STUDIES ON HAEMOLYMPH CONSTITUENTS OF *INDOPLANORBIS EXUSTUS* (DESHAYES) AND *LYMNAEA ACUMINATA* (LAMARCK) F. *RUFESCENS* (GRAY) AND THE EFFECTS OF COPPER ON THE ACTIVITY PATTERN OF SELECTED TRANSAMINASES AND PHOSPHATASES

THESIS

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BY

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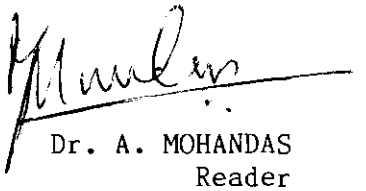
CERTIFICATE

This is to certify that this thesis is an authentic record of the research work carried out by Mr. SURESH, P.G., under my scientific supervision and guidance in the School of Environmental Studies, 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 Environmental Studies, and no part thereof has been presented for the award of any other degree, diploma, or associateship in any University.



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DECLARATION

I, Suresh, P.G., do hereby declare that this thesis entitled "STUDIES ON HAEMOLYMPH CONSTITUENTS OF INDOPLANORBIS EXUSTUS (DESHAYES) AND LYMNAEA ACUMINATA (LAMARCK) F. RUFESCENS (GRAY) AND THE EFFECTS OF COPPER ON THE ACTIVITY PATTERN OF SELECTED TRANSAMINASES AND PHOSPHATASES" 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.

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C O N T E N T S

C H A P T E R - I

INTRODUCTION

The phylum mollusca constitutes one of the major divisions of the animal kingdom, and is of unusual interest both in regard to the diversity of organization and in the multitude of living species. The molluscs greatly vary in form, structure, habits, and habitats. They are highly adaptive and occupy all possible aquatic and terrestrial habitats. The phylum includes animals of wide diversity in form, such as the common slugs and snails, slow moving chitons, oysters and clams, swift darting squids, slithering octopuses, and the chambered nautilus. Molluscs have particular importance in that they form valuable fisheries in various parts of India as they are being used as food, as a source of lime, pearls and decorative shells, and as constituents of medical preparations. Thus, molluscs in general, have occupied a marked place in the affairs of man for time immemorial in his affairs of state and economy, of mind and aesthetic values, and of religion and rites of worship. In more recent times they have come to occupy prominent position in heraldy and royal insignia, and more conspicuously in the economy of vast section of the people. The gastropod molluscs constitute an important part of the ecosystem, and many aquatic animals thrive on them. Gastropods, including slugs and snails are the most successful of all molluscs, and are of special concern in that they serve as intermediate and as paratenic hosts of a variety of helminth parasites causing diseases in man and domestic animals. It is still not very clear why certain snail species are refractory to infection

while yet others are susceptible, and even among the susceptible ones, only certain age group snails are infected. This aspect is fascinating to investigate because, of late, susceptibility to trematode infection is being correlated with weak defence mechanism and it has been proved beyond doubt that haemocytes play an important role in internal defence against foreign materials. Obviously, haemolymph also plays a significant role in the defence mechanisms. Yet another aspect is to find out whether haemolymph can be treated as an organ system because in most of the studies in the past dealing with biochemical, physiological and metabolic changes, particular attention was given to determine the level of changes in specific organs such as the muscles, mantle, gills, digestive gland etc., but haemolymph was seldom considered as an organ system. Of late, it has been, however, shown that several parameters of blood can be taken as reliable indicators for diagnostic purposes, and also to monitor environmental pollution. The present investigation was, therefore, carried out on two species of freshwater gastropods for the following reasons, (i) to find out whether age, biotic and abiotic factors bring about any change in the haemolymph constituents particularly in haemocyte numbers since the haemocytes play a very significant role in cellular defence mechanisms of molluscs, (ii) to understand the various aspects of molluscan blood since very little work has been done in India, and (iii) to monitor pollution in freshwater environment as it has been suggested that quantitative determination of the levels of lysosomal enzymes and transaminases

can be employed as reliable indicator of the stress by environmental pollution.

