end-products, such as succinate, lactate, octopine, and alanine, volatile fatty acids and N-carboxyethyl alanine have also been detected (Kluytmans et al., 1975, 1978; Collicutt and Hochachka, 1977).

Contributions from India relative to the effects of trace metal pollutants on bivalves have dealt, mostly, with acute toxicity and uptake kinetics. Very little information is available on metabolic end-products in specific organs under prevailing anoxic and/or hypoxic conditions subsequent to complete or partial valve closure as a result of exposure to pollutants. However, Lakshmanan and Nambisan (1985) have reported that in whole tissues of the bivalves Perna viridis and Villorita cyprinoides var. cochinensis the lactic acid levels increased but glycogen levels decreased with increasing lethal concentrations of Cu^{2+} and Hg^{2+} ions over a period of 2, 4 and 24 hrs.

Bivalves have an open circulatory system and the body tissues are continuously bathed in haemolymph. Since haemocytes and haemolmph are involved, among other functions, in nutrient digestion, transport, and excretion, end-products formed in one specific organ can be removed from the production site to other organs for storage or other cyclical activities. Thompson et al. (1978) have stated that haemolymph functions as a "sink" into which the tissues deposit ammonia and probably other metabolic end-products after a

period of anaerobiosis. Moreover, an analysis of end-products may provide initial clues as to the type of metabolism that occurs while under environmental stress. With these views in mind, and as a prelude to detailed studies on end-product accumulation in specific organs of bivalves under anoxic/hypoxic conditions, an experiment was designed to determine the levels of lactic acid in haemolymph of the marine clam Sunetta scripta exposed to two sub-lethal concentrations of Cu^{2+} and Hg^{2+}. The sub-lethal concentrations were determined as a result of pervious bioassays.

MATERIALS AND METHODS

Methods of collection of clams, rearing the clams, acclimatisation, mode of collection of haemolymph, dosing the clams with copper and the statistical analysis of the data were the same as described in detail in chapter II. But the concentration of copper employed was different. In addition to copper, mercury was also used in this study. Clams of the size group 35-40 mm were selected for the experiment.

To study the haemolymph lactic acid levels, 60 clams were transferred to each of the four 15-litre tanks. The four tanks were filled with filtered seawater and dosed with 1 and 3 ppm of copper, and 1 and 3 ppm of mercury ions, respectively. These four batches of clams constituted the experimental group. The 60 clams changed to the 15-litre tank filled with filtered seawater served as controls. The water was changed daily and the metal ion concentrations in the experimental tanks were maintained at their respective levels throughout the experimental period of 5 days. Feeding was stopped during the experimental period, and particular care was taken to ensure that in none of the tanks was crowding, oxygen, temperature and/or salinity acting

as limiting factors.

At every 24 hrs for 5 days, 1.0 to 1.5 ml of haemolymph was withdrawn from the adductor muscle of each of the 10 clams from the experimental group, and expelled into clean 5 ml centrifuge tubes and used within 2 min. Collection of haemolymph samples from clams comprising the control group was identically carried out.

ESTIMATION OF LACTIC ACID

The quantitative determination of lactic acid in the haemolymph, was done following the method of Barker (1957). A 0.5 ml sample of haemolymph from each clam of the control and experimental groups was discharged into 5-ml centrifuge tubes containing 1 ml of 5% TCA within 2 min of collection. The supernatant was decanded and 1 ml of this supernatant was taken into a centrifuge tube. To this, 1 ml of 20% $CuSO_4$ solution and 3 ml of distilled water were added and shaken well. Approximately 1 gm of powdered $Ca(OH)_2$ was added, and shaken well. The mixture was allowed to stand at room temperature for 30 min, with occassional shaking, and was then centrifuged. Duplicate aliquots of 1.0 ml of the supernatant fluid were carefully withdrawn from beneath any surface particles and the outside of the pipette wiped clean with a fresh filter paper and was transferred to a 20-ml test tube, 0.05 ml of 4% $CuSO_4$ solution was added, and the tube was chilled in an ice and water bath. Exactly 6.0 ml of concentrated H_2SO_4 was added slowly from a pipette and at the same time the contents of the tubes were mixed. The rack of tubes was then placed in a boiling water bath for 5 min, removed, and cooled to below 20° C. Then 2 drops of the p-hydroxy diphenyl solution were added from a pipette known to deliver 0.05 ml of this solution per drop.

The precipitated reagent was dispenced throughout the acid as quickly and uniformly as possible. The tubes were then placed in a beaker of water at 30° C and allowed to stand for at least 30 min. The precipitated reagent was redispersed by shaking at least once during the incubation period. Excess reagent was dissolved by heating the tubes in boiling water for 90 sec., then cooled them in cold water. The developed colour was read against the reagent blank at 560 nm. The average of the duplicate reading was used to calculate the lactate content of the aliquot by referring to a calibration curve prepared by using lithium lactate.

RESULT

Haemolymph lactic acid levels in S.scripta dosed with two sub-lethal concentrations of Hg^{2+} and Cu^{2+} are given in Table 20 A and Figure 13 A.

It was observed that the clams of the experimental group closed their valves and produced excess mucus, although there was no mortality. Statistical analysis of the data revealed the following. In the controls and in all the experimental groups, significant decrease ($P < 0.001$) was noticed on the 5th day values when compared with the 1st day values in their respective groups. Whereas in controls and copper-dosed clams, the decrease in lactic acid values started on the 2nd day ($P < 0.001$), in mercury-dosed clams it started on the 3rd day ($P < 0.05$). When compared with the controls, the values were not significant on all the 5 days for 1 ppm Hg^{2+} - and Cu^{2+}-dosed clams whereas they were significantly higher ($P < 0.05$) even on the 5th day for 3 ppm Hg^{2+}- and Cu^{2+}-dosed clams. In general, the values were found to be lower in Cu^{2+}-dosed clams than in Hg^{2+}-dosed ones.

Table 20A. Haemolymph lactic acid levels (μg/ml) in Sunetta scripta dosed with mercury and copper

		24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Control	N	10	10	10	10	10
	Mean value	7.20	5.80	4.96	5.06	3.10
	± SD	0.1897	0.4243	1.5510	1.5513	0.3847
	Range	6.9-7.4	5.2-6.5	3.4-7.8	3.8-7.2	2.6-3.7
1 ppm of Hg^{2+} dosed	N	10	10	10	10	10
	Mean value	7.26	6.56	4.88	4.96	3.70
	± SD	0.6119	0.7940	0.9765	0.5389	0.5899
	Range	6.3-8.1	5.6-8.0	3.5-6.3	4.3-5.6	3.1-4.6
3 ppm of Hg^{2+} dosed	N	10	10	10	10	10
	Mean value	7.30	7.32	4.68	5.06	4.16
	± SD	0.5514	0.2400	2.1683	0.9583	0.6468
	Range	6.7-8.3	6.9-7.6	3.3-9.0	4.3-6.9	3.6-5.0
1 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	7.14	5.94	4.50	3.92	3.94
	± SD	0.3878	0.5463	1.4339	0.7082	1.1412
	Range	6.7-7.7	5.4-6.7	3.2-7.2	3.4-5.3	3.1-6.2
3 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	7.50	6.48	3.72	4.96	3.66
	± SD	0.1265	0.3655	0.3429	0.9952	0.2577
	Range	7.3-7.7	6.0-6.9	3.2-4.2	3.7-6.1	3.4-4.1

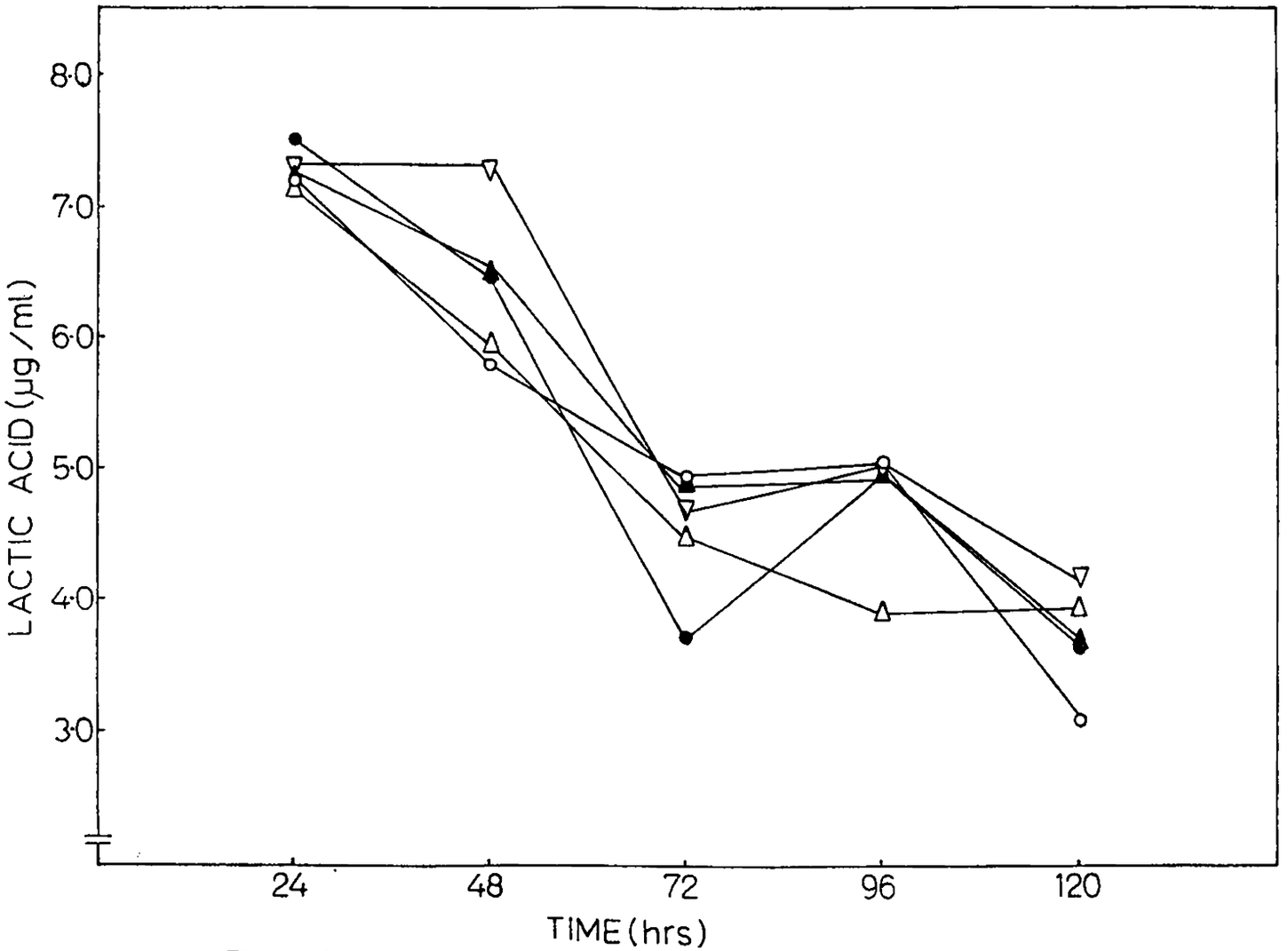

Fig. 13A. Haemolymph Lactic Acid (µg/ml) in Sunetta scripta dosed with two concentrations of mercury and copper. Control (o); 1 ppm mercury (▲); 3 ppm mercury (▽); 1 ppm copper (Δ); 3 ppm copper (●).

DISCUSSION

It is well known that certain heavy metal ions when present in marine ecosystems even at very low concentrations can severly affect the basic metabolism of marine organisms (Thurberg et al., 1974; Capuzzo and Sasner, 1977). Heavy metal ions either depress or elevate the rate of oxygen uptake in marine bivalves. When the rate of oxygen uptake decreases in such a situation, most often it is attributed to valve closure (Schlieper, 1955; Shapiro, 1964; Bayne et al., 1976; Phillips, 1977b), decreased ciliary activity and filtration rate (Brown and Newell, 1972; Abel, 1976; Lakshmanan, 1982; Mathew and Menon, 1984; Howell et al., 1984; Prebhudeva and Menon, 1985), direct metabolic effect (Shapiro, 1964; Phillips, 1977b), or to cellular damage of gills and ciliated cells (Prabhudeva and Menon, 1986). Both Cu^{2+} and Hg^{2+} act as respiratory depressants in bivalves as reported by Shapiro (1964), Brown and Newell (1972), Scott and Major (1972), Mathew and Menon (1983), Prebhudeva and Menon (1986), Baby and Menon (1986), among others.

Studies by Latha Thampuran (personal communication) in our laboratory have indicated that in S.scripta there is a decrease in oxygen uptake when dosed with Cu^{2+} at 1 and 2 ppm. Since there was no mortality as observed in the present study as well as in that by Thampuran, it is concluded that S.scripta can very well tolerate copper and mercury stress up to 3 ppm.

Several bivalves have well developed and efficient respiratory mechanisms that allow a sufficient uptake of oxygen even when present at low tensions (Bayne and Livingstone, 1977). This, however, does not mean that at reduced oxygen tensions the clams are still totally dependent on aerobic metabolism. On the contrary, in S.scripta the operation of aerobic and anaerobic pathways,

as suggested by Chen and Awapara (1969), Livingstone and Bayne (1974; 1977), de Zwaan and de Bont (1975), and Mangum and Burnett (1975) for other bivalves, may be occurring simultaneously. The present study on end-product accumulation shows the operation of anaerobic pathway also under hypoxic condition resulting in the accumulation of lactic acid in the haemolymph. Furthermore, the data presented indicate that the pathway leading to the production of lactic acid is operating effectively during the initial days, and also when the metal ion concentration is high.

Fairly high haemolymph lactic acid values were recorded in the controls and in all the experimental groups on the 1st day. As a result of transfer to new tanks at the begining of the experiment, certain amount of stress is imposed on all the clams. Those in the control group opened their valves within 3-5 hrs, protruded their siphons, and revealed high ciliary activities. All of these burst activities demand high energy resulting in initial functional hypoxia and the consequent lactic acid production on the first day. Significant reduction in haemolymph lactic acid levels in the subsequent days indicates that the clams could overcome the initial stress and rapidly attain homeostasis. Ellington (1983) has indicated that some animals may still retain an anaerobic component even after recovery from hypoxia.

In the experimental animals, the situation is entirely different as the clams produced excess mucus, closed their valves, and seldom protruded siphons. All of these features precede the virtual establishment of hypoxia within the shell. It is possible, however, that vigorous mechanical and metabolical activities are occurring to clear the toxic metabolic ions that have entered the systems. All these activities occurring under environmental hypoxic

conditions result in the accumulation of lactic acid in haemolymph at 24 hr. But the significant drop in values on the 2nd day in Cu^{2+}-dosed clams and on the 3rd day in Hg^{2+}-dosed clams in comparison with their respective 1st day values indicates that the clams still under hypoxic conditions have either reduced the mechanical and metabolic activities to tide over the prevailing situation or have switched over to some other pathway(s). The time difference is attributed to the difference in the metals used.

There were no significant differences in lactic acid values in 1 ppm Hg^{2+} and Cu^{2+}-dosed clams in comparison with those of the controls on all days but significantly higher values were recorded in 3 ppm Hg^{2+}- and Cu^{2+}- dosed clams even on the 5th day. It is noted that the mechanisms operating in the controls and the experimentals are totally different. Specifically, the controls, which were undergoing aerobic metabolism, were initially under functional hypoxia, but the experimentals were under environmental hypoxia throughout and resorted to anaerobic metabolism. The calms dosed with 1 ppm of the metals, although under less stress than those dosed with 3 ppm, might have adopted regulatory mechanisms or switched over to other pathway(s). When the stress is greater, as is the case in clams dosed with 3 ppm of the metal ions, the pathway leading to the production of lactic acid is still operating inspite of the fact that the 5th day values are significantly lower than the respective 1st day values but significantly higher than the 5th day value for the controls. The continued production of lactic acid by 3 ppm-dosed clams, however, does not mean that they have not adopted regulatory mechanisms or other pathway (s) is not operating.

The lower lactic acid values in Cu^{2+}-dosed clams than in Hg^{2+}-dosed clams,

in general, are attributed to the difference in the chemical nature of the metals used, and further the Cu^{2+}-dosed clams regulate their activities and achieve some other transition route(s) faster than the Hg^{2+}-dosed clams.

The two aspects of the present study that need further elaboration are: (1) whether lactic acid could be one of the end-products if not the major one in bivalves under environmental hypoxic conditions prevailing within the shell, and (2) whether haemolymph could be taken as an organ system in studies related to end-product accumulation in specific tissues of bivalves. For both, the answer is positive for the following reasons. While many molluscs are well adapted to survive hypoxic or anoxic conditions, there is only limited uniformity in the biochemical pathways of energy metabolism that are employed (Kluytmans et al., 1980). Moreover, in bivalves anaerobic glycogen breakdown results in multiple end-products whose formation differs considerably between the species, fluctuates quantitatively with time during anoxia, and is dependent on several factors (Zurburg and Kluytmans, 1980; Meinardus and Gäde, 1981). Lactate which is the classical end-product of carbohydrate fermentation, is normally not considered by some as a main anaerobic product during anoxia/hypoxia in bivalves where accumulation of succinate and/or alanine occurs (Hammen, 1966; Stokes and Awapara, 1968; de Zwaan and Zandee, 1972; de Zwaan and van Marrewijk, 1973). However, Ebberink et al. (1979), Gäde (1980), Meinardus and Gäde (1981), and Zurburg and Ebberink (1981) have reported that sluggish and sessile bivalves utilise arginine phosphate during anoxia but accumulate alanine or lactate. Lakshmanan and Nambisan (1985) have reported that in the bivalves P.viridis and V.cyprinoides var. cochinensis the tissue levels of lactic acid increased with increasing concentrations of Hg^{2+} and Cu^{2+} and

duration of exposure. These reports clearly indicate that in certain species of bivalves lactic acid is one of the end products during anoxia/hypoxia.

In most of the studies dealing with end-product accumulation in bivalves under anoxic/hypoxic conditions, particular attention was given to determining the levels of these products in specific organs such as the muscles, mantle, gills, digestive gland, etc., but haemolymph was seldom considered as an organ system. As bivalves have an open circulatory system, the body tissues are continuously bathed in haemolymph, and transport of end-products to the environment or to other organs from the site of origin occurs through it. Moreover, the concentration of end-products in haemolymph is a reliable initial indicator of the type of metabolic response occurring within the organism under stress. Hence, it is strongly argued that in studies related to end-product accumulation in specific tissues in bivalves, haemolymph should also be considered as an organ system. In support of this, it is noted that Crenshaw and Neff (1969) have observed that after 24 hr aerial exposure of Mercenaria mercenaria, succinate in total tissues and the combined mantle and extrapallial fluid accumulated at equal rates. Zurburg and Kluytmans (1980) have reported that in Mytilus edulis, during anoxia the concentrations of acetate and propionate in haemolymph were equal to those in other organs.

At this stage it is difficult to correlate the significant decrease in lactic acid levels in haemolymph of the experimentals with gluco(neo)genesis. However, in another experiment (Chapter II) I have observed that the haemolymph glycogen levels in S.scripta did not alter significantly in controls, and in those dosed with 1, 3, and 5 ppm of Cu^{2+}. Most bivalve organs and tissues store respectable amounts of glycogen and gluco(neo)genesis is probably a common

feature of all organs (de Zwaan, 1983) although information on the metabolic balance between the glucose utilisation and gluconeogenesis in marine bivalves is lacking (Gabbott, 1983). It is possible that lactic acid is transported via haemolymph to foot, hepatopancreas etc., from other tissues. Although supportive evidence for this speculation is limited, it includes the progressive decrease in haemolymph lactic acid levels as observed in the present study, my observation mentioned earlier (Chapter II) and the observation of Haeser and de Jorge (1971) in the pulmonate Strophocheilus oblongus musculatus.

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CHAPTER VIII

SUMMARY

In recent years there is increased awareness regarding the potentiality of molluscan fishery as a good resource to provide protein and mineral-rich food. For increased molluscan production, new methods are being adopted in mariculture programmes. In intensive molluscan culture programmes, set-backs because of bacterial, viral, protozoan and metazoan parasitic out-breaks are not uncommon. Bivalves are also peculiar in the sense that they have the capacity to accumulate potentially toxic heavy metals in their tissues far in excess of environmental levels. Likewise, it is also established that molluscs have the ability to concentrate micro-organisms from the environments in which it habits. There are reports indicating that they serve as vehicles for the transmisson of enteric diseases to man. A thorough knowledge about the mechanisms involved in these processes will also answer several problems of public health importance. Yet another important aspect that needs an emphasis is the utilisation of marine organisms as models for research directed at understanding the basic bio-medical problems that remain unresolved. The use of marine orgnisms has a practical advantage, Viz., many species of marine animals are readily available, usually at low cost. The effects of environmental variables on bivalves have been studied at the organismal, tissue and cellular levels. In the past, in studies dealing with sub-lethal effects of heavy metals, particular attention was given to specific organs such as mantle, gills, kidney, digestive gland, gonad etc., but haemolymph was seldom considered as an organ system. Therefore, in my studies, attention was given specifically to this system because bivalves have an open circulatory system and hence the body tissues are continuously bathed in haemolymph, the transport of materials occurs

through the circulatory system, and it can be a reliable initial indicator of the type of metabolic response occurring within the organism under stress.

In the present study, two species of clams, Sunetta scripta (Linné) and Villorita cyprinoides var. cochinensis (Hanley) were used. The thesis is divided into eight chapters. In the first chapter, opinions of different workers on the various aspects of bivalve haemolymph and haemocytes and their functional role including the part they play in defence mechanisms are brought together in the form of a review. The second chapter is on the haemocytes, and the third one deals with the levels of haemolymph glycogen and total carbohydrates in the four size groups of the two clam species, and also when subjected to abiotic and biotic challenges. The fourth chapter deals with variations in protein concentrations in the haemolymph under the same conditions as mentioned above. Variations in the haemolymph acid and alkaline phosphatases activities in the clams of the four size groups, and subsequent to abiotic and biotic challenges are outlined in the fifth and sixth chapters respectively. The seventh chapter contains observations on the end-product accumulation study in S.scripta exposed to sub-lethal concentrations of copper and mercury. Summary of the work forms the eighth chapter, followed by the list of references.

CHAPTER-I. In review, information giving emphasis on the types of haemolymph cells (haemocytes), haemopoiesis, ontogeny, cell behaviour and the functional role of haemocytes, such as shell repair, nutrient digestion and transport, excretion, wound repair and internal defence are given. In the past, there was no agreement on the classification of the types of the cells present in the haemolymph of bivalves. Earlier workers employed different criteria, such as the nature of the cytoplasm, presence or absence of granules, size of the

cell, staining reaction of the nucleus etc. and proposed different classifications, some identifying as many as seven types of cells. But recently these commonly employed criteria were rejected on specific grounds, and now it appears that there are only two categories of cells - granulocytes and agranulocytes/ hyalinocytes. However, there are detectable staining and other morphological alterations that occur during aging and functionalships during the life of cells. No satisfactory explanation has yet been given as to where haemopoises actually occurs in bivalves. It is generally believed that haemocytes arise from differentiation of connective tissue cells. The sequence of development (ontogeny) of bivalve haemocytes is also not clearly understood, and there are differences of opinion. In the haemolymph, the number of circulating haemocytes is dependent upon the amount of turbulence produced by cardiac action. These haemocytes when drawn from the body are irregularly oval, and form clusters. When allowed to stand, they adhere to glass and there is an exomigration of haemocytes from the clumps. They are also known to take up vital dyes such as Neutral Red, and Janus Green B. The haemocytes have been implicated to perform a variety of functions, and are involved in shell repair, nutrient digestion and transport, excretion, wound repair and internal defence against foreign materials. In shell repair process, the haemocytes are reported to transport calcium and other materials to the sites of shell repair. Though aggregates of haemocytes are observed in shell repair sites, the actual mechanism by which they contribute to the shell repair is not well understood. The involvement of haemocytes in nutrient digestion has been recognised long back and now there is a view that they transport nutrients into the body by picking digestible matter from the lumen of the intestine, and during movement

(by diapedesis) into deeper tissue intracellular digestion commences and the products of digestion are discharged into the exterior ie., to the deeper tissues. The role of haemocytes in excretion is mainly based on the report of the migration of foreign material-laden phagocytes across certain epithelial borders. Their role in wound repair has been recognised long back but as to the exact mechanisms and the extent of involvement, opinions differ.

The part played by the haemocytes in internal defence against foreign materials is important. The known types of phenotypic manifestations of innate cellular internal defence mechanisms in bivalves comprise such mechanisms as leucocytosis, encapsulation, nacrezation and phagocytosis. Of these, leucocytosis, is defined as an increase in the number of leucocytes or haemocytes and is a forerunner of phagocytosis and/or encapsulation, since the increased number of cells contributes to these active processes. Encapsulation involves the enveloping of an invading organism or experimentally introduced tissues too large to be phagocytosed by host cells. Nacrezation is the secretion of nacre around a foreign object. Phagocytosis is a well known type of internal defence mechanism in invertebrates. It involves the uptake of foreign materials by certain types of host cells and thus prevents direct contact of such materials, biotic or abiotic, with host's tissues. It has generally been agreed that the granulocytes are the most active from the stand-point of phagocytosis, and currently it is accepted that the mechanism of phagocytosis actually consists of four distinct stages: (1) Attraction of phagocytes to the non-self material, (2) Attachment of the non-self materials to the surface of the phagocyte, (3) Internalization, and (4) Intracellular digestion. Literature available on all these aspects are critically reviewed in this chapter.

CHAPTER-II. In this chapter, an attempt is made to find out the variation if any, in haemocyte number when the two species of clams, S.scripta and V.cyprinoides var. cochinensis were subjected to abiotic (copper ions) and biotic (Vibrio sp.) insults. In copper-dosed S.scripta no significant difference in the haemocyte number was observed between the experimentals and controls, where as in copper-dosed V.cyprinoides var. cochinensis, a significant drop in the number of haemocytes, which was to a certain extent dosage dependent, was observed. With the available knowledge, an explanation is provided for this observation. In Vibrio-injected S.scripta, a significant increase in the number of haemocytes started at 48 hrs and continued till 96 hrs whereas in V.cyprinoides var. cochinensis it started at 3 hrs and continued upto 12 hrs, indicating a clear interspecific difference in response to the presence of bacterium in the system. Since the cellular processes involved in stress caused by abiotic and biotic factors are entirely different, reasons for the variations in haemocyte number in clams injected with Vibrio sp. are explained in a different way.

CHAPTER-III. In bivalves, it is interesting to note that depending on the seasons there are differences in the energy reserves from which the energy is drawn to combat stress. Early studies on the biochemical composition of bivalves have shown that in most cases, changes in body weight are mainly due to changes in carbohydrate or glycogen content, and correspond to seasonal changes. Apart from this, very little information is available on the changes in the haemolymph glycogen and carbohydrate values during abiotic (heavy metal) and biotic (bacteria) stresses. And this aspect was investigated in the present study.

In copper-stressed S.scripta, the haemolymph glycogen values did not show any significant variation from the control values, whereas in V.cyprinoides var. cochinensis, the glycogen level was lower in the experimentals than in the controls, especially towards the end of the experimental period. In S.scripta and V.cyprinoides var. cochinensis the carbohydrate values were significantly higher in copper-dosed clams than in the controls. In bacteria-challenged S.scripta, the haemolymph glycogen values were found to be significantly higher than the values of the untampered controls at 3 hrs, whereas in V.cyprinoides var. cochinensis, this increase was noticed at 24 hrs. This increase in glycogen content is attributed to the glycogen synthesised through degradation of bacteria by the phagocytes. The time difference shown by the two clam species is attributed to the species difference. In S.scripta, subsequent to bacterial challenge, the haemolymph total carbohydrate showed significantly higher values upto 12 hrs, and the Vibrio-injected V.cyprinoides var. cochinensis showed higher values than the controls at all the time periods. The possible reasons and the probable mechanisms operating are explained to interpret the observed hyperglycemia in the haemolymph when the clams are under abiotic and biotic stresses.

CHAPTER-IV. In this chapter, variations in haemolymph protein values in S.scripta and V.cyprinoides var. cochinensis, under abiotic and biotic stress are reported. In bivalves, the haemolymph proteins have been implicated to perform a veriety of functions, such as transport of ions, detoxification of metal ions, and recognition of self and non-self materials etc. In S.scripta, the pattern in protein values shown by the copper-dosed clams was different depending on the concentration of copper employed. A similar trend in

haemolymph protein values was shown by copper-dosed V.cyprinoides var. cochinensis also. From the results it is evident that the protein values in the haemolymph compartment in copper-stressed condition is a matter of balance between entry and exit of protein into and out of the haemolymph compartment. In the light of available literature an explanation for the observed variations in protein values in copper-dosed clams is given. In Vibrio-injected clams of both the species, the hemolymph protein values did not show any significant variation between the experimentals and the controls during the entire experimental period. This aspect is also discussed in this chapter.

CHAPTER-V. Lysosomal system has been shown to be very sensitive to changes in the intra and extracellular environment, and subsequently to be involved directly or indirectly in regulating many physiological and pathological processes. In mussels and clams, lysosomal destabilisation has been demonstrated in response to xenobiotics, hypothermia and thermal death, starvation, changes in salinity, exposure to air, steroid hormone etc. Evaluation of the possible role of lysosomes in the deposition or depuration of pollutants including heavy metals, and in the degradation of micro-organisms like bacteria, could be utilised to quantify the changes in lysosomal latency which in turn could be monitored as an index for pollution caused by abiotic and biotic factors. An attempt in this direction was made by studying the activity pattern of one of the lysosomal marker enzymes, acid phosphatase, in the haemolymph compartment of the two clam species exposed to different concentrations of copper and challenged with bacteria. In copper-stress experiments with S.scripta and V.cyprinoides var. cochinensis, the pattern of peak activity clearly shows that in haemolymph the acid phosphatase activity is determined by its synthesis, its release into the haemolymph compartment and its final loss.

In the two clam species the differences in the time-periods showing the maximum and minimum mean activities are attributed to species difference, and to the concentrations of the metal ions used. In general, the higher the metal ions, the lesser will be the synthesis, storage, and release of the enzymes, and alteration in membrane permeability and/or membrane destabilisation caused by metal ions in higher concentrations will result in lesser enzyme turnover, ie., lesser synthesis, storage and release which will reflect as lesser enzyme activity in the haemolymph. In S.scripta injected with Vibrio sp. the acid phosphatase activity was significantly higher from 3 hrs upto 24 hrs, whereas in V.cyprinoides var. cochinensis it was from 3 hrs upto 12 hrs. This is interpreted as the release of this hydrolytic enzyme from the lysosomes in response to a biotic challenge. The observed increase is interpreted as the lysosomal enzymes released from the granulocytes into the serum to act on the bacterial cell walls to cause their partial degradation before being phagocytosed by the granulocytes. This is also discussed in this chapter.

CHAPTER-VI. Apart from the lysosomal detoxication systems, many organic xenobiotic are metabolised by the microsomal (smooth endoplasmic reticulum) cytochrome P-450 dependent monooxygenases. Though no such detoxication system has been implicated for the plasma membrane, its behaviour towards a xenobiotic insult is of interest. Plasma membrane which forms the bounding membrane will be the first membrane to confront a xenobiotic insult. Since it has been reported that destabilisation of the lysosomal membrane as a result of injury by xenobiotics bears a quantitative relationship to the magnitude of the stress response, it is of interest to study whether the same type of destabilisation occurs in the plasma membrane. For this, alkaline

phosphatase, which is considered as a marker enzyme for the plasma membrane, is selected and its activity followed during the stress imposed by abiotic (copper) and biotic (bacteria) agents for a period of 120 hrs.

In S.scripta, clams dosed with copper showed significantly higher haemolymph alkaline phosphatase activity but the onset of peak activity differed with the concentrations employed. In V.cyprinoides var. cochinensis, no significant variation in alkaline phosphatase activity was noticed. By comparing the activity patterns of acid and alkaline phosphatases for the same time periods in S.scripta and V.cyprinoides var. cochinensis, it is clear that the two enzymes need not be released simultaneously into the haemolymph compartment. Reasons for this are mentioned. This study also gives an insight into the efficient mechanism of compartmentalization of enzymes in different membrane systems. In S.scripta and V.cyprinoides var. cochinensis, Vibrio-injected clams showed significantly higher activity at 72 and 96 hrs, and 72, 96 and 120 hrs, respectively. This increase in alkaline phosphatase activity does not coincide with the increase in acid phosphatase activity described earlier, suggesting non-synchronised release of these enzymes from different membrane systems. This aspects are also discussed in this chapter.

CHAPTER-VII. In pollution bioassay studies, estimation of oxygen consumption is a routine practice to assess the metabolic rates of the test animals. But the lowest estimated rate based on oxygen uptake does not necessarily apply to situations in which metabolism is partly or totally anaerobic. Bivalves are known for their ability to engage in anaerobic metabolism during stress. Therefore, estimation of metabolic rates in these invertebrates during total or partial anaerobiosis must also involve the biochemical analysis of end-products

together with the determination of oxygen uptake. In most of the studies dealing with end-product accumulation in bivalves under anoxic/hypoxic conditions, particular attention was given to determining the levels of these products in specific organs such as the muscles, mantle, gills, digestive gland, etc., but haemolymph was seldom considered as an organ system. As bivalves have an open circulatory system, the body tissues are continuously bathed in haemolymph, and transport of end-products to the environment or to other organs from the site of origin occurs through it. Moreover, the concentration of end-products in haemolymph is a reliable initial indicator of the type of metabolic response occurring within the organism under stress. In this chapter, the lactic acid levels in the haemolymph of S.scripta when exposed to copper and mercury are reported with emphasis on two aspects:

1) whether lactic acid could be one of the end-products if not the major one in bivalves under environmental hypoxic conditions prevailing within the shell, and

2) whether haemolymph could be taken as an organ system in studies related to end-product accumulation in specific tissues of bivalves.

For both, the answer is positive and the reasons for this conclusion are also discussed in this chapter.

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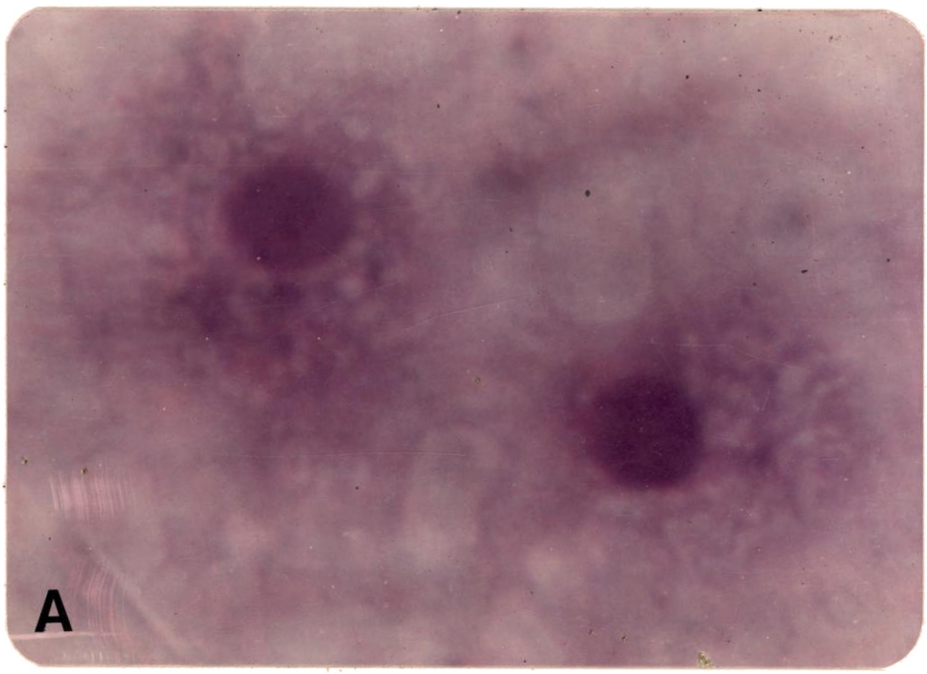

Fig. A. Granulocyte of Sunetta scripta.

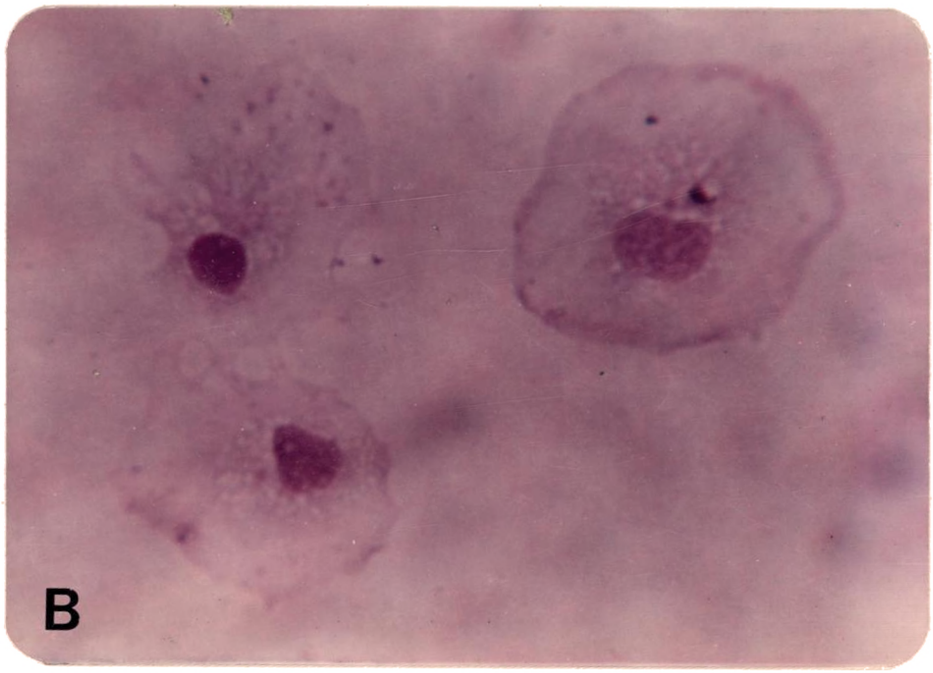

Fig. B. Granulocyte of Villorita cyprinoides
var. cochinensis.