The snail species selected for the present study are Indoplanorbis exustus (Deshayes), and Lymnaea acuminata (Lamarck) f. rufescens (Gray). Both the species serve as intermediate host for a large number of digenetic trematodes. A large number of cercariae are recorded from I. exustus, and a large number of trematodes of domestic and wild animals pass their larval stages through this molluscan species. L. acuminata f. rufescens is also recorded as the intermediate host of many flukes, and several cercarial forms have been recorded from this snail species (Rao, 1989).

It was reported that species of Lymnaea, L. stagnalis, is suitable as a test organism in toxicological studies (Canton and Sloof, 1977). This mollusc is easy to handle and culture, and is available in various quantities and stages of embryonic development. Canton and Sloof (1977) reported that L. stagnalis is a good biological indicator for establishing ecological limits for pollutants in surface waters. The adults are often used as test organism in acute toxicity experiments to measure mortality, immobility, and heart rate as criteria of toxicity (Batte et al., 1951; Patrick and Cairns, 1968; Sheanon and Trama, 1972; Knauf and Schulze, 1973; Polster, 1973).

Since gastropods have open circulatory system and the organ systems are bathed in haemolymph, any change in relation to abiotic or biotic stress is immediately reflected in blood and hence in the present study various haematological parameters of the two snail species were investigated in normal as well as in those under stress conditions. The haemolymph parameters studied were total haemocyte number, packed cell volume, haemoglobin (in I. exustus), and inorganic and organic constituents in three size groups of both the snail species. Moreover, the influence of various biotic and abiotic factors on total cell count was also investigated considering the importance of haemocytes in cellular defence mechanisms. To study the effect of pollution, copper was chosen as the pollutant because copper is the active ingredient in almost all molluscicide formulations, and the effect of copper toxicity was measured in terms of total haemocyte counts, and the activity pattern of selected phosphatases and transaminases.

The impact of pollutants on an organism is realized as perturbations at different levels of functional complexity (Moore, 1985). Xenobiotic induced sublethal cellular pathology reflects perturbations of function and structure at the molecular levels. In most cases the easiest detectable changes are associated with a particular type of subcellular organelle such as lysosomes, endoplasmic reticulum, and mitochondria. Cellular and subcellular responses to a variety of pollutants have been reported from a wide

range of animals. In molluscs, such responses reported include hepatopancreatic epithelial reduction in bivalves (Lowe et al., 1981; Couch, 1984), lysosomal disruption in mussels in response to copper and phenanthrene (Pickwell and Steinert, 1984, Moore et al., 1984), and inhibition of cellular immunity (Cheng and Sullivan, 1984). There are numerous studies in relation to accumulation and toxic effects of heavy metal ions including copper in molluscs (Menzel, 1979; Cunningham, 1979; Moore, 1985; Viarengo, 1985; Livingstone, 1985). Haemocytes are believed to play significant role in heavy metal metabolism, i.e., in the uptake, distribution to various tissues, and in the intralysosomal storage of metals (Galtstoff, 1964; Cunningham, 1979). However, very little information is available regarding the effects of toxic environmental chemicals on the immune functions in gastropods particularly when haemocytes are also involved in internal defence, and hence it was thought worthwhile (i) to study the effects of copper, ions on haemocyte number, and (ii) to examine the activity levels of selected lysosomal as well as non-lysosomal enzymes as xenobiotics can induce lysosomal destabilisation on molluscan cells, and quantitative measurements of the levels of both marker lysosomal and non-lysosomal enzymes have been indicated as reliable indicator of the presence of pollutants in the immediate environment.

Many trace metals are important in plant and animal nutrition, and as micronutrients they play an aessential role in tissue metabolism and growth. Among the essential trace metals copper has