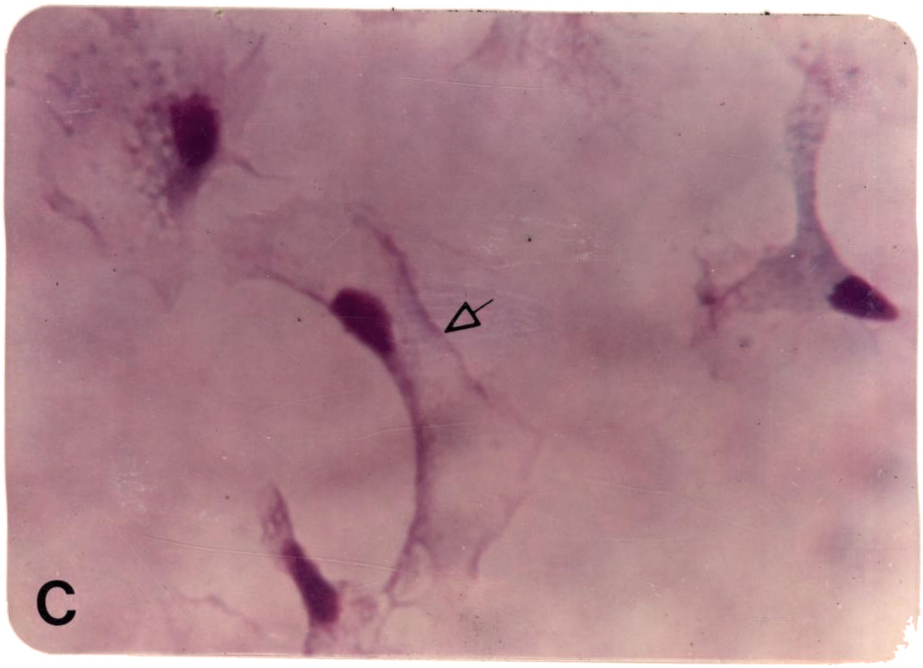

Fig. C. Agranulocyte of Sunetta scripta.
(Arrow mark)

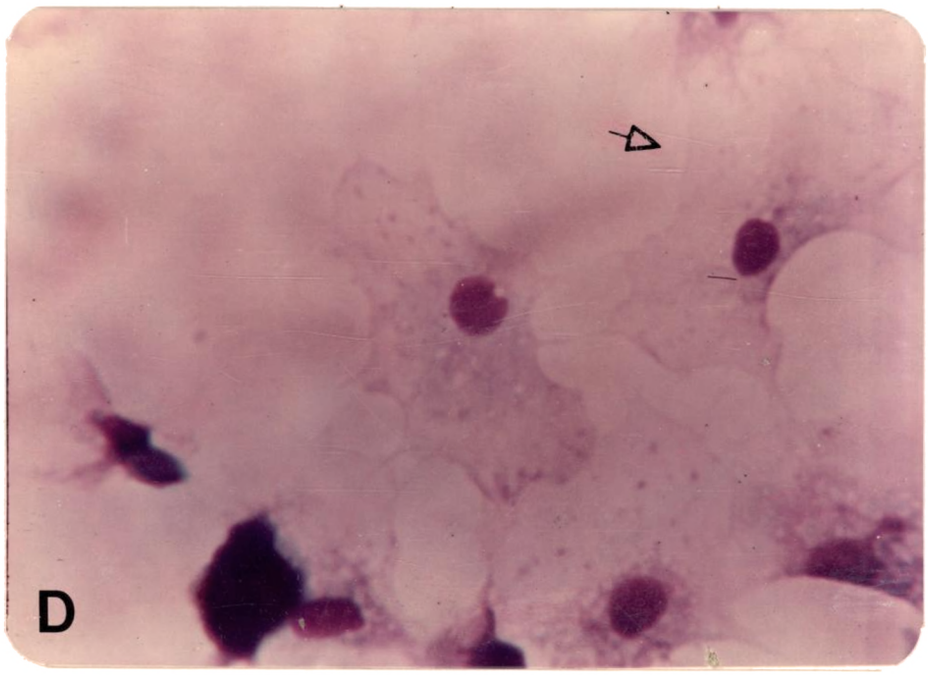

Fig. D. Agranulocyte of Villorita cyprinoides
var. cochinensis (Arrow mark)

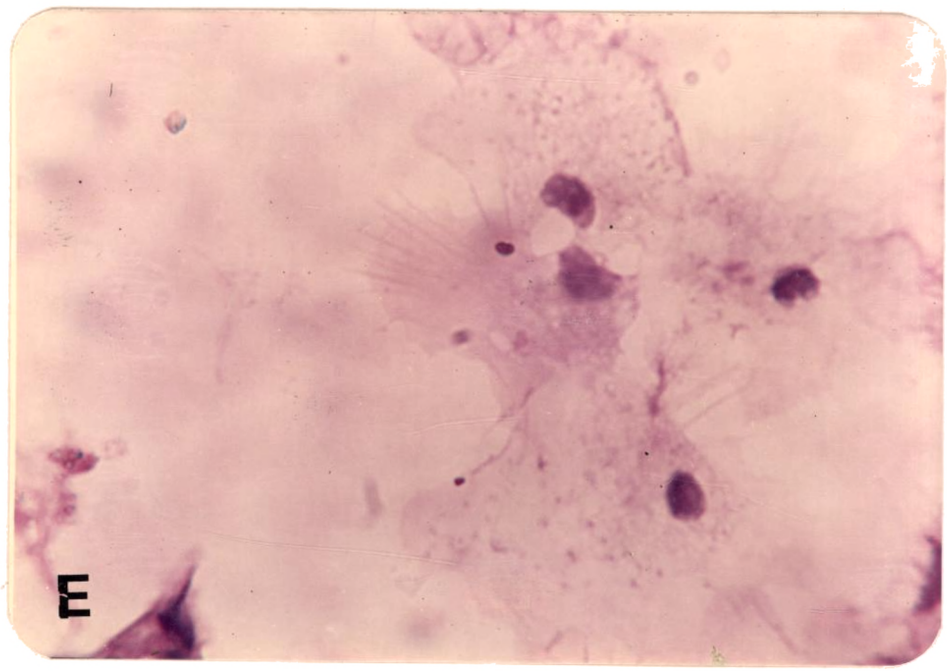

Fig. E. Filopodia of the haemocytes of Sunetta scripta with rib-like structure.

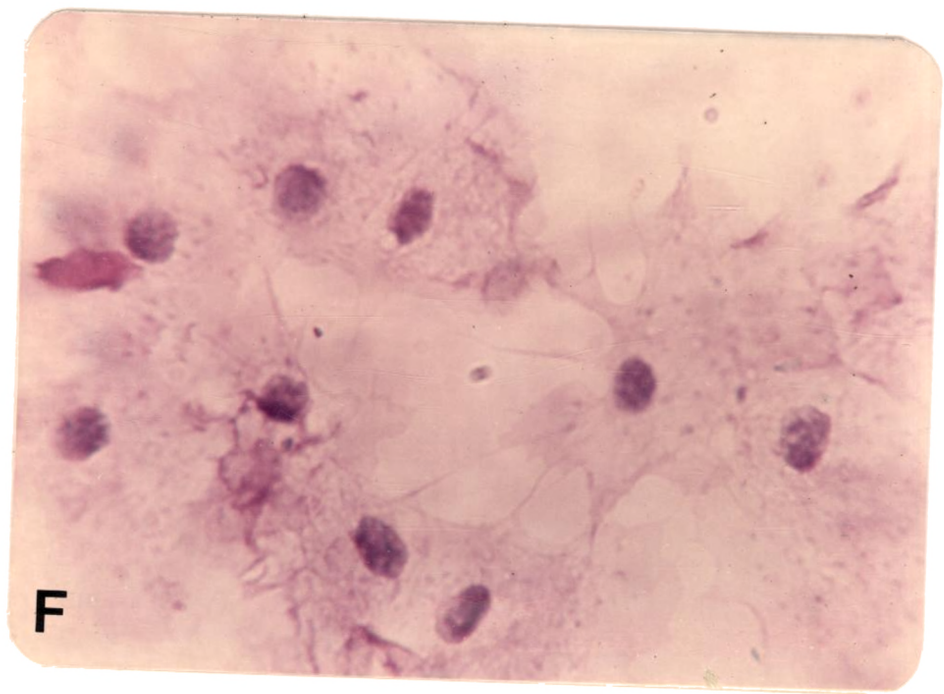

Fig. F. Filopodia of the haemocyte of Villorita cyprinoides var. cochinensis with rib-like structure.

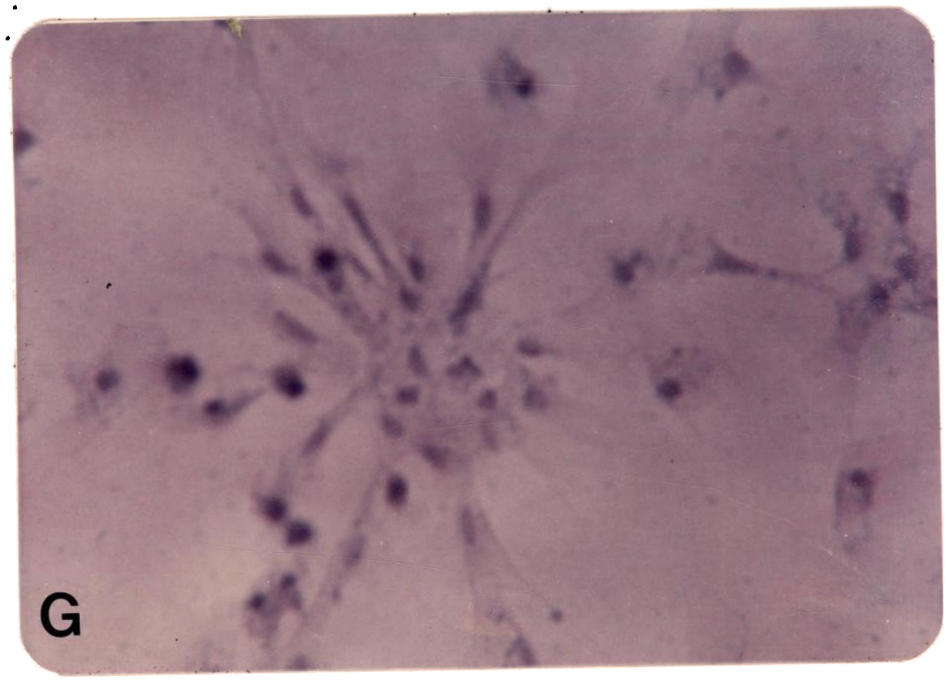

Fig. G. Clump formation of haemocytes in Sunetta scripta.

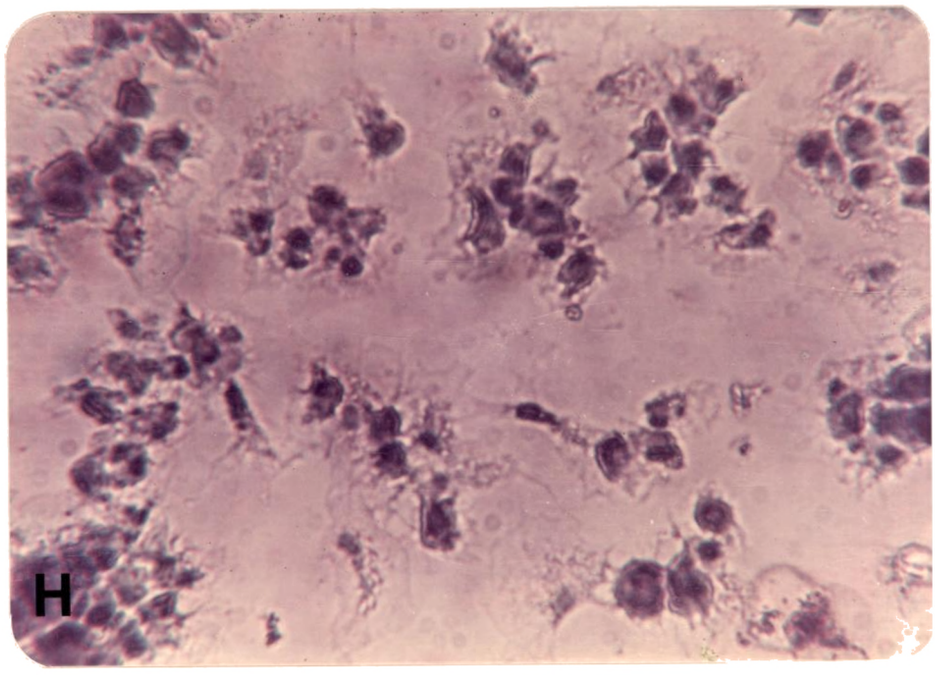

Fig. H. Clump formation of haemocytes in Villorita cyprinoides var. cochinensis.

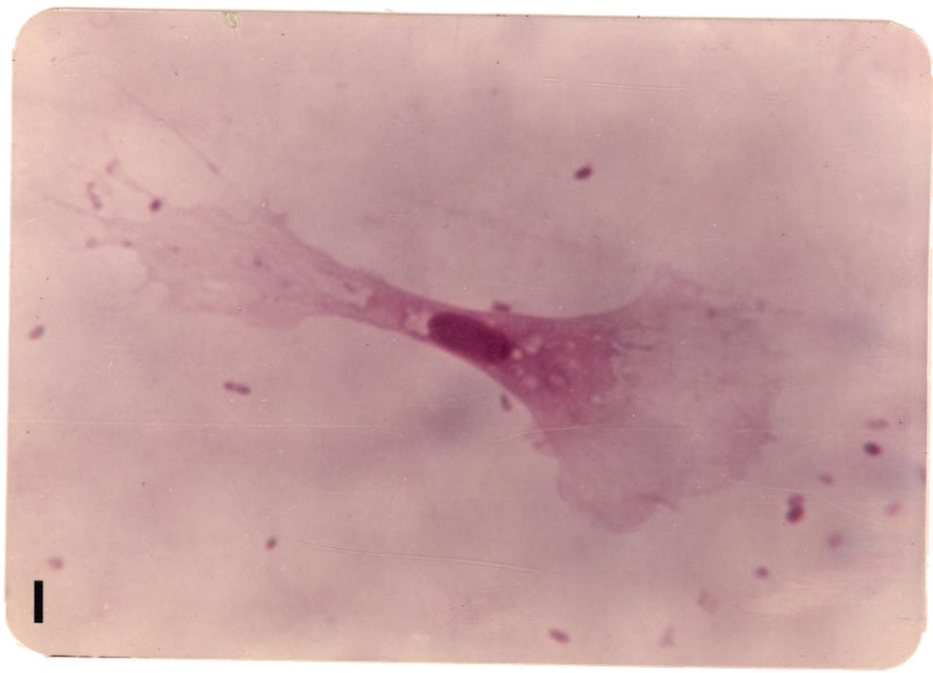

Fig. I. Uptake of bacteria by the granulocyte of Sunetta scripta with the involvement of filopodia.

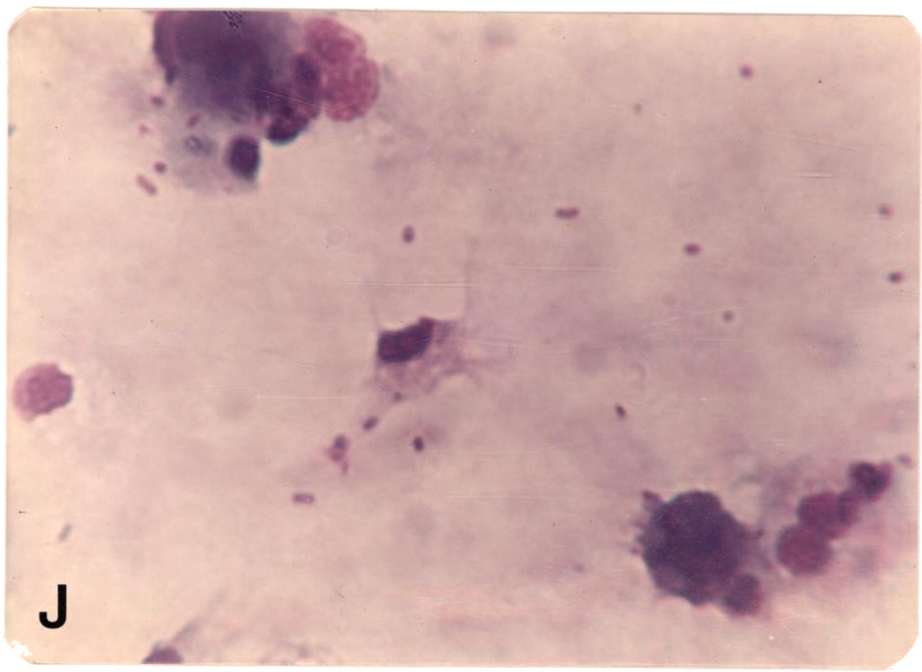

Fig. J. Uptake of bacteria by the granulocyte of Villorita cyprinoides var. cochinensis with the involvement of filopodia.

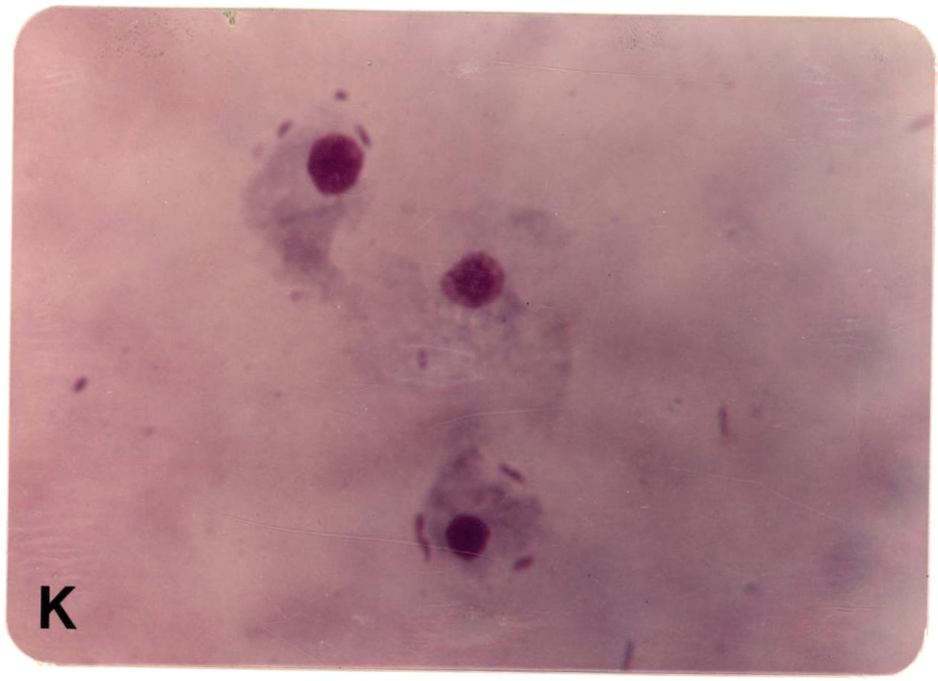

Fig. K. Second uptake mechanism of bacteria by the granulocyte of Sunetta scripta.

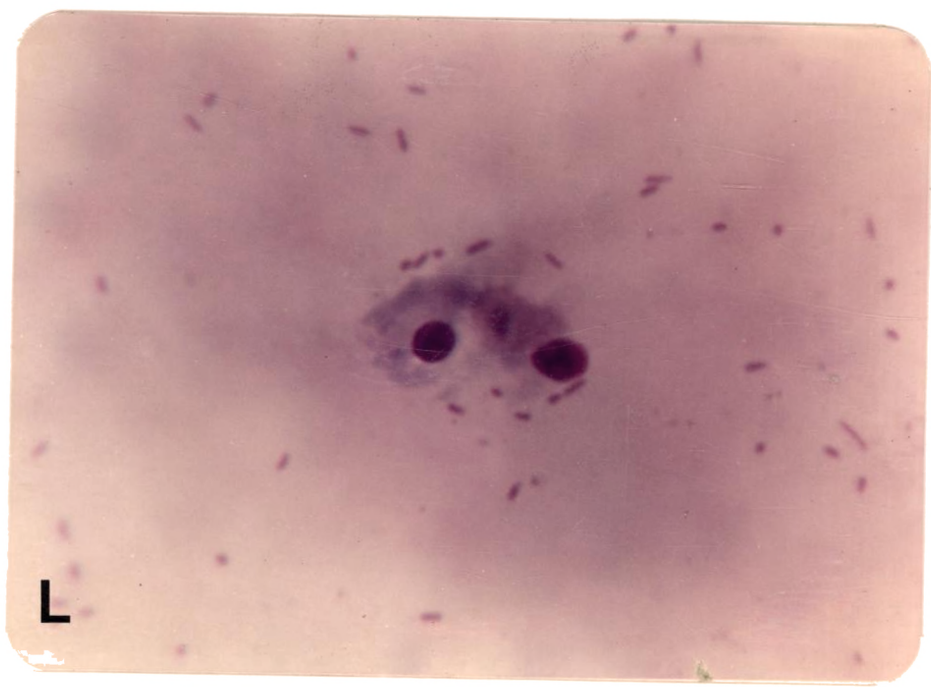

Fig. L. Second uptake mechanism of bacteria by the granulocyte of Villorita cyprinoides var. cochinensis.

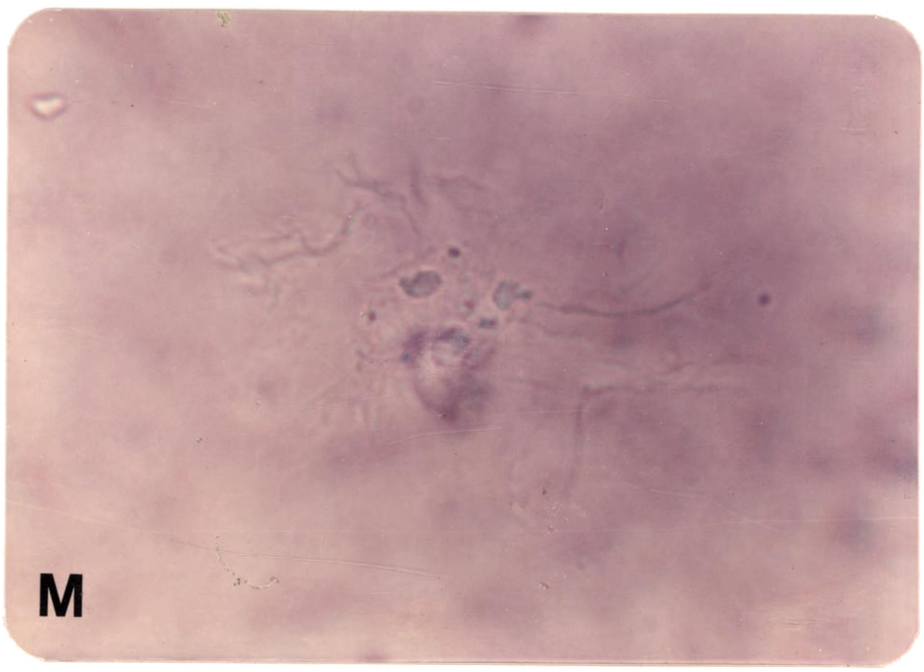

Fig. M. Uptake of bovine serum albumin by the haemocytes of Sunetta scripta.