an important role and it is an essential component of many enzymes. However, not all of these enzyme activities are decreased in copper deficiency to the level that they are metabolically limiting. Copper is known to become toxic to aquatic organisms when the concentration exceeds tolerable limits. Copper sulphate is used in aquaculture for the treatment of ectoparasites and to eradicate certain diseases. Copper compounds are commonly used as molluscicides, and among them copper sulphate is the most important one. Copper sulphate is a less expensive molluscicide and is found effective in destroying the molluscan intermediate hosts of a variety of trematodes. However, it was reported that like all other major molluscidides copper sulphate has also certain disadvantages (see Ritchie, 1973). It was found to be totally or partially inactivated in natural waters due to adsorption by soil and organic materials, and is ineffective at alkaline pH's, and is toxic to other non-target organisms especially young fishes and certain aquatic vegetation (Cheng and Sullivan, 1975). In the present study, copper was chosen as the toxicant to study the effect of heavy metal pollution on the haemolymph for the following reasons: (i) copper is the main ingredient in almost all the molluscicides now in use, and (ii) very little is known about the pathophysiology and toxic mechanisms of cupric ions on gastropods and particularly so on total haemocyte counts, and hence in defence mechanisms.

The concept of haematological manifestation in response to abiotic and/or biotic stress is applied widely for identification

of stress factors and much of the information regarding the haemocytes and their variation due to stress has come from studies involving insects, crustaceans and molluscs. Molluscan haemocytes have been implicated in diverse functions such as wound repair, nutrient digestion and transport, excretion, and internal defence which include phagocytosis and encapsulation. Although the haemocytes in bivalve molluscs have been classified into granulocytes and agranulocytes (Cheng, 1981) differences continue to exist. Regarding gastropods, while Ottaviani (1983) reported two distinct haemocyte types, spreading and round, in Planorbis corneus which are not different maturational stages of a single cell type, Sminia et al. (1983) reported round and spreading haemocytes in L. stagnalis, and considered these cells as different maturational stages of a single cell. Renwrantz et al. (1979), Cheng (1980), Mohandas (1985), and Cheng and Downs (1988) have reported the occurrence of subpopulations of haemocytes in molluscs. Differences were also observed between blood cells of juvenile and adult specimens of L. stagnalis (and this difference was attributed to be one of the reasons for varying susceptibility to infection by larval trematodes) (Dikkeboom et al., 1984), and also in two different strains of Biomphalaria glabrata (Stumpf and Gilbertson, 1978). Several biotic as well as abiotic factors are reported to affect the number and distribution of circulating haemocytes. These factors include infection, snails size, age, host strain difference, temperature, wounding, heavy metal stress etc. (Sminia, 1981). In the present study the total

haemocyte counts in three size groups-juveniles, intermediate, and adults- of both the snail species were studied. Moreover, the effect of various abiotic and biotic factors on total haemocyte number was also investigated. The various factors selected for the study were temperature, pH, snail-conditioned water, and heavy metal - copper-stress.

It was reported that the organic in organic composition of molluscan haemolymph is variable (Bayne, 1973; Thompson, 1977). Burton (1983) reported that several factors shell size/age such as temperature, rainfall, photoperiodism, hibernation, starvation, oviposition etc. affect the biochemical composition of the haemolymph in molluscs. On gastropods, very little information is available concerning the influence of shell size/age on plasma metabolites, and hence to generalize the inorganic and organic composition of the haemolymph such a study on different age group snails is needed. In the present investigation the various inorganic and organic constituents in the haemolymph of the two snails species in relation to size/age were analysed and reported. The various inorganic constituents studied were haemolymph sodium, potassium, calcium, chloride, and ammonia while the organic constituents were urea, total carbohydrate, glycogen, total protein, and total lipids.

Molluscs generally have low enzyme level and information on their specific roles is sparse (Fried and Levin, 1973). Enzymes by themselves are not present in the haemolymph, unless they belong

either to the haemocytes or leak from intracellular confines of the damaged tissues and hence serum enzymes levels were considered to be of diagnostic value (Jyothirmayi and Rao, 1987). In the present study, activity levels of lysosomal and non-lysosomal enzymes - phosphatases and transaminases- in the haemolymph of normal and copper exposed snails were estimated to understand the effect of copper on the activity pattern of enzymes and also to examine its diagnostic value. The enzymes selected for the present study were acid phosphatase, alkaline phosphatase, glutamate-oxaloacetate transaminase, and glutamate-pyruvate transaminase. Lysosomes and cell membrane are the first target of pollutants because lysosomes are concerned with the disintegration of foreign materials and the cell membrane is the first barrier to a xenobiotic agent. Acid phosphatase is a lysosomal marker enzyme and alkaline phosphatase is considered by some as lysosomal enzyme and by others as plasma membrane enzyme. During the period of stress, in the most likely event of lysosomal membrane disruption, these enzymes are released into the haemolymph thus increasing the enzyme level there. Hence, these two enzymes can be treated as reliable indicators of stress. It was reported that when copper accumulates in mammalian tissues, a significant rise in serum transaminases and lactic dehydrogenase occurs. Determination of the activity levels of serum glutamate oxaloacetate transaminase and glutamate pyruvate transaminase has therefore been proposed as an aid in detection of chronic copper poisoning (Metz and Sagone, 1972).

The thesis is arranged in six chapters. The general introduction forms the first chapter. The second chapter is on total haemocyte counts and the factors affecting variability. In the third chapter the inorganic and organic constituents of haemolymph are reported. The effect of copper on the activity patterns of phosphatases, and transaminases forms the subject matter of chapters four and five. Summary of the work forms the sixth chapter, followed by the list of references.

C H A P T E R - II

TOTAL HAEMOCYTE COUNTS AND FACTORS AFFECTING VARIABILITY IN *INDOPLANORBIS EXUSTUS* AND *LYMNAEA ACUMINATA* F. *RUFESCENS*

2.1 INTRODUCTION

The morphology and functions of the cells present in the haemolymph of gastropod molluscs have been investigated by many (Tripp, 1970; Sminia, 1972; Sminia et al., 1973, 1974; Yoshino, 1976; Cheng and Garrabrant, 1977). The blood cells of gastropods are often called leucocytes, other names used are haemocytes, amoebocytes, granulocytes, lymphocytes, and macrophages (Wagge, 1955; Cheng et al., 1969; Davies and Partridge, 1972; Cheng and Auld, 1977). Although many studies have been undertaken on the structure and functions of gastropod blood cells, there still exists no agreement on the number of blood cell types present in gastropods (Sminia, 1972). This disagreement is mainly due to the use of different cell type concepts (Sminia et al., 1983). Many authors consider cells with slight morphological variations as different cell types, others, distinguish different cell types on the basis of small variations in diameter, while still others point to the importance of functional characteristics. Ultrastructural and histochemical studies on gastropod blood cells have failed to solve the question of the number of blood cell types. A number of investigators are of the opinion that gastropods possess two distinct types of blood cells, granulocytes and hyalinocytes (Harris, 1975; Yoshino, 1976; Cheng and Auld, 1977). On the other hand, Sminia

(1972) who has done extensive work on Lymnaea stagnalis blood cells has indicated that there is only one type of blood cell in gastropods, the amoebocytes, which are either round or spreading.

Considering haemotopoiesis, it was reported that molluscs in general lack well-defined haematopoietic organs (Cheng and Rifkin, 1970; Narain, 1973). In gastropods, parts of connective tissue or of epithelia have been reported to function primarily as proliferation sites of blood cells. The epithelium and the connective tissue of the mantle in Helix aspersa (Wagge, 1955); kidney wall in Biomphalaria glabrata (Pan, 1958); the ventricle and the connective tissue of the mantle in Bulinus africanus and B. truncatus (Kinoti, 1971), and in L. stagnalis the connective tissue (Muller, 1956), especially around the lung cavity (Sminia et al., 1983). Wolmarans and Yssel (1988) reported that in B. glabrata oxygen tension in water could also result in haemocytosis, but source was not indicated. It has been suggested that the blood cells originate (a) from precursor cells (amoeboblasts, Kinoti, 1971; Jeong et al., 1983), (b) from mantle amoebocytes (George and Ferguson, 1950; Brown and Brown, 1965), or (c) fibroblasts, epithelial or endothelial cells (Wagge, 1951, 1955; Pan, 1958). In B. glabrata, Lie et al. (1975) found an organ located between the pericardium and the posterior epithelium of the mantle cavity that appeared to be responsible for the production of amoebocytes; and later this was confirmed by Jeong et al. (1983) through EM

studies. Three mechanisms of proliferation have been proposed, mitosis, amitosis, and cytoplasmic fragmentation (Wagge, 1955; Cheng and Rifkin, 1970; Sminia, 1974).