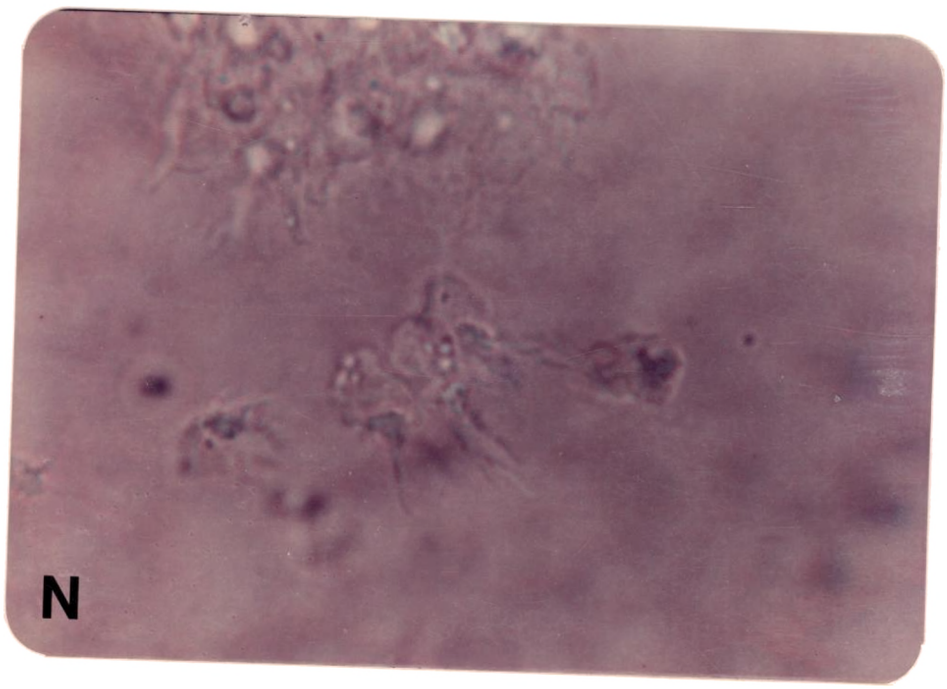

Fig. N. Uptake of bovine serum albumin by the haemocytes of Villorita cyprinoides var. cochinensis.

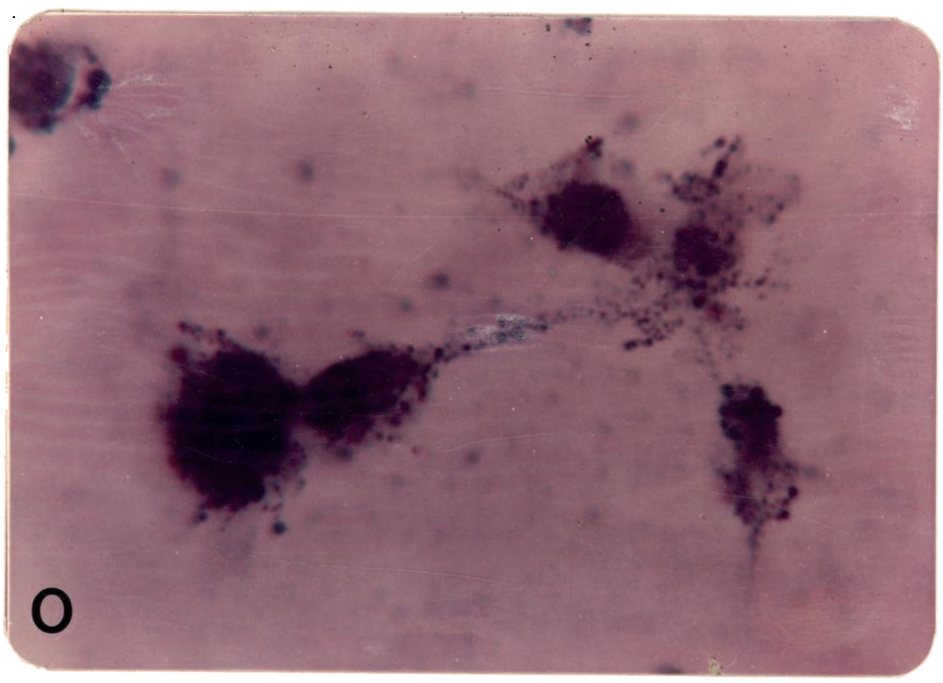

Fig. O. Uptake of oil (Tween 80) by the haemocytes of Sunetta scripta.

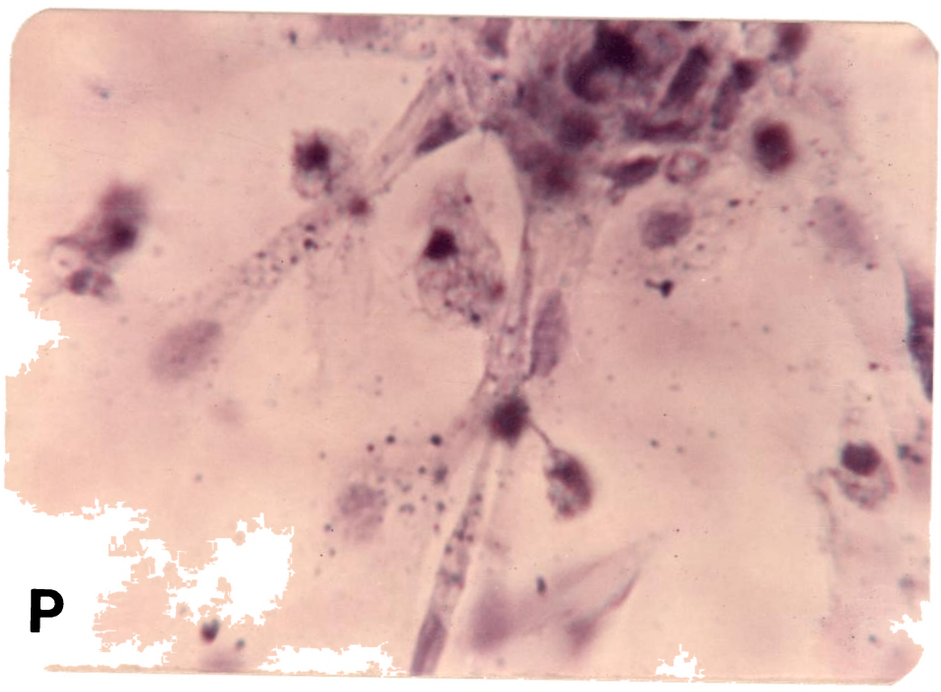

Fig. P. Uptake of oil (Tween 80) by the haemocytes of Villorita cyprinoides var. cochinensis.

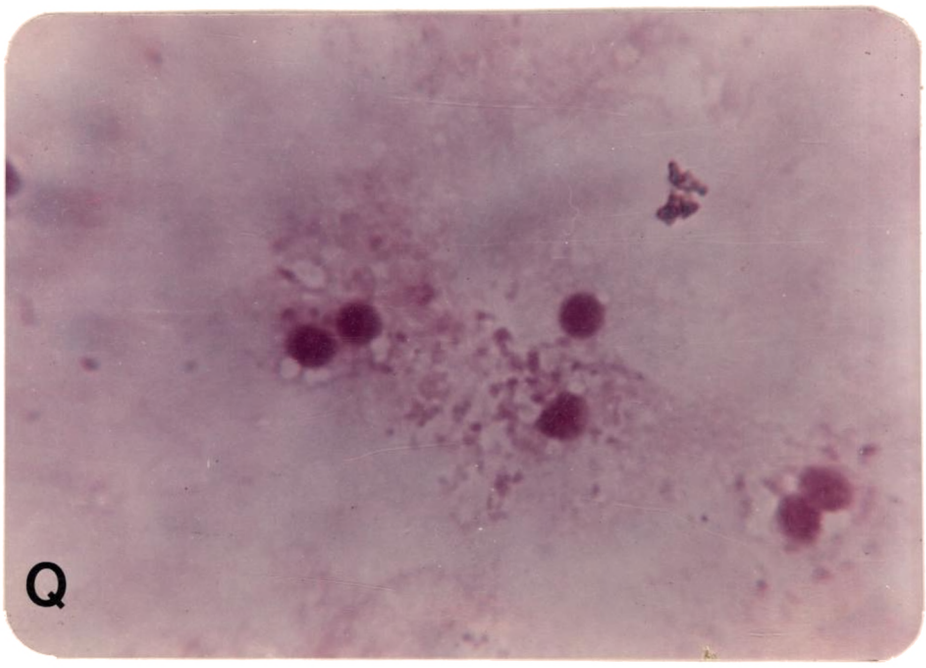

Fig. Q. Glycogen granules in the granulocytes of Sunetta scripta.

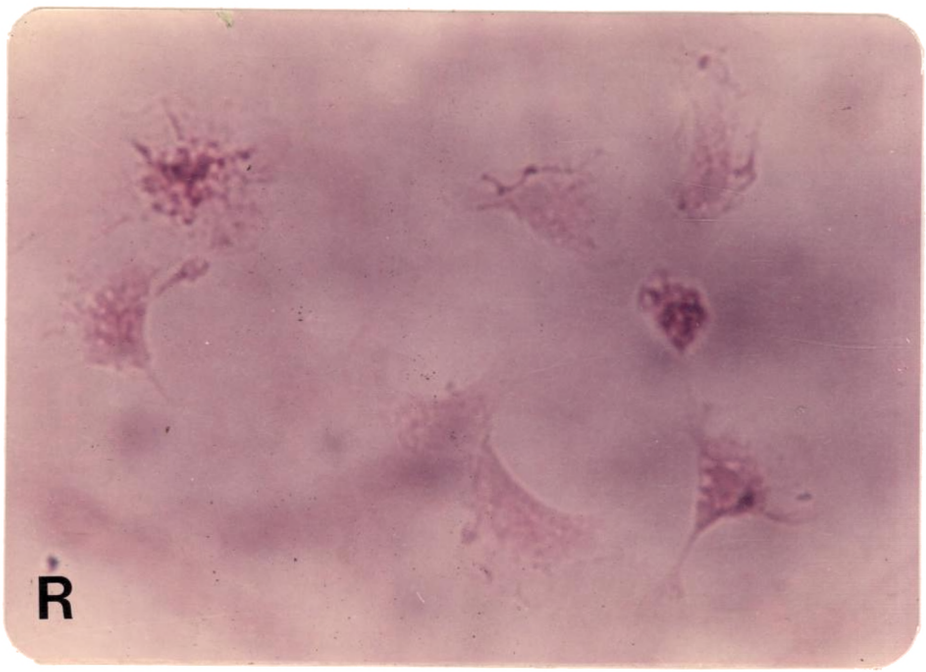

Fig. R. Glycogen granules in the granulocytes of Villorita cyprinoides var. cochinensis.

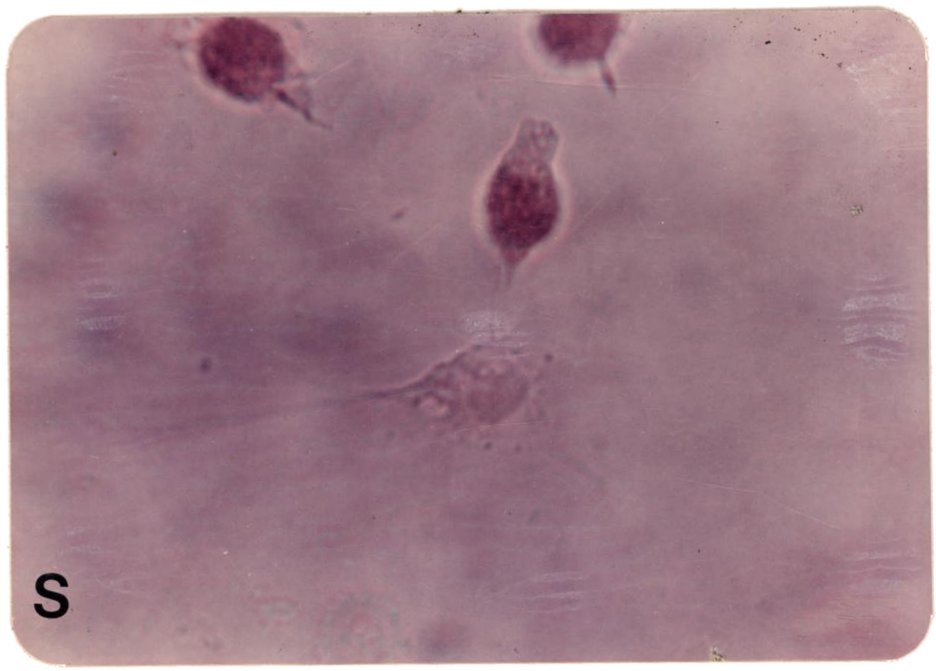

Fig. S. Uptake of Neutral Red by the granulocytes of Sunetta scripta. Note the agranulocyte does not take up the stain.

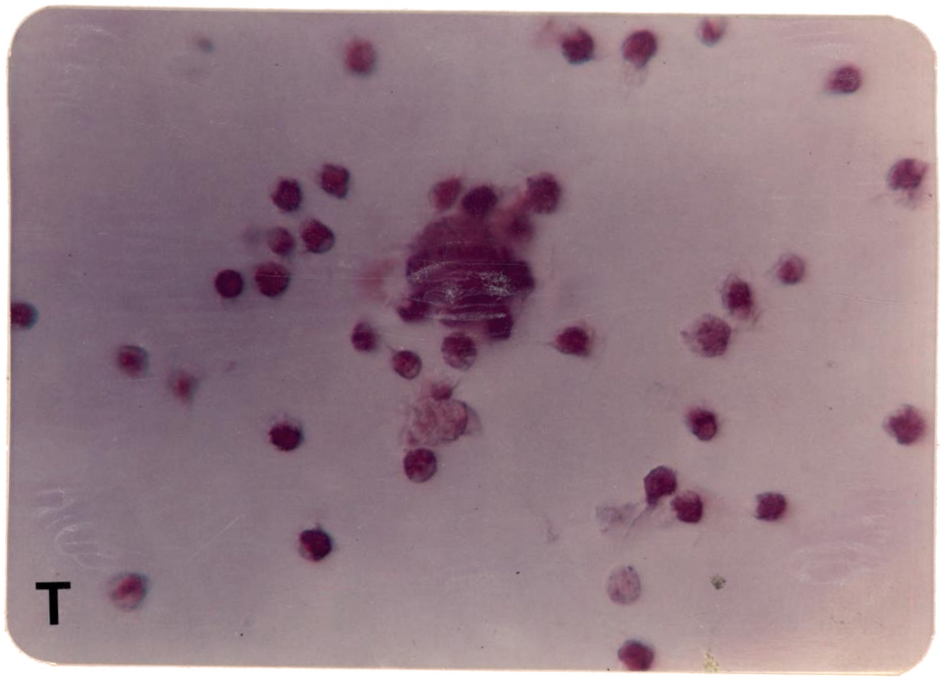

Fig. T. Uptake of Neutral Red by the granulocytes of Villorita cyprinoides var. cochinensis. Note the agranulocyte does not take up the stain.

REFERENCES

REFERENCES

Abel,P.D. 1976. Effect of some pollutants on the filtration rate of Mytilus. Mar. Pollut. Bull., 7: 228-231.

Abolins-Krogis,A. 1963. The histochemistry of the mantle of Helix pomatia (L) in relation to the repair of the damaged shell. Ark. Zool., 15: 461-474.

Abolins-Krogis,A. 1972. The tubular endoplasmic reticulum in the amoebocytes of the shell-regenerating snail, Helix pomatia (L). Z. Zellforsch. Mikrosk. Anat., Bd. 128, S. 58-72.

Abolins-Krogis,A. 1973. Fluorescence and histochemical studies of the calcification-initiating lipofuscin type pigment granules in the shell-repair membrane of the snail, Helix pomatia (L). Z. Zellforsch. Mikrosk. Anat., Bd. 142,S. 205-221.

Abolins-Krogis,A. 1976. Ultrastructural study of the shell-repair membrane in the snail, Helix pomatia (L). Cell Tissue Res., 172: 455-476.

Addink,A.D.F., and P.R.Veenhof. 1975. Regulation of mitochondrial matrix enzymes in Mytilus edulis (L). Proc. 9th. Europ. Mar. Biol. Symp., 109-119. Aberdeen Univ. Press.

Akberali,H.B., and E.R.Trueman. 1985. Effects of environmental stress on marine bivalve molluscs. In:"Advances in Marine Biology". 22: 101-198.

Akberali,H.B., M.J. Earnshaw., and K.R.M.Marriott. 1984. The action of heavy metals on the gametes of the marine mussel, Mytilus edulis (L). I. Copper-induced uncoupling of respiration in the

unfertilized eggs. Comp. Biochem. Physiol., C 77: 289-294.

Allison,A.C. 1969. Lysosomes and cancer. In: "Lysosomes in Biology and Pathology". (Eds. J.T.Dingle and H.B.Fell), Elsevier, Amsterdam, London, New York. Vol.II. 178-204.

Arcisz,W., and C.B.Kelly. 1955. Self-purification of the soft clam, Mya arenaria. Pub. Health. Rep., 70: 605-614.

Baby,K.V., and N.R.Menon. 1986. Oxygen uptake in the brown mussel Perna indica (Kuriakose and Nair) under sublethal stress of Hg, Cd, and Zn. Indian J. Mar. Sci., 15: 127-128.

Baccino,F.M. 1978. Selected patterns of lysosomal response in hepatocytic injury. In:"Biochemical Mechanisms of Liver injury". (Ed. T.F.Slater), Academic Press, New York, London. 518-557.

Baldo,B.A., G.Uhlenbruck., and G. Steinhausen. 1977. Invertebrate antigalactans. A comparative study of agglutinins from the clam Tridacna maxima, the marine sponge Axinella polypoides and the anemone Cerianthus membranaceus. Comp. Biochem. Physiol., A56: 343-351.

Bang,F.B. 1956. A bacterial disease of Limulus polyhemus. Bull. Johns. Hopkins. Hosp., 98: 325-351.

Bang,F.B. 1961. Reaction to injury in the oyster (Crassostrea virginica) Biol. Bull., 121: 57-68.

Bang,F.B. 1967. Summary - Defense mechanisms of invertebrates. Fed. Proc., 26: 1713-1715.

Barker,S.B. 1957. Preparation and colorimetric determination of lactic acid. In: "Methods in Enzymology" (Eds. S.P.Colewick and N.O.Kaplan)

Academic Press, New York, Vol.III. 241-246.

Barondes,S.H. 1981. Lectins. Their multiple endogenous cellular functions. Ann. Rev. Biochem., 50: 207-231.

Barry,R.J.C., and K.A.Munday. 1959. Carbohydrate levels in Patella. J. Mar. Biol. Assoc. U.K., 38: 81-95.

Bayne,B.L. 1973. Aspects of the metabolism of Mytilus edulis during starvation. Neth. J. Sea. Res., 7: 399-410.

Bayne,B.L. 1975. Aspects of physiological condition in Mytilus edulis (L) with special reference to the effects of oxygen tension and salinity. Proc. 9th. Europ. Mar. Biol. Symp. (Ed. H.Barnes) Aberdeen Univ. Press. Scotland. 213-238.

Bayne,B.L., and D.R.Livingstone. 1977. Responses of Mytilus edulis (L) to low oxygen tension: Acclimation of the rate of oxygen consumption. J. Comp. Physiol., 114: 129-142.

Bayne,B.L., and C.Scullard. 1977. Rates of nitrogen excretion by species of Mytilus (Bivalvia:Mollusca). J. Mar. Biol. Assoc. U.K. 57: 355-369.

Bayne,B.L., K.R.Clarke., and M.N.Moore. 1981. Some practical considerations in the measurement of pollution effects on bivalve molluscs and some possible ecological consequences. Aqua. Toxicol., 1: 159-174.

Bayne,B.L., J.Widdows., and R.J.Thompson. 1976. Physiology II. In: "Marine Mussels: Their Ecology and Physiology". (Ed. B.L.Bayne), Cambridge Univ. Press, London/New York. 207-260.

Bayne,B.L., M.N.Moore., J.Widdows., D.R.Livingstone., and P.Salked. 1979a.

Measurement of the responses of individuals to environmental stress and pollution studies with bivalve molluscs. Trans. Roy. Soc. Lon. Series B 286: 561-563.

Bayne,B.L., J.Widdows., M.N.Moore., P.Salkeld., C.M.Worrall., and P.Donkin. 1982. Some ecological consequences of the physiological and biochemical effects of petroleum compounds on marine molluscs. Phil. Trans. Roy. Soc. Lond., B 297: 219-239.

Bayne,B.L., K.R.Clarke., D.R.Dixon., A.Ivanovici., D.R.Livingstone., D.M.Lowe., M.N.Moore., A.R.D.Stebbing., and J.Widdows. 1985. "The effects of stress and pollution on marine animals". Praeger Scientific, New York. 384.