The concept of haematological manifestations in response to stress, either biotic or abiotic, is well known and is applied widely for identification of stress factors. One of the known manifestations of stress in molluscs is significant fluctuations in total haemocyte counts, and besides stress several other factors also affect the number and distribution of circulating haemocytes. These factors include infection, snail size, host strain difference, temperature, wounding, oxygen tension etc. The number of cells per ml of haemolymph varies from species to species, and even within a species shows large variations. The number of blood cells per ml is reported to be 3.8 - 7.2 x 10^6 in Bullia laevissima (Brown and Brown, 1965); 0.2 x 10^6 in H. pomatia (Bayne, 1974); 1.0 - 9.0 x 10^6 in Patella vulgata (Davies and Partridge, 1972), 0.5 - 4 x 10^6 in L. stagnalis (Sminia, 1972), and 0.1 - 1 x 10^6 in B. glabrata (Jeong and Heyneman, 1976; Cheng and Auld, 1977; Stumpf and Gilbertson, 1978). The number of blood cells varies considerably in blood collected from different body parts. Thus, Brown and Brown (1965) reported that in B. laevissima, haemolymph samples taken from heart and arteries were richer in blood cells than in those taken from veins, while sinuses showed a still lower cell number. The number of blood cells will also vary in snails living under

different conditions. While studying intraspecific variability of certain chemical parameters in the haemolymph of B. glabrata, Michelson and Dubois (1975) found an association between the parameters and the size and strain of the snail. Feng found (1965) no relation between the haemocyte number and size of the oyster, Crassostrea virginica. The number of circulating blood cells in B. glabrata and L. stagnalis (Stumpf and Gilbertson, 1978; Dikkeboom et al., 1984) seems to be related to the age of the animal. The density of circulating amoebocytes in the haemolymph of juvenile L. stagnalis is 3 to 4 times lower than in that of adult snails. A temperature dependent variation in haemocyte number was reported by Davies and Partridge (1972), and Stumpf and Gilbertson (1976). In P. vulgata, Davies and Partridge (1972) reported that the concentration of circulating blood cells varies from about 1×10^6 cells per ml at $5^{\circ}C$ to 9×10^6 cells per ml at $25^{\circ}C$. In B. glabrata, the number of circulating cells increases rapidly when the temperature rises (Stumpf and Gilbertson, 1978). The number of circulating blood cells of H. pomatia was found to decrease after injection of foreign particles (Bayne, 1974), but in B. laevissima after this initial decrease, the number increases within 7 days to about 2 - 5 times the original number (Brown and Brown, 1965). Sminia (1972) reported an increase in haemocyte number within 1 hr in L. stagnalis after haemolymph withdrawal. The haemocyte counts in B. glabrata have been determined after the snails were kept for two hours in snail-conditioned water, and under immobilized

and anaerobic conditions (Wolmarans and Yssel, 1988). In anaerobic conditions, the haemocyte number increased significantly after 2 hr. Infection is another factor which influences the number of haemocytes. Michelson and Dubois (1975) and Stumpf and Gilbertson (1980) reported an increase in the haemocyte number in freshwater molluscs after infection with parasites.

Dikkeboom et al. (1984) who studied blood cells of L. stagnalis reported the following differences between the juvenile and adult snails. Juvenile snails contain fewer circulating amoebocytes per ml haemolymph. The number of circulating amoebocytes as well as haemolymph volume are found to be much lower than in adult snails. However, a higher percentage of these cells shows mitotic activity. The cells of juvenile snails are small and round with few inclusions having a high nucleocytoplasm ratio, and a high pyronin stainability. The enzymes acid phosphatase, non-specific esterase, and alkaline phosphatase are present in all amoebocytes of juvenile and adult snails. The activity of peroxidase differed in two size groups, in juveniles a lower percentage of the cells are positive, and the granules that contain the activity are less abundant than in amoebocytes of adults. It was reported that the activity of the internal defence system in juvenile L. stagnalis is on a lower level than that in adult snails. Dikkeboom et al. (1985) reported that the phagocytic capacity of the circulating amoebocytes of juvenile L. stagnalis is lower than that of adult snails.