Bayne.C.J. 1983. Molluscan immunobiology. In: "The Mollusca" (Eds. A.S.M.Saleuddin and K.M.Wilbur) Academic Press, New York. Vol. 5: 407-486.

Bayne,C.J., M.N.Moore., T.H. Carefoot., and R.J. Thompson. 1979b. Hemolymph functions in Mytilus californianus: The cytochemistry of hemocytes and their responses to foreign implants and hemolymph factors in phogocytosis. J. Invertebr. Pathol., 34: 1-20.

Beedham.G.E. 1965. Repair of the shell in species of Anodonta. Proc. Zool. Soc. London., 145: 107-125.

*Betances,L.M. 1922. Les cellules du sang de quelques Lamellibranches. Arch. D'Anat. Microscop., 18: 309-327.

Bishop,S.H., D.E.Greenwalt., and J.M.Burcham. 1981. Amino acid cycling in ribbed mussel tissues subjected to hyperosmotic shock.

(* not seen in original)

J. Exp. Zool., 215: 277-287.

Bogitsh,B.J. 1974. Histochemistry, some techniques and applications. Bios., XLV: 23-30.

Bohuslav,P. 1933a. Die Gewebezuchtung des postembryonalen Verdauungstraktus, der Glandula salivalis und des Receptaculum seminis bei Mollusken aus der Familie Helicidae. Arch. Exptl. Zellforsch. Gewebezucht., 13: 673-708.

Bohuslav,P. 1933b. Die Explanation des reinen postembryonalen Herzbindegewebes aus Helix pomatia. Arch. Exptl. Zellforsch. Gewebezucht., 14: 139-151.

Booth,C.E., and C.P.Mangum. 1978. Oxygen uptake and transport in the lamellibranch Modiolus demissus. Physiol. zool., 51: 17-32.

*Bouquegneau J.M., and M.Martoja. 1982. La teneur en Cuivre et son degre de complexation chez quatre Gasteropodes marins. Donn'ees sur le cadmium et le zinc. Oceanologica Acta 5: 219-228.

Bourne,G. 1935. Notes on some experiments with snail tissue in culture. Austral. J. Exptl. Biol. Med. Sci., 13: 43-48.

Boyd,W.C., and E.Shapleigh. 1954. Antigenic relations of blood group antigens as suggested by tests with lectins. J. Immunol., 73: 226-231.

Brown,A.C., and R.J.Brown. 1965. The fate of thorium dioxide injected into the pedal sinus of Bullia (Gastropoda:prosobranchiata). J. Exp. Biol., 42: 509-519.

Brown,B.E., and R.C. Newell. 1972. The effect of copper and zinc on the metabolism of the mussel Mytilus edulis. Mar. Biol., 16: 108-118.

Brown,A.D., and J.R.Simpson. 1972. Water relations of sugar-tolerant yeasts: The role of intracellular polyols. J. Gen. Microbiol., 72: 589-591.

Bryan,G.W. 1973. The occurrence and seasonal variation of trace metals in the scallops Pecten maximus (L.) and Chlamys opercularis (L) J. Mar. Biol. Assoc. U.K., 53: 145-166.

Bryan,G.W. 1979. Bioaccumulation of marine pollutants. Philo. Trans. Roy. Soc, London., B 286: 483-505.

Bubel,A. 1973. An electron microscope study of periostracum repair in Mytilus edulis. Mar. Biol., 20: 235-244.

Bubel, A., M.N.Moore., and D.Lowe. 1977. Cellular responses to shell damage in Mytilus edulis (L). J.Exp. Mar. Biol. Ecol., 30: 1-27.

Buchner,P. 1965. "Endosymbiosis of animals with plant microorganisms". Interscience, New York.

Cabelli,V.J., and W.P.Heffernan. 1970. The accumulation of E.coli by the northern quahaug. Appl. Microbiol., 19: 239-244.

Calabrese,A., J.R.Mac Innes., D.A.Nelson., R.A.Greig., and P.P.Yevich. 1984. Effects of long term exposure to silver and copper on growth, bioaccumulation, and histopathology in the blue mussel Mytilus edulis. Mar. Environ. Res. 11: 253-274.

* Canegallo,M.A. 1924. I leucociti, l'intestino e le branchie nell'alimentazione dell'unio. Riv. Biol., 614.

Capuzzo,J.M., and J.J.Sasner Jr. 1977. The effect of chromium on filtration rates and metabolic activity on Mytilus edulis (L) and Mya arenaria (L). In: "Physiological Responses of Marine Biota

to pollutants." (Eds. F.J.Vernberg., A.Calabrese., F.P.Thurberg., and W.B.Vernberg), Academic Press, New York/London. 225-237.

Carmichael,N.G., K.S.Squibb., D.W.Engel., and B.A.Fowler. 1980. Metals in the molluscan kidney:uptake and subcellular distribution of ^{109}Cd, ^{54}Mn and ^{65}Zn by the clam Mercenaria mercenaria. Comp. Biochem. Physiol., A 65: 203-206.

Casterline,J.L.(Jr)., and G.Yip. 1975. The distribution and binding of cadmium in oyster, soybean and rat liver and kidney. Arch. Environ. Contam. Toxicol., 3: 319-329.

Chandy,J.P., and B.Patel. 1985. Do selenium and glutathione (GSH) detoxify mercury in marine invertebrates? Effects on lysosomal response in the tropical blood clam Anadara granosa. Dis. Aquat. Org., 1: 39-47.

Chaplin,A.E., and J.Loxton. 1976. Tissue differences in the response of the mussel Mytilus edulis to experimentally induced anaerobiosis. Biochem. Soc. Trans., 4: 437-441.

Chen,C., and J.Awapara. 1969. Effect of oxygen on the end-products of glycolysis in Rangia cuneata. Comp. Biochem. Physiol., 31: 395-401.

*Cheney,D.P. 1969. "The morphology, morphogenesis and reactive responses of ^{3}H-thymidine labelled leucocytes in the Manila clam, Tapes semidecussata (Reeve)." Ph.D. Thesis, University Washington, Seattle, Washington.

Cheney,D.P. 1971. A summary of invertebrate leucocyte morphology with

emphasis on blood elements of the Manila clam,Tapes semidecussata. Biol. Bull., 140: 353-368.

Cheng,T.C. 1967. Marine molluscs as hosts for symbiosis: with a review of known parasites of commercially important species. Adv. Mar. Biol., 5: 1-424.

Cheng,T.C. 1975. Functional morphology and biochemistry of molluscan phagocytes. Ann. N.Y. Acad. Sci., 266: 343-379.

Cheng,T.C. 1976a. Aspects of substrate utilization and energy requirement during molluscan phagocytosis. J. Invertebr. pathol., 27: 263-268.

Cheng,T.C. 1976b. Beta-glucuronidase in the serum and hemolymph cells of Mercenaria mercenaria and Crassostrea virginica (Mollusca: pelecypoda). J. Invertebr. Pathol., 27: 125-128.

Cheng,T.C. 1977a. The role of hemocytic hydrolases in the defence of molluscs against invading parasites. Haliotis. 8: 193-209.

Cheng,T.C. 1977b. Biochemical and ultrastructural evidence for the double role of phagocytosis in molluscs: defence and nutrition. Comp. Pathobiol., 3: 21-30.

Cheng,T.C. 1978. The role of lysosomal hydrolases in molluscan cellular response to immunologic challenge. Comp. Pathobiol., 4: 59-71.

Cheng,T.C. 1981. Bivalves. In: "Invertebrate blood cells." (Eds. N.A. Ratcliffe and A.F.Rowley). Academic Press, London. Vol.I: 233-300.

Cheng,T.C. 1983a. The role of lysosomes in molluscan inflammation. Amer. Zool., 23: 129-144.

Cheng,T.C. 1983b. Triggering of immunologic defence mechanisms of molluscan shellfish by biotic and abiotic challenge and its applications. Mar. Tech. Soc. J., 17: 18-25.

Cheng.T.C. 1984. A classification of molluscan hemocytes based on functional evidences. Comp. Pathobiol., 6: 111-146.

Cheng,T.C. 1985. Evidences for molecular specificities involved in molluscan inflammation. Comp. Pathobiol., 8: 129-142.

Cheng,T.C. 1986. General parasitology. Second Edn. Academic Press, Orlando, USA.

Cheng,T.C., and M.S.Butler. 1979. Experimentally induced elevations of acid phosphatase activity in hemolymph of Biomphalaria glabrata (Mollusca). J. Invertebr. Pathol., 34: 119-124.

Cheng,T.C., and A.Call. 1974. An electron microscope study of the fate of bacteria phagocytized by granulocytes of Crassostrea virginica. Contem. Top. Immunobiol., 4: 25-35.

Cheng,T.C., and D.A.Foley. 1975. Hemolymph cells of the bivalve mollusc Mercenaria mercenaria: An electron microscopical study. J. Invertebr. Pathol., 26: 341-351.

Cheng,T.C., and P.C.Galloway. 1970. Transplantation immunity in mollusks: the histoincompatibility of Helisoma duryi normale with allografts and xenografts. J. Invertebr. Pathol., 15: 177-192.

Cheng,T.C., and K.H.Howland. 1979. Chemotactic attraction between haemocytes of the oyster, Crassostrea virginica, and bacteria. J. Invertebr. Pathol., 33: 204-210.

Cheng,T.C., and K.H.Howland. 1982. Effects of colchicine and cytochalasin

B on chemotaxis of oyster (Crassostrea virginica) hemocytes. J. Invertebr. Pathol., 40: 150-152.

Cheng,T.C., and A.Mohandas. 1985. Effect of high dosages of bacterial challenge on acid phosphatase release from Biomphalaria glabrata hemocytes. J. Invertebr. Pathol., 45: 236-241.

Cheng,T.C.,and E.Rifkin. 1970. Cellular reactions in marine molluscs in response to helminth parasitism. In: A symposium on diseases of fishes and shellfishes. American Fisheries Society,Special Publication No. 5: 443-496.

Cheng,T.C.,and G.E.Rodrick. 1974. Identification and characterization of lysozyme from the hemolymph of the soft-shelled clam Mya arenaria. Biol. Bull., 147: 311-320.

Cheng,T.C., and G.E.Rodrick. 1975. Lysosomal and other enzymes in the hemolymph of Crassostrea virginica. J. Invertebr. Pathol., 52: 443-447.

Cheng,T.C., and G.E.Rodrick. 1980. Nonhemocyte sources of certain lysosomal enzymes in Biomphalaria glabrata (Mollusca: Pulmonata). J. Invertebr. Pathol., 35: 107-108.

Cheng,T.C., and B.M.Rudo. 1976a. Chemotactic attraction of Crassostrea virginica hemolymph cells to Staphylococcus lactus. J.Invertebr. Pathol., 27: 137-139.

Cheng T.C.,and B.M.Rudo. 1976b. Distribution of glycogen resulting from degradation of ^{14}C-labelled bacteria in the American oyster, Crassostrea virginica. J. Invertebr. Pathol., 27: 259-262.

Cheng,T.C., and J.T.Sullivan. 1974. Mode of entry, action and toxicity of

copper molluscicides. In: "Molluscicides in Shistosomiasis Control" (Ed: T.C.Cheng), Academic Press, New York.89-153.

Cheng,T.C., and J.T.Sullivan. 1984. Effects of heavy metals on phagocytosis by molluscan hemocytes. Mar. Environ. Res., 14: 305-315.

Cheng,T.C., and T.P.Yoshino. 1976a. Lipase activity in the serum and hemolymph cells of the soft-shelled clam, Mya arenaria, during phagocytosis J. Invertebr. Pathol., 27: 243-245.

Cheng,T.C., and T.P.Yoshino. 1976b. Lipase activity in the hemolymph of Biomphalaria glabrata (Mollusca) challenged with bacterial lipids. J. Invertebr. Pathol., 28: 143-146.

Cheng,T.C., A.Cali., and D.A.Foley. 1974. Cellular reactions in marine pelecypods as a factor influencing endosymbiosis. In: "Symbiosis in the Sea". (Ed. W.B.Vernberg) Univ. South. Carolina Press, S.C. 61-91.

Cheng,T.C., M.J.Chorney., and T.P.Yoshino. 1977. Lysozyme-like activity in the hemolymph of Biomphalaria glabrata challenged with bacteria. J. Invertebr. Pathol., 29: 170-174.

Cheng,T.C., V.G.Guida., and P.L.Gerhart. 1978a. Aminopeptidase and lysozyme activity levels and serum protein concentrations in Biomphalaria glabrata (Mollusca) challenged with bacteria. J. Invertebr. Pathol., 32: 297-302.

Cheng,T.C., J.J.Marchalonis., and G.R.Vasta. 1984. Role of molluscan lectins in recognition processes. In: "Recognition Proteins, Receptors and Probes: Invertebrates" Alan.R.Liss, New York. 1-15.

Cheng,T.C., C.N.Shuster.Jr., and A.H.Anderson. 1966. Effects of plasma and

tissue extracts of marine pelecypods on the cercaria of Himasthla quissetensis. Exptl.Parasitol., 19: 9-14.

Cheng,T.C., A.S.Thakur., and E.Rifkin.1969. Phagocytosis as an internal defense mechanism in mollusca: with an experimental study of the role of leucocytes in the removal of ink particles in Littorina scabra Linn. In: Symposium on Mollusca-Proceedings of the Marine Biology. Association of India, Part II, Bangalore, India. 546-563.

Cheng,T.C., K.J.Lie., D.Heyneman.,and C.S.Richards. 1978b. Elevation of aminopeptidase activity in Biomphalaria glabrata (Mollusca) parasitized by Echinostoma lindoense (Trematoda). J.Invertebr. Pathol., 31: 57-62.

Cheng,T.C., G.E.Rodrick., D.A.Foley., and S.A.Koehler. 1975. Release of lysozyme from hemolymph cells of Mercenaria mercenaria during phagocytosis. J.Invertebr. Pathol., 25: 261-265.

Cheng,T.C., G.E.Rodrick., S.Moran., and W.A.Sodeman. 1980a. Notes on Theba pisana and quantitative determinations of lysosomal enzymes and transaminations from the head-foot of this gastropod. J. Invertebr. Pathol., 36: 1-5.

Cheng,T.C., M.N.Bui., K.H.Howland., D.A.Schoenberg., and J.T.Sullivan. 1981. Effect of preinjection of Crassostrea virginica with bacteria on subsequent chemotactic response by its hemocytes. J. Invertebr. Pathol., 38: 122-126.

Cheng,T.C.,J.W.Huuang., H.Karadogan.,L.R.Renwrantz., and T.P.Yoshino. 1980b. Separation of oyster hemocytes by density gradient

centrifugation and identification of their surface receptors. J. Invertebr. Pathol., 36: 35-40.

Cherian,M.G., and R.A.Goyer. 1978. Metallothioneins and their role in the metabolism and toxicity of metals. Life Sci., 23: 1-10.

Chipman,W.A. 1966. Uptake and accumulation of chromium^{51} by the clam Tapes decussatus in relation to physical and chemical form. In: "Disposal of Radiactive Waste into Seas, Oceans and Surface Waters". Int. At. Energy Agency, Vienna. 571-582.

Cohn,Z.A. 1972. Properties of macrophages. In: "Phagocytic Mechanisms in Health and Disease". (Eds.R.C.Williams,Jr., and H.H.Fudenberg) Intercontinental Medical Book Corp., New York. 39-49.

*Collicutt,J.M. 1975. Anaerobic metabolism in the oyster heart. M.Sc Thesis, University of British Columbia.

Collicutt,J.M., and P.W.Hochachka. 1977. The anaerobic oyster heart: Coupling of glucose and aspartate fermentation. J. Comp. Physiol., 115: 147-157.

Conney,A.H, and J.J.Burns. 1972. Metabolic interactions among environmental chemicals and drugs. Science, 178: 576-586.

Coombs,T.L. 1974. The nature of Zinc and copper complexes in the oyster Ostrea edulis. Mar. Biol. (Berlin), 28: 1-10.

Coombs,T.L. 1977. Uptake and storage mechanisms of heavy metals in marine organisms. Proc. Anal. Div. Chem. Soc., 14: 219-221.

Couch,J.A. 1984. Atrophy of diverticular epithelium as an indicator of environmental irritant in the oyster, Crassostrea virginica. Mar. Environ. Res., 14: 525-526.

Cousins,R.J. 1985. Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol. Rev., 65: 238-309.

Crawford,G.N.C., and R.Barer. 1951. The action of formaldehyde on living cells as studied with phase-contrast microscopy. Quart. J. Microsc. Sci., 92: 403-452.

Crenshaw,M.A., and J.M.Neff. 1969. Decalcification at the mantle-shell interface in molluscs. Amer. Zool., 9: 881-885.

* Crovario,P. 1958. Some observation on the depuration of mussels infected with poliomyelitis virus. Ig. Mod., 51: 22-32.

* Cuenot,L. 1891. Études sur le sang et les glandes lymphatiques dans la série animale (2 Portie: Invertebres). Arch. Z. Exper., 9: 19-54.

* Dakin,W.J. 1909. Pecten. Liverp. Mar. Biol. Comm. Mem., 17: 1-144.

Dean,R.T. 1977. "Lysosomes". The Institute of Biology's Studies in Biology, No. 84. Edward Arnold. 54.

De Zwaan,A. 1977. Anaerobic energy metabolism in bivalve molluscs. Oceanogr. Mar. Biol., 15: 103-187.

De Zwaan,A. 1983. Carbohydrate catabolism in bivalves. In: "The Mollusca" (Ed. P.W.Hochachka), Academic Press, New York. Vol. I. 137-175.

De Zwaan,A., and A.M.T. De Bont. 1975. Phosphoenolpyruvate carboxykinase from adductor muscle tissue of the sea mussel Mytilus edulis (L). J. Comp. Physiol., 96: 85-94.

De Zwaan,A., and W.J.A. Van Marrewijk. 1973. Anaerobic glucose degradation in the sea mussel Mytilus edulis (L). Comp. Biochem. Physiol.,

B 44: 429-439.

De Zwaan,A., and T.C.M.Wijsman. 1976. Anaerobic metabolism in bivalvia (Mollusca). Comp. Biochem. Physiol., B 54: 313-324.

De Zwaan,A., and D.Zandee. 1972. Body distribution and seasonal changes in the glycogen content of the common sea mussel Mytilus edulis. Comp. Biochem. Physiol., A 43: 53-58.

De Zwaan,A., R.J.Thompson., and D.R.Livingstone. 1980. Physiological and bichemical aspects of valve snaps and valve closure responses in the giant scallop Placopecten magellanicus. II.Biochemistry. J. Comp. Physiol., 137: 105-115.

Des Voigne,D.M., and A.K.Sparks. 1968. The process of wound healing in the Pacific oyster, Crassostrea gigas. J. Invertebr. Pathol., 12: 53-65.

Des Voigne,D.M., and A.K.Sparks. 1969. The reaction of the Pacific oyster, Crassostrea gigas, to homologous tissue implants. J. Invertebr. Pathol., 14: 293-300.

Dixon,D.R. 1982. Aneuploidy in mussel embroys Mytilus edulis L. originating from a polluted dock. Mar. Biol. Lett., 3: 155-161.

Dixon,M., and E.C.Webb. 1967. "Enzymes." Longmans, London.

Dodgson,R.W. 1928. Report on mussel purification. Min. Agr. Fish. Ser.,11:10.

Dougherty,W.J., and R.Altman. 1962. Viral hepatitis in New Jersey. Am. J. Med., 32: 704-736.

Drew,G.H. 1910. Some points in the physiology of lamellibranch blood-corpuscles. Quart. J. Microsc. Sci., 54: 605-621.

Dubois,M., K.A.Gilles., J.K.Hamilton., P.A.Rebers., and F.Smith. 1956.

Colorimetric method for determination of sugars and related substances. Anal. Chem., 28: 350-356.

Dunachie,J.F. 1963. The periostracum of Mytilus edulis. Trans. R. Soc. Edinburg. 65: 383-410.

Dundee,D.S. 1953. Formed elements of the blood of certain fresh water mussels. Trans. Am. Microsc. Soc: 72: 254-264.

Durham,H.E. 1891. On wandering cells in echinoderms, etc. more specially with regard to excretory functions. Quart. J. Microsc. Sci., 33: 81-121.

Durning,W.C. 1957. Repair of a defect in the shell of the snail Helix aspersa. J.Bone Jt. Surg. Am. A 39: 377-393.

*Ebberink,R.H.M. 1980. Regulation of anaerobic carbohydrate degradation in the sea mussel Mytilus edulis (L). Ph.D. Thesis, Univ. of Utrecht, Utrecht.

Ebberink,R.H.M., W.Zurburg., and D.I.Zandee. 1979. The energy demand of the posterior adductor muscle of Mytilus edulis in catch during exposure to air. Mar. Biol. Lett., 1: 23-31.

Eble,A.F. 1966. Some observations on the seasonal distribution of selected enzymes in the American oyster as revealed by enzyme histochemistry. Proc. Natl. Shellfish. Assoc., 56: 37-42.

Eble,A.F. 1969. A histochemical demonstration of glycogen, glycogen phosphorylase and branching enzyme in the American oyster. Proc. Natl. Shellfish. Assoc., 59: 27-34.

Eble,A.F., and M.R.Tripp. 1969. Oyster leucocytes in tissue culture: A functional study. Proc. Natl. Shellfish. Assoc., (1968 Natl. Shellfish.

Assoc. Convention) 59: 2-3.

Ellington,W.R. 1983. Recovery from anaerobiosis. J. Exp. Zool., 228: 431-444.

Engel,D.W. 1987. Metal regulation and moulting in the blue crab, Callinectes sapidus: Copper, Zinc and Metallothionein. Biol. Bull., 172: 69-82.

Erdman,I.E., and A.D.Tennant. 1956. The self-cleansing of soft clams: bacteriological and public health aspects. Can. J. Public Health., 47: 196-202.

Ericsson,J.L.E. 1969. Mechanism of cellular autophagy. In: "Lysosomes in biology and pathology", (Eds. J.T.Dingle and H.B.Fell) North-Holland Publishing Co. Amsterdam and London. Vol. II. 345-394.

Farley,C.A. 1968. Minchinia nelsoni (Haplosporida) disease syndrome in the American oyster Crassostrea virginica. J. Protozool., 15: 585-599.

Feng,S.Y. 1959. Defense mechanism of the oyster. Bull. N.J. Acad. Sci. 4:17.

Feng,S.Y. 1965a. Heart rate and leucocyte circulation in Crassostrea virginica (Gamelin). Biol. Bull., 128: 198-210.

Feng,S.Y. 1965b. Pinocytosis of proteins by oyster leucocytes. Biol. Bull., 129: 95-105.

Feng,S.Y. 1966a. Experimental bacterial infections in the oyster Crassostrea virginica. J. Invertebr. Pathol., 8: 505-511.

Feng,S.Y. 1966b. Biological aspects of hard clam purification. Proc. Natl. Shellfish. Assoc., 56: 3.

Feng,S.Y. 1967. Responses of molluscs to foreign bodies with special reference

to the oyster. Fed. Proc., 26: 1685-1692.

Feng,S.Y., and J.S.Feng. 1974. The effect of temperature on cellular reactions of Crassostrea virginica to the injection of avian erythrocytes. J. Invertebr. Pathol., 23: 22-37.

Feng,S.Y., and L.A.Stauber. 1968. Experimental hexamitiasis in the oyster Crassostrea virginica. J. Invertebr. Pathol., 10: 94-110.

Feng,S.Y., J.S.Feng., and T.Yamasu. 1977. Roles of Mytilus coruscus and Crassostrea gigas blood cells in defense and nutrition. Comp. Pathobiol., 3: 31-67.

Feng,S.Y., J.S.Feng., C.N.Burke., and L.H.Khairallah. 1971. Light and electron microscopy of the leucocytes of Crassostrea virginica (Mollusca:Pelecypoda). Z.Zellforsch., 120: 222-245.

Fisher,W.S. 1986. Structure and functions of oyster hemocytes. In: "Immunity in Invertebrates". (Ed. Michel Brehélin). Springer-Verlag Berlin, Heidelberg, New York, Tokyo., 25-35.

*Foley,D.A. 1974. "Studies on Hemolymph Cells of Marine Pelecypods". Ph.D. Dissertation. Lehigh University, Bethlehem, Pennsylvania.

Foley,D.A., and T.C.Cheng. 1972. Interaction of molluscs and foreign substances: The morphology and behaviour of hemolymph cells of the American oyster, Crassostrea virginica, in vitro. J. Invertebr. Pathol., 19: 383-394.

Foley,D.A., and T.C.Cheng. 1974. Morphology, hematologic parameters and behaviour of hemolymph cells of the quahaug clam, Mercenaria mercenaria. Biol. Bull., 146: 343-356.

Foley,D.A., and T.C.Cheng. 1975. A quantitative study of phagocytosis by

hemolymph cells of the pelecypods Crassostrea virginica and Mercenaria mercenaria. J. Invertebr. Pathol., 25: 189-197.

Foley,D.A., and T.C.Cheng. 1977. Degranulation and other changes of molluscan granulocytes associated with phagocytosis. J. Invertebr. Pathol., 29: 321-325.

Font,W.F. 1980. Effects of hemolymph of the American oyster, Crassostrea virginica, on marine cercariae. J. Invertebr. Pathol., 36: 41-47.

Fowler,S.W., and G.Benayoun. 1974. Experimental studies on cadmium flux through marine biota. In: "Radioactivity in the Sea", Int. At. Energy Agency, Vienna. 44: 159-178.

Fowler,B.A., D.A.Wolfe., and W.F.Hettler. 1975. Mercury and iron uptake by cytosomes in mantle epithelial cells of quahaug clams (Mercenaria mercenaria) exposed to mercury. J. Fish. Res. Board. Can., 32: 1767-1775.

Frankenne,F., F.Noel-Lambot., and A.Disteche. 1980. Isolation and characterization of metallothioneins from cadmium-loaded mussel Mytilus edulis. Comp. Biochem. Physiol., C66: 179-182.

Fretter,V. 1939. The structure and function of the alimentary canal of some tectibranch molluscs, with a note on excretion. Trans. Roy. Soc. Edinburgh., 59: 599-646.

Fries,C.R., and M.R.Tripp. 1970. Uptake of viral particles by oyster leucocytes in vitro. J. Invertebr. Pathol., 15: 136-137.

Fries,C.R., and M.R.Tripp. 1980. Depression of phagocytosis in Mercenaria

following chemical stress. Develop. Comp. Immunol., 4: 233-244.

Gabbott,P.A. 1976. Energy metabolism In: "Marine Mussels": Their ecology and physiology. (Ed. B.L.Bayne). Cambridge University Press, Cambridge. 293-355.

Gabbott,P.A. 1983. Developmental and seasonal metabolic activities in marine molluscs. In: "The Mollusca" Vol.II. Environmental biochemistry and physiology. (Ed. P.W.Hochachka). Academic Press, New York. 165-217.

Gabbott,P.A., and B.L.Bayne. 1973. Biochemical effects of temperature and nutritive stress on Mytilus edulis (L). J. Mar. Biol. Assoc. U.K., 53: 269-286.

Gäde,G. 1980. The energy metabolism of the foot muscle of the jumping cockle Cardium tuberculatum: Sustained anoxia versus muscular activity. J. Comp. Physiol., 137: 177-182.

*Galtsoff,P.S. 1964. The American oyster Crassostrea virginica (Gmelin). Fisher. Bull. Fish. Wild. Serv., 64: 1-480.

Gatenby,J.B. 1932. Absence of mitosis in tissue culture and regeneration in Helix aspersa. Nature. 130: 628.

Gatenby,J.B., and J.C.Hill. 1934. Improved technique for non-aseptic tissue culture of Helix aspersa, with notes on molluscan cytology. Quart. J. Microsc. Sci., 76: 331-352.

Gatenby,J.B., J.C.Hill., and T.J.Mac Doughald. 1934. On the behaviour of explants of Helix aspersa in asceptic and non-asceptic tissue culture. Quart. J. Microsc. Sci., 77: 129-155.

George,S.G. 1983a. Heavy metal detoxication in the mussel, Mytilus edulis: Composition of Cd-containing kidney granules (tertiary lysosomes). Comp. Biochem. Physiol., C76: 53-58.

George,S.G. 1983b. Heavy metal detoxication in Mytilus kidney- an in vivo study of Cd- and Zn- binding to isolated tertiary lysosomes. Comp. Biochem. Physiol., C76: 59-65.

George,S.G., and T.L.Coombs. 1975. A comparison of trace metal and metalloenzyme profiles in different molluscs and during development of the oyster. Proc. Eur. Symp. Mar. Biol., 9th Aberdeen, Scot., 1974. 433-449.

George,S.G., and T.L.Coombs. 1977. The effects of chelating agents on the uptake and accumulation of cadmium by Mytilus edulis. Mar. Biol. (Berlin)., 39: 261-268.

George,S.G., and B.J.S.Pirie. 1980. Metabolism of zinc in the mussel Mytilus edulis. A combined ultrastructural and biochemical study. J. Mar. Biol. Assoc. U.K., 60: 575-590.

George,S.G., T.L.Coombs., and B.J.S.Pirie. 1982. Characterisation of metal-containing granules from the kidney of common mussel, Mytilus edulis. Biochem. Biophys. Acta., 716: 61-71.

George,S.G., B.J.S.Pirie., and T.L.Coombs. 1976. The kinetics of accumulation and excretion of ferric hydroxide in Mytilus edulis (L) and distribution in the tissues. J. Exp. Mar. Biol. Ecol., 23: 71-84.

George,S.G., B.J.S.Pirie., J.M.Frazier., and J.D.Thomson. 1984. Interspecies differences in heavy metal detoxication in oysters. Mar. Environ. Res., 14: 462-464.

George,S.G., E.Carpene., T.L.Coombs., J.Overnell., and A.Y.Youngson. 1979. Characterisation of cadmium-binding proteins from mussels, Mytilus edulis (L) exposed to cadmium. Biochem. Biophys. Acta., 580: 225-233.

George,S.G., B.J.S.Pirie., A.R.Cheyne., T.L.Coombs., and P.T.Grant. 1978. Detoxification of metals by marine bivalves: An ultra-structural study of the compartmentation of copper and zinc in the oyster Ostrea edulis. Mar. Biol. (Berlin)., 45:147-156.

George.W.C. 1952. The digestion and absorption of fat in lamellibranchs. Biol. Bull., 102: 118-127.

*Goetsch,W., and L.Scheuring. 1962. Parasitismus und Symbiose der Algengattung Chlorella. Zeit. Morph. Okol. Tiere., 7: 220-253.

Goldberg,E.D. 1975. The mussel watch-A first step in global marine pollution monitering- Mar. Poll. Bull., 6: 111.

Goodrich,E.S. 1919. Pseudopodia of leucocytes of invertebrates. Quart. J. Microsc. Sci., 64: 19-27.

Götz,P. 1986. Encapsulation in arthropods. In "Immunity in invertebrates" (Ed. M.Breholin), Springer-Verlag, Berlin Heidelberg. 153-170.

Gresson,R.A.R. 1937. Studies on the cultivation of pieces of the mantle of Modiolus modiolus., Quart. J. Microsc. Sci. 79: 659-678.

Guida,V.G., and T.C.Cheng. 1980. Lead Hematoxylin-Basic Fuchsin: New stain for molluscan hemocytes. Trans. Amer. Micros. Soc., 99: 135-140.

Gurski,E and A.F.Eble 1973. Studies of blood cells of the hard clam,

Mercenaria mercenaria., Bull. N.J.Acad. Sci., 18: 20.

*Haeckel,E. 1862. "Die Radiolarian" George Reiner, Berlin.

Haeser,P.E., and F.B.De Jorge. 1971. Anoxic anoxia in Strophocheilus (Pulmonata, Mollusca). Comp. Biochem. Physiol., B 38: 753-757.

Hammen,C.S. 1966. Carbon dioxide fixation in marine invertebrates. V. Rate and pathway in oyster. Comp. Biochem. Physiol., 17: 289-296.

Hardy,S.W., A.W.Thomson., and T.C.Fletcher. 1978. Effect of haemolymph and agglutinins from the Pacific oyster (Crassostrea gigas) on cultured human and rabbit lymphocytes. Comp. Biochem. Physiol., A 60: 473-477.

Harrison,R.L. 1969. Accumulation and distribution of ^{54}Mn and ^{65}Zn in freshwater clams. In: "Radioecology" (Eds. D.J.Nelson and F.C.Evans), CONF. 670503, U.S. Dep. Commer., Springfield, Virginia. 198-220.

Harrison,F.L., and R.Berger. 1982. Effects of copper on the latency of lysosomal hexosaminidase in the digestive cells of Mytilus edulis. Mar. Biol., 68: 109-116.

Harrison,P.M., and R.J.Hoare. 1980. "Metals in Biochemistry" Chapman and Hall, London.

Hart,J.C. 1945. Typhoid fever from clams. Comm. Health. Bull., 59: 289-292.

Hartland,B.J., and J.F.Timoney. 1979. In vivo clearance of enteric bacteria from the haemolymph of the hard clam and the American

oyster. Appl. Environ. Microbiol., 37: 517-520.

Haughton,I. 1934. Note on the amoeboid elements in the blood of Helix aspersa. Quart. J. Microsc. Sci., 77: 157-166.

Have,N. 1969. Sulphydryl inhibitors and pancreatic islets tissue. Acta. Endocrinol. Suppl., 139: 1-231.

Hazleton,B.J., and G.R.Isenberg. 1977. Formed blood elements of the mussel Elliptio complanatum. Proc. Pa. Acad. Sci., 51: 54-56.

Hedstrom,C.E., and E.Lycke. 1964. An experimental study on oysters as virus carriers. Am. J. Hyg., 79: 134-144.

Hoffstein,S., and G.Weissman. 1975. Arthritis Rheum. 18: 153-164.

Holwerda,D.A., and A. de Zwaan. 1980. On the role of fumarate reductase in anaerobic carbohydrate catabolism of Mytilus edulis (L). Comp. Biochem. Physiol., B 67: 447-453.

Houser,L.S. 1964. Depuration of shellfish. J. Environ. Health., 27: 477-480.

Howell,R., A.M.Grant., and N.E.Maccoy. 1984. Effect of treatment with reserpine on the change in filtration rate of Mytilus edulis subjected to dissolved copper. Mar. Pollut. Bull., 15: 436-439.

Howland,K.H., and T.C.Cheng. 1982. Identification of bacterial chemo-attractants for oyster (Crassostrea virginica) hemocytes. J. Invertebr. Pathol., 39: 123-132.

Huffman,J.E., and M.R.Tripp. 1982. Cell types and hydrolytic enzymes of soft-shell clam (Mya arenaria) hemocytes. J. Invertebr. Pathol., 40: 68-74.

Humason,G.L. (1967). Animal tissue techniques, 2nd ed. W.H.Freeman & Co. San Francisco. 569.

Humason.G.L (1979.) Animal Tissue Techniques 4th Ed. W.H. Freeman and Co., San Francisco.

Iordăchesw,D., I.P.Dumitru., and S.N.Niculescu. 1978. Activation by copper ions of Mytilidases - Acid proteolytic enzymes obtained from Mytilus galloprovincialis. Comp. Biochem. Physiol., B 61: 119-122.

Jackim,E. 1974. Enzyme responses to metals in fish. In: "Pollution and physiology of Marine Organisms" (Eds. F.J.Vernberg and W.B.Vernberg), Academic Press, New York. 59-65.

Jackim,E., J.M.Hamlin., and S.Sonis. 1970. Effects of metal poisoning on five liver enzymes in the Killifish (Fundulus heteroclitus). J.Fish. Res. Bd. Can., 27: 383-390.

Jeffries,H.P. 1972. A stress syndrome in the hard clam, Mercenaria mercenaria. J. Invertebr. Pathol., 20: 242-251.

Jenkin,C.R., and D.Rowley. 1970. Immunity in Invertebrates. The purification of haemagglutinin to rat and rabbit erythrocytes from the hemolymph of the Murray mussel (Velesunio ambiguus). Aust. J. Exp. Biol. Med. Sci., 48: 129-137.

Jeong,K.H., K.J.Lie., and D.Heyneman. 1980. Leucocytosis in Biomphalaria glabrata sensitized and resensitized to Echinostoma lindoense. J. Invertebr. Pathol., 35: 9-13.

Jeong,K.H., K.J.Lie., and D.Heyneman. 1983. The ultrastructure of the amoebocyte-producing organ in Biomphalaria glabrata. Dev. Comp. Immunol., 7: 217-228.

Johnson,H.M. 1964. Human blood group. A specific agglutinin of the

butter clam Saxidomus giganteus. Science, 146: 548-549.

Joky,A., M.M.Gondran., and J.Benex. 1985. Response to the amoebocyte-producing organ of sensitized Biomphalaria glabrata after exposure to Echinostoma caproni miracidia. J. Invertebr. Pathol., 45: 28-33.

Jordon,H.E. 1952. "A text book of histology" Appelton-Century-Crofts., New York.

Jyothirmayi,G.N., and P.V.Rao. 1987. Diagnostic value of haemolymph enzyme activity profile in infected snail Lymnaea luteola (Lamarck). Indian J. Exp. Biol., 25: 132-134.

Kaplow,L.S. 1973. Clinical significance of leucocyte alkaline phosphatase determination. Rev. Roum. Med. Int., 10: 365-372.

Kapur,S.P., and A.S.Gupta. 1970. The role of amoebocytes in the regeneration of shell in the land pulmonate, Euplecta indica (Pfieffer). Biol. Bull. 139: 502-509.

Kawaguti,S., and N.Ikemoto. 1962. Electron microscopy on the mantle of a bivalve Musculus senhousia, during regeneration of shell. Biol. J. Okayama Univ., 8: 31-42.

Kelly,C.B. 1956. Bacteriological examination as an indicator of sanitary quality of market shellfish. Proc. Shellfish. Sanitation Workshop, U.S. Dep. Health Educ. Welf. Public Health Serv., 85-94.

Kinoti,G.K. 1971. Observation on the infection of bulinid snails with Schistosoma mattheei. II. The mechanism of resistance to infection. Parasitology, 62: 161-170.

Kiremidjian-Schumacher,L., G.Stotzky., R.A.Dickstein., and J.Schwartz. 1981. Influence of cadmium, lead and zinc on the ability of guinea pig macrophages to interact with macrophage migration inhibitory factor. Environ. Res. 24: 106-116.

Kluytmans,J.H., P.R.Veenhof., and A.de Zwaan. 1975. Anaerobic production of volatile fatty acids in the sea mussel Mytilus edulis (L). J. Comp. Physiol., 104: 71-78.

Kluytmans,J.H., A.M.T.de Bont., J.Janus., and T.C.M.Wijsman. 1977. Time dependent changes and tissue specificities in the accumulation of anaerobic fermentation products in the sea mussel Mytilus edulis (L). Comp. Biochem. Physiol., B 58: 81-87.

Kluytmans,J.H., M.van Graft., J.Janus., and H.Pieters. 1978. Production and excretion of volatile fatty acids in the sea mussel Mytilus edulis (L). J. Comp. Physiol., 123: 163-167.

Kluytmans.J.H., D.I.Zandee., W.Zurburg., and H.Pieters. 1980. The influence of seasonal changes on energy metabolism in Mytilus edulis (L). III. Anaerobic energy metabolism. Comp. Biochem. Physiol., B 67: 307-315.

Koenig,H. 1963. Intravital staining of lysosomes by basic and metallic ions. J. Histochem. Cytochem., 11: 120-121.

Koller,L.D. 1980. Effects of lead, cadmium and Methyl mercury on immunological memory. J. Environ. Pathol. and Toxicol., 4: 47-52.

Lackie,A.M. 1980. Invertebrate immunity. Parasitology, 80: 393-412.

Lakshmanan,P.T. 1982. Investigations on the chemical constituents and trace metal interactions in some bivalve molluscs of the Cochin

backwaters. Ph.D. Thesis, University of Cochin.

Lakshmanan,P.T., and P.N.K.Nambisan. 1977. Toxicity of copper on the bivalve Villorita cyprinoides var. cochinensis. Indian J. Mar. Sci., 6: 83-85.

Lakshmanan,P.T., and P.N.K.Nambisan. 1985. Tissue lactic acid and glycogen level of molluscs exposed to Cu and Hg. Curr. Sci., 54: 478-479.

Lauwerys,R., and J.P.Buchet. 1972. Study on the mechanism of lysosome labilization by inorganic mercury in vitro. Eur. J. Biochem., 26: 535-542.

Lewis,W.H. 1931. Pinocytosis. Bull. Johns Hopkins Hosp., 49: 17-27.

Li,T.Y., A.J.Kramer., C.F.Shaw., and D.H.Petering. 1980. Ligand substitution reactions of metallothionein with EDTA and apo-carbonic anhydrase. Proc. Natl. Acad. Sci. USA. 77: 6334-6338.

Lie,K.J., and D.Heyneman. 1976. Studies on resistance in snails. 3. Tissue reactions to Echinostoma lindoense sporocysts in sensitized and resensitized Biomphalaria glabrata. J. Parasitol., 62: 51-58.