The occurrence of subpopulations of haemocytes in molluscs is reported by Cheng et al. (1980b), Mohandas (1985), Dikkeboom et al. (1985), and Cheng and Downs (1988). Cheng et al. (1980b) reported that in Crassostrea virginica four subpopulations of granulocytes and one population of hyalinocyte exist. Dikkeboom et al. (1985) also developed a panel of monoclonal antibodies directed against L. stagnalis haemocytes, which enabled to distinguish antigenically different subpopulations some of which occur in different proportions in juvenile, adult, and infected snails. Monoclonal antibodies that react with membrane antigens were used to separate haemocyte subpopulations. Those monoclonal antibodies that react with haemocytes of juvenile snails or those of adults were used to study the ontogeny of the haemocyte system.

Gastropods possess a well developed innate defence system where blood cells and/or haemolymph play a predominant role. Several functions have been attributed to the blood cells of molluscs. These cells have been suggested to play a role in defence reactions such as phagocytosis (Tripp, 1961; Feng, 1967; Sminia, 1972), encapsulation and infiltration of foreign tissues (Pan, 1965; Cheng and Galloway, 1970; Cheng and Rifkin, 1970; Tripp, 1970), in the synthesis, uptake, and transport of substances, such as glycogen and calcium granules (Kapur and Gupta, 1970; Abolins-Krogis, 1961, 1972), and in wound healing (Des Voigne and Sparks, 1968; Pauley and Heaton, 1969; Armstrong et al., 1971; Ruddel, 1974).

Gastropod molluscs possess a very effective immune system which can dispose a variety of foreign particles and organisms (Michelson, 1975; Sminia, 1981). Snails like B. glabrata, H. pomatia and L. stagnalis are able to clear large doses of foreign injected material rapidly from their haemolymph (Tripp, 1961; Bayne, 1980; Renwrantz et al., 1981; Van der Knaap et al., 1981). Phagocytosis and encapsulation are the two major processes involved in the cellular internal defence of gastropods. Phagocytic cells are found in the haemolymph and tissues of gastropods and have been considered to be an important component of the cellular defence system (Tripp, 1961; Cheng et al., 1970). Sminia (1980) studied the phagocytic cells of L. stagnalis, and reported one type of free phagocyte (amoebocytes), and two types of fixed phagocytes (reticulam cells, and pore cells). Morphological and ultrastructural studies have shown that amoebocytes phagocytose foreign particles by (i) extending pseudopodia which engulf the foreign particles, or (2) by forming invaginations of the plasma membrane. Both processes result in the particle becoming enclosed in phagosomes, and digested in the lysosomal system.

Encapsulation is the isolation of invading organisms and foreign bodies from host tissue by the formation of a fibrous envelope or capsule. Encapsulation of experimentally introduced abiotic as well as biotic material was studied in L. stagnalis (Sminia et al., 1974). It appears that abiotic materials are encapsulated within 24 hr by a large number of amoebocytes. All

biotic implants, autografts and allografts, are not encapsulated, only a transient amoebocyte reaction occurs on the cut surfaces of these grafts while xenografts are encapsulated and infiltrated by amoebocytes. On the basis of these results, it is concluded that L. stagnalis is able to discriminate between different types of implant.

Tissue repair is another major function performed by the molluscan blood cells. Wound healing in L. stagnalis was studied by Sminia et al. (1973). It was reported that immediately after incision the wound areas are infiltered by a large number of amoebocytes. These cells which clear the wound area of invading micro organisms and cell debris, aggregate into small thrombi which together form a large amoebocyte plug. The wound is closed by the formation of a cell plug and by contraction of the muscles of the body wall. During further process, the size of the plug diminishes, and the round amoebocytes in the wound area transform into flattened cells which form the collagenous connective tissue fibrils. As a result, the amoebocyte plug has been replaced by collagenous connective tissue. Thus the amoebocytes are involved in the initial clearing and repairing phase of wound healing.