Lie,K.J., D.Heyneman., and P.Yau. 1975. The origin of amoebocytes in Biomphalaria glabrata. J. Parasitol., 63: 574-576.

Liu,O.C., H.R.Seraichekas., and B.L.Murphy. 1966. Fate of polio virus in northern quahaugs. Proc. Soc. Exp. Biol. Med., 121: 601-607.

Livingstone,D.R. 1982. Energy production in the muscle tissues of different kinds of molluscs. In: "Exogenous and endogenous influences

on metabolic and neural control of activity and energy supply in muscles" (Eds. A.D.F.Addink and N.Spronk). Pergamon, Oxford. 257-274.

Livingstone,D.R., and B.L.Bayne. 1974. Pyruvate kinase from the mantle tissue of Mytilus edulis (L). Comp. Biochem. Physiol., B48: 481-497.

Livingstone,D.R., and B.L.Bayne. 1977. Responses of Mytilus edulis (L) to low oxygen tensions: Anaerobic metabolism of the posterior adductor muscle and mantle tissues. J. Comp. Physiol., 114: 143-155.

Lowe,D.M., and M.N.Moore. 1978. Cytology and quantitative cytochemistry of a proliferative atypical hemocytic condition in Mytilus edulis (Bivalvia,Mollusca). J. Natur. Cancer Inst., 60: 1455-1459.

Lowe,D.M., and M.N.Moore. 1979a. The cytology and occurrence of granulocytomas in mussels. Mar. Pollut. Bull., 10: 137-141.

Lowe,D.M., and M.N.Moore. 1979b. The cytochemical distribution of zinc (ZnII) and iron (FeIII) in the common mussel Mytilus edulis, and their relationship to lysosomes. J. Mar. Biol. Assoc. U.K., 59: 851-858.

Lowe,D.M., M.N.Moore., and B.L.Bayne. 1982. Aspects of gametogenesis in the marine mussel Mytilus edulis. J. Mar. Biol. Assoc. U.K., 62: 133-145.

Lowe,D.M., M.N.Moore., and K.R.Clarke. 1981. Effects of oil on digestive cells in mussels: quantitative alterations in cellular ı and

lysosomal structure. Aquat. Toxicol., 1: 213-226.

Lowry,O.H., N.J.Rosebrough., A.L.Farr., and R.J.Randall. 1951. Protein measurement with Folin-Phenol reagent. J. Biol. Chem., 177: 751-766.

Mackin,J.G. 1951. Histopathology of infection of Crassostrea virginica by Dermocystidium marinum. Bull. Mar. Sci. Gulf and Caribb., 1: 72-87.

Mahler,H.R. 1961. Interrelationships with enzymes. In: Mineral metabolism, an advanced treatise (Eds. C.C.Comar., and F.Bronner). Academic Press, New York. 745-879.

Malek,E.A., and T.C.Cheng. 1974. "Internal defence mechanisms" In: Medical and Economic Malacology. Academic Press. Inc., New York. 188-203.

Mangum,C.P., and L.E.Burnett. 1975. The extraction of oxygen by estuarine invertebrates. In: "Physiological Ecology of Estuarine Organisms" (Ed. F.J.Vernberg). Univ. of South Carolina Press, Columbia. SC. 147-163.

Manohar,L., and P.V.Rao. 1975. Experimental induction of hyperglycemia in Lymnaea luteola (Lamarck). Indian J. Exp. Biol., 13: 583-585.

Marshall,A.T., and V.Talbot. 1979. Accumulation of cadmium and lead in the gills of Mytilus edulis: X-ray microanalysis and chemical analysis. Chem. Biol. Interactions., 27: 111-123.

*Martin,A.S. 1970. Cytology, cytochemistry and enzyme cytochemistry of the amoebocytes of the Atlantic surf clam, Spisula solidissima.

Master's thesis, Trenton State College, Trenton, New Jersey.

Mathew,R., and N.R.Menon. 1983. Oxygen consumption of two tropical bivalves exposed to heavy metals. Indian J. Mar. Sci., 12: 57-59.

Mathew.R., and N.R.Menon. 1984. Filteration in Perna viridis and Meretrix casta under heavy metal stress. Mahasagar, 17: 183-186.

Mc Dade,J.E., and M.R.Tripp. 1970. Lysozyme in the hemolymph of the oyster, Crassostrea virginica. J. Invertebr. Pathol., 9: 531-535.

Mc Gee-Russel,S.M. 1957. Tissue for assessing histochemical methods for calcium. Quart. J. Microsc. Sci., 98: 1-8.

Meenakshi,V.R., P.L.Blackwelder., and K.M.Wilbur. 1973. An ultrastructural study of shell regeneration in Mytilus edulis (Mollusca:Bivalvia). J. Zool. Lond., 171: 475-484.

Meinardus,G., and G.Gäde. 1981. Anaerobic metabolism of the common cockle Cardium edule. IV. Time dependent changes of metabolites in the foot and gill tissue induced by anoxia and electrical stimulation. Comp. Biochem. Physiol., B 70: 271-277.

Metcalf,T.G., and W.C.Stiles. 1965. The accumulation of the enteric viruses by the oyster, Crassostrea virginica. J. Infec. Dis., 115: 68-76.

Michelson,E.H. 1961. An acid-fast pathogen of freshwater snails. Am. J. Trop. Med. Hyg., 10: 423-427.

Michelson,E.H. 1975. Cellular defence mechanisms and tissue alterations in gastropod molluscs. In: "Invertebrate Immunity" (Eds. K.Maramorosch., and R.Shope). Academic Press, New York. 181-195.

Millott,N. 1937. On the structure and function of the wandering cells in the wall of the alimentary canal of nudibranchiate mollusca. J. Exptl. Biol., 14: 405-412.

Mitchell,J.R., M.W.Presnell., E.W.Akin., J.M.Cummins., and O.C.Liu. 1966. Accumulation and elimination of polio virus by the eastern oyster. Am. J. Epidemiol., 84: 40-50.

Mix,M.C. 1976. A general model for leucocyte renewal in bivalve mollusks. Mar. Fisher. Rev., 38: 37-41.

Mix,M.C., J.W.Hawkes., and A.K.Sparks. 1979. Observations on the ultrastructure of large cells associated with putative neoplastic disorders of mussels, Mytilus edulis, from Yaquina Bay, Oregon. J. Invertebr. Pathol., 34: 41-56.

Mohandas,A. 1985. An electron microscope study of endocytosis mechanisms and subsequent events in Mercenaria mercenaria granulocytes. Comp. Pathobiol., 8: 143-162.

Mohandas,A., and T.C.Cheng. 1985a. Release pattern of aminopeptidase from Biomphalaria glabrata hemocytes subjected to high-level bacterial challenge. J. Invertebr. Pathol., 45: 298-303.

Mohandas,A., and T.C.Cheng. 1985b. An electron microscope study of the structure of lysosomes released from Mercenaria mercenaria granulocytes. J. Invertebr. Pathol., 46: 332-334.

Mohandas,A., T.C.Cheng., and J.B.Cheng. 1985. Mechanism of lysosomal enzyme release from Mercenaria mercenaria granulocytes: A scanning electron microscope study. J. Invertebr. Pathol., 46: 189-197.

Montgomery,R. 1957. Determination of glycogen. Arch. Biochem. Biophys., 67: 378-386.

Moore,C.A. and A.F.Eble. 1977. Cytochemical aspects of Mercenaria mercenaria hemocytes. Biol. Bull., 152: 105-119.

Moore,C.A., N.Drake., and A.F.Eble. 1972. Cytology and cytochemistry of the amoebocytes of Mercenaria mercenaria. Bull. N. J. Acad. Sci., 17: 44.

Moore,M.N. 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel, Mytilus edulis, and changes induced by thermal stress. Cell Tissue Res., 175: 279-287.

Moore,M.N. 1977. Lysosomal responses of environmental chemicals in some marine invertebrates. In: "Pollution Effects on Marine Organisms" (Ed. C.S.Giam), Heath, Toronto. 143-154.

Moore,M.N. 1980. Cytochemical determination of cellular responses to environmental stressors in marine organisms. In: "Biological Effects of Marine Pollution and the Problems of Monitoring organisms". (Eds. A.D.Mc Intyre., and J.B.Pearce). Rapp. P. V. Reun. Cons. Int. Explor. Mer., 179: 7-15.

Moore,M.N. 1982. Lysosomes and environmental stress. Mar. Pollut. Bull., 13: 42-43.

Moore,M.N. 1985. Cellular responses to pollutants. Mar. Pollut. Bull., 16: 134-139.

Moore,M.N., and K.R.Clarke. 1982. Use of microstereology and quantitative cytochemistry to determine the effects of crude oil-derived

aromatic hydrocarbons on lysosomal structure and function in a marine bivalve molluscs, Mytilus edulis. Histochem. J., 14: 713-718.

Moore,M.N., and S.V.Farrar. 1985. Effects of polynuclear aromatic hydrocarbons on lysosomal membranes in molluscs. Mar. Environ. Res., 17: 222-225.

Moore,M.N., and D.W.Halton. 1977. The cytochemical localisation of lysosomal hydrolases in the digestive cells of littorinids and changes induced by larval trematode infection. Z. Parasitenk., 53: 115-122.

Moore,M.N., and D.M.Lowe. 1977. The cytology and cytochemistry of the haemocytes of Mytilus edulis and their responses to experimentally injected carbon particles. J. Invertebr. Pathol., 29: 18-30.

Moore,M.N., and D.M.Lowe. 1985. Cytological and cytochemical measurements. In: "The Effects of stress and Pollution on Marine Animals". (Ed. B.L.Bayne et al.). Praeger Scientific, New York. 46-74.

Moore,M.N., and A.R.D.Stebbing. 1976. The quantitative cytochemical effects of three metal ions on a lysosomal hydrolase of a hydroid. J. Mar. Biol. Assoc. U.K., 56: 995-1005.

Moore,M.N., D.M.Lowe., and S.L.Moore. 1979. Induction of lysosomal destabilisation in marine bivalve molluscs exposed to air. Mar. Biol. Lett., 1: 47-57.

Moore,M.N.., R.K.Pipe., S.V.Farrar., S.Thomson., and P.Donkin. 1985. Lysosomal and microsomal responses in Littorina littorea: further investigations of environmental effects in the vicinity of the

Sullom Voe Oil Terminal and the effects of experimental exposure to phenanthrene. In: "Oceanic Processes in Marine Pollution- Biological Processes and waste in the Ocean". (Eds. J.M.Capuzzo., and D.R.Kester), Krieger Publishing, Melborne (Florida). Vol.I.

Moore,M.N., J.Widdows., J.J.Cleary., R.K.Pipe., P.N.Salkeld., P.Donkin., S.V.Farrar., S.V.Evans., and P.E.Thomson. 1984. Responses of the mussel Mytilus edulis to copper and phenanthrene: Interactive effects. Mar. Environ. Res., 14: 167-183.

*Morton,B.S. 1980. The anatomy of the "living fossil" Pholadomya candida Sowerby 1823 (Mollusca:Bivalvia:Anomalodesmata). Videnск. Medd. Dansk. Natur. Foren., 142: 7-102.

Mukundan,C. 1968. Molluscs in Indian tradition and economy. Proc. Symp. Molluscs. Mar. Biol. Assoc. India. Part I. 28-35.

*Müller,G. 1956. Morphologie lebenslauf, und bildungsort der blutzellen von Lymnaea stagnalis (L). Z. Zellforsch., 44: 519-556.

Nakahara,H., and G.Bevelander. 1969. An electron microscope study of ingestion of thorotrast by amoebocytes of Pinctada radiata. Tex. Rep. Biol. Med., 27: 101-109.

Narain,A.S. 1968. A note on the formed elements of the blood of certain fresh water bivalves. Gorakhpur Univ. Res. J., 2: 46.

*Narain,A.S. 1969. "Studies on the Heart and Blood of Lamellidens corrianus (Lea)". Ph.D. Thesis, University Gorakhpur, India.

Narain,A.S. 1972a. Formed elements of the blood of the freshwater mussel,

Lamellidens corrianus. J. Morph., 137: 63-70.

Narain,A.S. 1972b. Blood chemistry of Lamellidens corrianus. Experientia., 28: 507.

Narain,A.S. 1973. The amoebocytes of lamellibranch molluscs with special reference to the circulating amoebocytes. Malacol. Rev. 6: 1-12.

National Seminar on Mussel Watch. 1986. Cochin University. 1-186.

Nayar,K.N., and K.S.Rao. 1985. Molluscan fisheries of India. Marine Fisheries Information Service. No:61.

Noël-Lambot,F. 1976. Distribution of Cd, Zn, and Cu in the mussel Mytilus edulis. Existence of Cd- binding proteins similar to metallothioneins. Experientia, 32: 324-325.

*Noël-Lambot,F., J.M.Bouquegneau., F.Frankenne., and A.Disteche. 1978. Le role des metallothioneins dans le stockage des metaux lourds chez les animaux marins. Rev. Int. Oceanog. Med., XLIX: 13-20.

Norseth,T. 1968. The intracellular distribution of mercury in rat liver after a single injection of mercuric chloride. Biochem. Pharmacol., 17: 581-593.

*Ohuye,T. 1937. On the coelomic corpuscles in the body fluid of some invertebrates. Sci. Rep. Tohoku. Imp. Univ. Ser., 4,12: 203-209.

Olafsen,J.A. 1986. Invertebrate lectins- Biochemical heterogeneity as a possible key to their biological function. In: "Invertebrate Immunity" (Ed. M.Brehelin), Springer-Verlag, Berlin, Heidelberg. 94-111.

*Orton,J.H. 1923. Cause or causes of the unusual mortality among oysters in English oyster beds during 1920 & 1921. Fish. Invest. Lond. Ser., 2, 6: 3.

Owen,G. 1966. Digestion. In: "Physiology of Mollusca" (Eds. K.M.Wilbur and C.M.Yonge). Academic Press, New York/London. Vol.II. 53-96.

Pal,S.G., and S.Modak. 1981. Histophysiology of amoebocytes of marine and estuarine bivalves. Biol. Bull. India. Vol.3: 110-116.

Pal,S.G., S.Modak., S.Haldar., and T.Das. 1983. Amoebocytes and the histodynamics of the digestive tubules of Modiolus striatulus. (Bivalvia; Fam. Mytiladea). Biol. Bull. India., 5: 173-181.

Pan,C.T. 1958. The general histology and topographic microanatomy of Australorbis glabratus. Bull. Mus. Comp. Zool. Harvard., 119: 237-299.

Parke,D.V. 1981. Cytochrome P-450 and the detoxication of environmental chemicals. Aquat. Toxicol., 1: 367-376.

Patel,B., and S.Patel. 1964. Studies on the haemoglobin of Anadara sps. (Marine Lamellibranchs). J. Anim. Morphol., 11: 143-161.

Patel,S., and B.Patel. 1971. Effect of ionising radiation on haemoglobin of marine lamellibranchs. Mar. Biol., 10: 272-279.

Patel,S., and B.Patel. 1985. Effect of environmental parameters on lysosomal marker enzymes in the tropical blood clam Anadara granosa. Mar. Biol., 85: 245-252.

Pauley,G.B., and L.H.Heaton. 1969. Experimental wound repair in the number of circulating hemocytes in the California sea hare, Aplysia

californica. J. Invertebr. Pathol., 13: 241-249.

Pauley, G.B., and A.K.Sparks. 1965. Preliminary observations on the acute inflammatory reaction in the Pacific oyster, Crassostrea gigas (Thunberg). J. Invertebr. Pathol., 7: 248-256.

Phillips,D.J.H. 1976a. The common mussel Mytilus edulis as an indicator of pollution by zinc, cadmium, lead and copper. I. Effects of environmental variables on uptake of metals. Mar. Biol., 38: 59-69.

Phillips,D.J.H. 1976b. The common mussel Mytilus edulis as an indicator of pollution by zinc, cadmium, lead and copper.II. Relation of metal in the mussel to those discharged by industry. Mar. Biol., 38: 71-80.

Phillips,D.J.H. 1977a. The use of biological indicator organisms to monitor metal pollution in marine and estuarine environments- A review. Environmental Pollution, 13: 281-317.

Phillips,D.J.H. 1977b. The common mussel Mytilus edulis as an indicator of trace metals in Scandinavian waters. I. Zinc and cadmium. Mar. Biol., 43: 283-291.

Pickwell,G.V., and S.A.Steinert. 1984. Serum biochemical and cellular responses to experimental cupric ion challenge in mussels. Mar. Environ. Res., 14: 245-265.

Pipe,R.K., and M.N.Moore. 1985. Ultrastructural changes in the lysosomal vacuolar system in digestive cells of Mytilus edulis as a response to increased salinity. Mar. Biol., 87: 157-163.

Pirie,B.J.S., S.G.George., D.G.Lytton., and J.D.Thomson. 1984. Metal containing

blood cells of oysters: Ultrastructure, histochemistry and X-ray micro-analysis. J. Mar. Biol. Assoc. U.K., 64: 115-123.

Prabhudeva,K.N., and N.R.Menon. 1985. Rate of filteration by Perna viridis pre-exposed to heavy metals. Fish. Technol., 22: 102-104.

Prabhudeva,K.N., and N.R.Menon. 1986. Oxygen consumption under copper and zinc stress in Perna viridis. Fish. Technol., 23: 24-26.

Prytherch,H.F. 1940. The life cycle and morphology of Nematopsis ostrearum sp. Nov., a gregarine parasite of the mud crab and oyster. J. Morph., 66: 39-65.

Purchon,R.D. 1968. "The Biology of the Mollusca" Pergamon Press, London. 560.

Rasalam,E.J., and M.J.Sebastian. 1976. The lime-shell fisheries of the Vembanad Lake, Kerala. J. Mar. Biol. Assoc. India., 18: 323-355.

Rasmussen,L.P.D., E.Hage., and O.Karlog. 1985. An electron microscope study of the circulating leucocytes of the marine mussel, Mytilus edulis. J. Invertebr. Pathol., 45: 158-167.

Reade,P., and E.Reade. 1972. Phagocytosis in invertebrates. II. The clearance of carbon particles by the clam, Tridacna maxima. J. Reticuloendothel. Soc., 12: 349-360.

Reddy,N.M., and P.V.Rao. 1983. A possible mechanism of detoxification of copper, in the freshwater mollusc, Lymnaea luteola. Ind. J. Physiol. Pharmac., 27: 283-288.

Renwrantz,L.R., and A.Stahmer. 1983. Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of haemocytes from

Mytilus edulis. J. Comp. Physiol., 149: 535-546.

Renwrantz,L.R., T.P.Yoshino., T.C.Cheng., and K.R.Auld. 1979. Size determination of haemocytes from the American oyster, Crassostrea virginica, and the description of a phagocytosis mechanism. Zool. J. Physiol., 83: 1-12.

Reynolds,E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol., 17: 208-212.

Richardson,C.A., D.J.Crisp., and N.W.Runham. 1981. Factors influencing shell deposition during a tidal cycle in the intertidal bivalve Cerastoderma edule. J. Mar. Biol. Assoc. U.K., 61: 465-476.

Ridlington,J.W., and B.A.Fowler. 1979. Isolation and partial characterization of a cadmium-binding protein from the American oyster (Crassostrea virginica). Chem. Biol. Interactions, 25: 127-138.

Rifkin,E., and T.C.Cheng. 1968. On the formation, structure and histochemical characterization of the encapsulating cysts in Crassostrea virginica parasitized by Tylocephalum metacestodes. J. Invertebr. Pathol., 10: 51-64.

Rifkin,E., T.C.Cheng., and H.R.Hohl. 1969. An electron microscope study of the constituents of encapsulating cysts in Crassostrea virginica found in response to Tylocephalum metacestodes. J. Invertebr. Pathol., 14: 211-226.

Roberts,D. 1976. Mussels and Pollution. In: "Marine Mussels: Their Ecology and Physiology". Cambridge University Press, Cambridge. 67-80.

Robohm,R. 1984. In vitro phagocytosis by molluscan hemocytes: A survey

and critique of methods. Comp. Pathbiol., 6: 148-172.

Rodrick,G.E., and T.C.Cheng. 1974. Kinetic properties of lysozyme from the hemolymph of Crassostrea virginica. J. Invertebr. Pathol., 24: 41-48.

Rodrick,G.E., and S.A.Ulrich. 1984. Microscopical studies on the haemocytes of bivalves and their phagocytic interaction with selected bacteria. Helg. Meeres., 37: 167-176.

Roesijadi,G. 1980. Influence of copper on the clam Protothaca staminea. Effects on gills and occurrence of copper-binding proteins. Biol. Bull., 158: 233-247.

Roesijadi,G. 1980-81. The significance of low molecular weight, metallothionein-like proteins in marine invertebrates: Current status. Mar. Environ. Res., 4: 167-179.

Roesijadi,G., and R.E.Hall. 1981. Characterization of mercury-binding proteins from the gills of marine mussels exposed to mercury. Comp. Biochem. Physiol., C70: 59-64.

Ruddell,C.L. 1971a. The fine structure of oyster agranular amoebocytes from regenerating mantle wounds in the Pacific oyster, Crassostrea gigas. J. Invertebr. Pathol., 18: 260-268.

Ruddell,C.L. 1971b. The fine structure of the granular amoebocytes of the Pacific oyster, Crassostrea gigas. J. Invertebr. Pathol., 18: 269-275

Ruddell,C.L. 1971c. Elucidation of the nature and function of the granular oyster amoebocytes through histochemical studies of normal and traumatized oyster tissues. Histochemie., 26: 98-112.

Ruddell,C.L., and D.W.Rains. 1975. The relationship between zinc, copper and the basophils of two Crassostreid oysters Crassostrea gigas and Crassostrea virginica. Comp. Biochem. Physiol., A51: 585-591.

Saleuddin,A.S.M. 1967. The histochemistry of the mantle during the early stages of shell repair. Proc. Malacol. Soc. London, 37: 371-380.

Saleuddin,A.S.M. 1970. Electron microscopic study of the mantle of normal and regenerating Helix. Can. J. Zool., 48: 409-416.

Saleuddin,A.S.M. 1980. Shell formation in molluscs with special references to periostracum formation and shell regeneration. In: "Pathway in Malacology" (Eds. S.Van der Spoel., A.C.Van Bruggen., and J.Lever), Schellema and Holkema, Utrecht, The Netherlands. 47-81.

Salih,K.Y.M. 1979. Studies on the biochemical composition of the clam Meretrix casta (Chemnitz) off Cochin Barmouth. Bull. Dep. Mar. Sci. Univ. Cochin., 10: 47-73.

Schellens,J.P.M., W.T.Daems., J.J.Emeis., P.Brederoo., W.C.De Bruijn., and E.Wisse. 1977. Electron microscopical identification of lysosomes In: "Lysosomes, A Laboratory Handbook" (Ed. J.T.Dingle), North-Holland, The Netherlands. 147-208.

Schlieper,C. 1955. Die Regulation des Merzschlagens der Meismuschel Mytilus edulis (L). bei geöffneten und bei geschlossenen. Schalen. Kiel. Meeresforsch., 11: 139-148.

Schulz-Baldes,M. 1974. Lead uptake from seawater and food and lead loss in the common mussel Mytilus edulis. Mar. Biol. (Berlin), 25: 177-193.

Scott,D.M., and C.W.Major. 1972. The effect of copper (II) on survival, respiration and heart rate in the common blue mussel Mytilus edulis. Biol. Bull., 143: 679-688.

Shapiro,A.Z. 1964. The effects of certain inorganic poisons on the respiration of Mytilus galloprovincialis (L). Tr. Sevaastop. Biol. St. Akad. Nauk. Ukr. SSR., 17: 334-341.

Sharon,N., and H.Lis. 1972. Lectins: cell agglutinating and sugar specific proteins Science, 177: 949-959.

Shuster,D.N., and B.H.Pringle. 1969. Trace metal accumulation by the American eastern oyster, Crassostrea virginica. Proc. Natl. Shellfish. Assoc., 59: 91-103.

Sigma Technical Bulletin- No: 104.

Simkiss,K., and A.Z.Mason. 1983. Metal ions: Metabolic and Toxic Effects. In: "The Mollusca" (Ed. P.W.Hochachka), Academic Press, New York. 101-164.

Simkiss,K., and A.Z.Mason. 1984. Cellular responses of molluscan tissues to environmental metals. Mar. Environ. Res., 14: 103-118.

Simkiss,K., and M.Taylor. 1981. Cellular mechanisms of metal ion detoxification and some new indices of pollution. Aquat. Toxicol., 1: 279-290.

Simkiss,K., and K.M.Wilbur. 1977. The molluscan epidermis and its secretions. Symp. Zool. Soc. London., 39: 35-76.

Simkiss,K., M.Taylor., and A.Z.Mason. 1982. Review: Metal detoxification and bioaccumulation in molluscs. Mar. Biol. Lett., 3: 187-201.

Sivankutty Nair,G., and C.S.Shynamma. 1975. Studies on the salinity tolerance

of Villorita cyprinoides var. cochinensis (Hanley). Bull. Dep. Mar. Sci. Univ. Cochin., 7: 537-542.

Slanetz,L.W., C.W.Bartley., and K.W.Stanley. 1968. Coliforms, fecal Streptococci and Salmonella in seawater and shellfish. Health Lab. Sci., 5: 66-78.

Slater,T.E. 1978. Biochemical studies on liver injury. In: "Biochemical Mechanisms of Liver Injury". (Ed. T.F.Slater), Academic Press, New York. 2-44.

Sminia,T. 1972. Structure and function of blood and connective tissue cells of the fresh-water pulmonate Lymnaea stagnalis studied by electron microscopy and enzyme histochemistry. Z. Zellforsch., 130: 497-526.

Sminia,T., and W.P.W.Van der Knaap. 1986. Immunorecognition in invertebrates with special reference to molluscs. In: "Immunity in Invertebrates (Ed. M.Brehelin), Springer-Verlag, Berlin Heidelberg. 112-124.

Sparks,A.K. 1972. "Invertebrate Pathology: Non-communicable Diseases". Academic Press, New York.

Sparks,A.K., and G.B.Pauley. 1964. Studies of the normal postmortem changes in the oyster, Crassostrea gigas (Thunberg). J. Insect. Pathol., 6: 78-101.

Sporn,A., I.Dinu., and I.Stvensecu. 1970. Influence of cadmium administration on the carbohydrate and cellular energetic metabolism in the rat liver. Rev. Roum. Biochem., 7: 299-305.

Spronk,N., F.Tilders., and R.F.van Hoek. 1973. Copper in Lymnaea stagnalis. III. Uptake from freshwater and the role of the shell. Comp.

Biochem. Physiol., A45: 257-272.

Stauber,L.A. 1950. The fate of India ink injected into the oyster Ostrea virginica Biol. Bull., 98: 227-241.

Stauber,L.A. 1961. Immunity in invertebrates with special reference to the oyster. Proc. Natl. Shellfish. Assoc., 50: 7-20.

Sternlieb,I., and S.Goldfischer. 1976. Heavy metals and lysosomes. In: "Lysosomes in Biology and Pathology" (Eds. J.T.Dingle., and R.T.Dean), Elsevier, Amsterdam. Vol.V., 185-200.

Stokes,T., and J.Awapara. 1968. Alanine and succinate as end-products of glucose degradation in the clam Rangia cuneata. Comp. Biochem Physiol., 25: 883-892.

Stumpf,J.L., and D.E.Gilbertson. 1978. Hemocytes of Biomphalaria glabrata: Factors affecting variability. J. Invertebr. Pathol., 32: 177-181.

Stumpf,J.L., and D.E.Gilbertson. 1980. Differential leukocytic responses of Biomphalaria glabrata to infection with Schistosoma mansoni. J. Invertebr. Pathol., 35: 217-218.

Summer,A.T. 1969. The distribution of some hydrolytic enzymes in the cells of the digestive gland of certain lamellibranchs and gastropods. J. Zool. Lond., 158: 277-291.

*Takatsuki,S.I. 1934a. On the nature and functions of the amoebocytes of Ostrea edulis. Quart. J. Microsc. Sci., 76: 377-436.

*Takatsuki,S.I. 1934b. Beitrage Zur Physiologie des Austerherzens V. uber den Ban des Herzens unter besonderer Beruch sichtigung seiner physiologischen Reaktionen. Sci. Rept. Tokyo Bunrika Daigaku.,

Sec. B2: 55-62.

Talbot,V., and J.Magee. 1978. Naturally occurring heavy metal binding proteins in invertebrates. Arch. Environ. Contam. Toxicol., 7: 73-81.

Tanaka,K., T.Takasugi., and H.Maoka. 1961. Morphological characteristics of the blood corpuscles of the common oyster, Gryphea gigas. Bull. Jap. Soc. Sci. Fish., 27: 365-371.

*Theede,H. 1963. Experimentelle Untersuchungen Über due Filtrations leistung der Miermuschel Mytilus edulis. Kiel. Meeresforsch., 19: 20-41.

Thompson,R.J., and B.L.Bayne. 1972. Active metabolism associated with feeding in the mussel Mytilus edulis (L). J. Exp. Mar. Biol. Ecol., 9: 111-124.

Thompson,R.J., N.A.Ratcliffe., and B.L.Bayne. 1974. Effects of starvation on structure and function in the digestive gland of the mussel (Mytilus edulis L.). J. Mar. Biol. Assoc. U.K., 54: 699-712.

Thompson,R.J., C.J.Bayne., M.N.Moore., and T.H.Carefoot. 1978. Haemolymph volume, changes in the biochemical composition of the blood, and cytochemical responses of the digestive cells in Mytilus californianus Conrad, induced by nutritional, thermal and exposure stress. J. Comp. Physiol., 127: 287-298.

Thurberg,F.P., A.Calabrese., and M.A.Dawson. 1974. Effects of silver on oxygen consumption of bivalves at various salinities. In: "Pollution and Physiology of Marine Organisms". (Eds. F.J.Vernberg and W.B.Vernberg), Academic Press, New York. 67-78.

Timmermans,L.P. 1973. Mantle activity following shell injury in the pond snail,

Lymnaea stagnalis (L). Malacologia, 14: 53-61.

Tripp,M.R. 1958a. Disposal by the oyster of intracardially injected vertebrate red blood cells. Proc. Natl. Shellfish. Assoc., 48: 143-147.

Tripp,M.R. 1958b. Studies on the defence mechanism of the oyster. J. Parasitol., 44: 35-36.

Tripp,M.R. 1960. Mechanisms of removal of injected micro-organisms from the American oyster, Crassostrea virginica (Gmelin). Biol. Bull., 119: 210-223.

Tripp,M.R. 1966. Hemagglutinin in the blood of the oyster Crassostrea virginica. J. Invertebr. Pathol., 8: 478-484.

Tripp,M.R., C.G.Fries., M.A.Craven., and C.E.Grier. 1984. Histopathology of Mercenaria mercenaria as an indicator of pollutant stress. Mar. Environ. Res., 14: 521-524.

Tsujii,T. 1976. An electron microscopic study of the mantle epithelial cells of Anodonta sp. during shell regeneration. In: "The Mechanisms of Mineralization in the Invertebrates and Plants" (Eds. N.Watabe and K.M.Wilbur), Univ. of South Carolina Press, Columbia. 339-353.

Udom,A,O., and F.O.Brady. 1980. Reactivation in vitro of zinc-requiring apo-enzymes by rat liver zinc-thionein. Biochem. J., 187: 329-335.

Ulmer,D.D. 1970. Effects of metals on protein strsucture: In: "Effects of Metals on Cells, Subcellular Elements and Macromolecules" (Eds. J.Maniloff., J.R.Coleman., and M.W.Miller), Charles C. Thomas Publishers, Springfield. 11-35.

Vallee.B.L. 1979. Metallothionein: Historical Review and Perspectives. In: "Metallothionein" (Eds. J.H.R.Kägi., and M.Nordberg), Birkhäuser Verlag, Basel, Boston, Stuttgart. 19-40.

Vallee,B.L., and D.D.Ulmer. 1972. Biochemical effects of mercury, cadmium and lead. Ann. Rev. Biochem., 41: 103-108.

Vallee,B.L., and W.E.Wacker. 1970. Metalloproteins. In: "The Proteins" (Ed. H.Neurath), Second Edition, Academic Press, New York. Vol.V.

Vasta,G.R., T.C.Cheng., and J.J.Marchalonis. 1984. A lectin on the hemocyte membrane of the oyster (Crassostrea virginica). Cell Immunol., 88: 475-488.

Vasta,G.R., J.T.Sullivan., T.C.Cheng., J.J.Marchalonis., and G.W.Warr. 1982. A cell membrane-associated lectin of the oyster hemocyte. J. Invertebr. Pathol., 40: 367-377.

Verity,M.A., and A.Reith. 1967. Effects of mercurial compounds on structure linked latency of lysosomal hydrolases. Biochem. J., 105: 685-690.

Viarengo,A., G.Zanicchi., M.N.Moore., and M.Orunesu. 1981a. Accumulation and detoxication of copper by the mussel Mytilus galloprovincialis Lam.: A study of the subcellular distribution in the digestive gland cells. Aquat. Toxicol., 1: 147-157.

Viarengo,A., M.Pertica., G.Mancinelli., R. Caplli., and M.Orunesu. 1980a. Effects of copper on the uptake of amino acids, on protein synthesis, and on ATP content in different tissues of Mytilus galloprovincialis Lam. Mar. Environ. Res., 4: 145-152.

Viarengo,A., M.Pertica., G.Mancinelli., S.Palmero., and M.Orunesu. 1982. Effects of Cu^{2+} on nuclear RNA polymerase activities in the mussel digestive gland. Mar. Biol. Lett., 3: 345-352.

Viarengo,A., M.Pertica., G.Mancinelli., G.Zanicchi., and M.Orunesu. 1980b. Rapid induction of copper-binding proteins in the gills of metal exposed mussels. Comp. Biochem. Physiol., C 67: 215-218.

Viarengo,A., M.N.Moore., M.Pertica., G.Mancinelli., G.Zanicchi.,and R.K.Pipe. 1985. Detoxification of copper in the cells of the digestive gland of mussel: The role of lysosomes and thioneins. The Science of the Total Environment, 44: 135-145.

Viarengo,A., M.Pertica., G.Mancinelli., S.Palmero., G.Zanicchi., and M.Orunesu. 1981b. Synthesis of Cu-binding proteins in different tissues of mussels exposed to the metal. Mar. Pollut. Bull., 12: 347-350.

Viarengo,A., M.Pertica., G.Mancinelli., G.Zanicchi., J.M.Bouquegneau., and M.Orunesu. 1984a. Biochemical characterization of Cu-thioneins isolated from the tissues of mussels exposed to the metal. Mol. Physiol., 5: 41-52.

Viarengo,A., M.Pertica., G.Mancinelli., M.Orunesu., G.Zanicchi., M.N.Moore., and R.K.Pipe. 1984b. Possible role of lysosomes in the detoxication of copper in the digestive gland cells of metal-exposed mussels. Mar. Environ. Res., 14: 469-470.

Wagge,L.E. 1951. The activity of amoebocytes and of alkaline phosphatases during the regeneration of the shell in snail, Helix aspersa. Quart. J. Microsc. Sci., 92: 307-321.

Wagge,L.E. 1955. Amoebocytes. Int. Rev. Cytol., 4: 31-78.

Wagge,L.E., and T.Mittler. 1953. Shell regeneration in some British molluscs. Nature, 171: 528-529.

Waldichuk,M. 1979. Review of the problems. Philosophical Transactions of the Royal Society, London. Series B 286: 399-424.

Watabe,N. 1983. Mollusca: Shell. In: "Biology of the Integument"- Invertebrates. (Ed. K.S.Richards), Springer-Verlag, New York/Berlin. Vol.I.

Watabe,N., and P.L.Blackwelder. 1976. Studies on shell regeneration in molluscs. III. Effects of inorganic ions on shell minerology; ultrastructural changes of mantle epithelium. Am. Zool., 16: 249.

*Watabe,N., and P.L.Blackwelder. 1980. Ultrastructural and calcium localization in the mantle epithelium of the fresh water gastropod Pamacea paludosa during shell regeneration. In: "The mechanisms of Biomineralization in Animals and Plants" (Eds. M.Omori., and N.Watabe), Tokai Univ., Press, Tokyo, Japan. 131-144.

Webb,J.L. 1966. "Enzyme and Metabolic Inhibitors", Academic Press, New York. Vol. II, III and IV.

*Wells,W.F. 1916. Artificial purification of oysters. Pub. Health. Rep., 31: 1648-1852.

Weser,U., and L.Hubner. 1970. Cd^{2+}, Mn^{2+}, and Zn^{2+} -induced synthesis of nuclear RNA in the livers of normal and adrenalectomized rats. FEBS Lett., 10: 169-174.

Widdows,J., and B.L.Bayne. 1971. Temperature acclimation of Mytilus edulis with reference to its energy budget. J. Mar. Biol. Assoc. U.K., 51: 827-843.

Wilbur,K.M. 1964. Shell formation and regeneration. In: "Physiology of Mollusca" (Eds. K.M.Wilbur., and C.M.Yonge), Academic Press, New York. Vol.I. 243-282.

Wilbur,K.M., and A.S.M.Saleuddin. 1983. Shell formation. In: The Mollusca. Academic Press, New York. Vol.IV., Part 1: 248.

Wittke,M., and L.Renwrantz. 1984. Quantification of cytotoxic hemocytes of Mytilus edulis using a cytotoxicity assay in agar. J. Invertebr. Pathol., 43: 248-253.

Wood,P.C. 1961. The principles of water sterilization by ultraviolet, and their application in the purification of oysters. Fish. Invest. Min. Agr. Fish. Food. (Gt. Brit.). Ser.1. Sea Fish., 23: 1-48.

Yonge,C.M. 1923. Studies on the comparative physiology of digestion. I. The mechanism of feeding, digestion, and assimilation in the lamellibranch, Mya. Br. J. Exp. Biol., 1: 15-63.

Yonge,C.M. 1926. Structure and physiology of the organs of feeding and digestion in Ostrea edulis. J. Mar. Biol. Assoc. U.K., 14: 295-386.

Yonge,C.M., and H.M.Nicholas. 1940. Structure and function of the gut and symbiosis with Zooxanthellae in Tridacna crispata (Oerst). Pap. Tortugas. Lab., Carnegie Inst., 32: 287-301.

Yoshino,T.P. 1976. Histopathological effects of larval Digenea on the digestive epithelia of the marine prosobranch Cerithidea californica: fine structural changes in the digestive gland. J. Invertebr. Pathol., 28: 209-216.

Yoshino,T.P., and T.C.Cheng. 1976a. Fine structural localizaion of acid

phosphatase in granulocytes of the pelecypod Mercenaria mercenaria. Trans. Am. Microsc. Soc., 95: 215-220.

Yoshino,T.P., and T.C.Cheng. 1976b. Experimentally induced elevations of aminopeptidase activity in hemolymph cells of the American oyster, Crassostrea virginica. J. Invertebr. Pathol., 27: 367-370.

Yoshino,T.P., and T.C.Cheng. 1977. Aminopeptidase activity in the hemolymph and body tissues of the pulmonate gastropod Biomphalaria glabrata. J. Invertebr. Pathol., 30: 76-79.

Yoshino,T.P., and W.O.Granath.Jr. 1985. Surface antigens of Biomphalaria glabrata (Gastropoda) hemocytes: Functional heterogeneity in cell sub-populations recognised by a monoclonal antibody. J. Invertebr. Pathol., 45: 174-186.

Yoshino,T.P., L.R.Renwrantz., and T.C.Cheng. 1979. Binding and redistribution of surface membrane receptors for concanavalin A on oyster hemocytes. J. Exp. Zool., 207: 439-450.

Yoshino,Y., T.Mozai., and K.Nakao. 1966. Biochemical changes in the brain in rat poisoned with an alkyl-mercury compound; with special reference to the inhibition of protein synthesis in brain cortex slices. J. Neuro. Chem., 13: 1223-1230.

Zaba,B.N., and E.J.Harris. 1978. Accumulation and effects of trace metal ions in fish liver mitochondria. Comp. Biochem. Physiol., C 61: 89-93.

Zacks,S.L. 1955. The cytochemistry of the amoebocytes and intestinal epithelium of Venus mercenaria (Lamellibranchiata) with remarks on a

pigment resumbling ceroid. Quart. J. Microsc. Sci., 96: 57-71.

Zacks,S.L., and J.H.Welsh. 1953. Cholinesterase and lipase in the amoebocytes, intestinal epithelium and heart muscle of the quahog, Venus mercenaria. Biol. Bull., 105: 200-211.

Zar,J.H. 1974. "Biostatistical Analysis", Prentice Hall, Englewood Cliffs, N.J.

Zurburg,W., and R.H.M.Ebberink. 1981. The anaerobic energy demand of Mytilus edulis. Organ specific differences in ATP -supplying processes and metabolic routes. Mol. Physiol., 1: 153-164.

Zurburg,W., and J.H.Kluytmans. 1980. Organ specific changes in energy metabolism due to anaerobiosis in the sea mussel Mytilus edulis (L). Comp. Biochem. Physiol., B67: 317-322.

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