Gastropod blood cells are also involved in functions such as digestion, distribution of food materials to the organs, shell repair, and regeneration. Fat droplets and glycogen particles are occassionally present in the blood cells and thus it is assumed

that the blood cells are engaged in digestion and transport of food materials. Blood cells are also said to play a role in shell repair and regeneration by transporting calcium and other substances from the digestive gland to the shell. This hypothesis is based on the fact that (a) calcium rich granules have been observed within blood cells and in lime cells, and (b) large number of blood cells occur at sites of shell repair (Sminia, 1981).

Haemoglobins and haemocyanins are the respiratory pigments reported in molluscs. Haemoglobins are found in molluscs in the body tissues or in the circulatory fluids, dissolved in haemolymph or contained in the haemocoelic erythrocytes (Ghiretti and Ghiretti-Magaldi, 1972). Haemoglobins circulating in the blood have the function of oxygen carrier. Sminia et al. (1972) reported that pore cells present in the connective tissue of B. glabrata and Planorbarius corneus synthesize haemoglobins. Similarly, electronmicrographs of pore cells of L. stagnalis suggest that these cells produce and store haemocyanin (Sminia and Boer, 1973; Skelding and Newell, 1975). The haemocyanin occurs free in the blood plasma (Scheer, 1967; Ghiretti, 1968). Sminia (1977) reported a detailed account on the fine structure and function of haemocyanin producing cells in gastropods.

The percentage of haemolymph volume occupied by cells is termed packed cell volume (PCV) but the literature concerned with

PCV is lacking in gastropods. Foley and Cheng (1974) reported the PCV of M. mercenaria from two geographical location as 0.46% and 0.34%,respectively.

In the present chapter, total haemocyte counts as well as factors affecting haemolymph number in I. exustus and L. acuminata f. rufescens are reported. The PCV and haemoglobin levels in I. exustus are also reported.

2.2 MATERIALS AND METHODS

2.2.1 Test Animals

The freshwater snails selected for the present study were Indoplanorbis exustus, and Lymnaea acuminata f. rufescens.

2.2.1.1 Indoplanorbis exustus

I. exustus is a common freshwater snail found in stagnant waters, ponds and paddy fields. Specimens of I. exustus were collected from paddy fields of Thevara, Ernakulam. This snail species serves as an intermediate host for a number of digenetic trematode parasites. After thorough check, only infection-free snails were used.

2.2.1.2 Lymnaea acuminata f. rufescens

L. acuminata f. rufescens, is another common freshwater snail found mainly in permanent water bodies with abundant

vegetation, and is distributed throughout India. This snail species is also an intermediate host for a number of larval trematode parasites. They attain a maximum shell height of 23 ± 1 mm, and the specimens used for the present study were collected from paddy fields near South Kalamassery, Ernakulam. The snails were thoroughly checked for possible larval trematode infections, and only infection-free snails were used.

2.2.2 Laboratory Conditioning of Test animals

After collection, the snails were immediately transported in polythene bags filled with water from the collection site to the laboratory with least disturbance. In the laboratory they were maintained in fibreglass tanks of 50 L capacity containing well aerated, dechlorinated water having a pH range 7.0 to 7.5, and temperature 30 ± 1.5°C. The snails were acclimated for 48 to 96 hours, and during this period of acclimation water was changed every 24 hrs. I. exustus were fed with Lemna sp., while Lymnaea acuminata f. rufescens with Hydrilla sp. All snails used for any set of experiment belonged to the same population.

2.2.3 Selection of Animal Groups

In order to study the total haemocyte number in relation to shell size, three different size groups of snails were selected. They were 7 ± 1 mm, 11 ± 1 mm, and 15 ± 1 mm for Indoplanorbis exustus, and 13 ± 1 mm, 18 ± 1 mm, and 21 ± 1 mm for L. acuminata f. rufescens. In both the snail species the abundant group in

natural population was the intermediate size group, i.e., 11 ± 1 mm, and 18 ± 1 mm shell size for I. exustus and L. acuminata f. rufescens, respectively. In both cases, the required quantity of haemolymph could be collected from individual specimens of this size group. Hence, for studies on total haemocyte number under varying abiotic and biotic conditions, the intermediate size group snails of both species were used.

2.2.4 Collection of Haemolymph

2.2.4.1 Indoplanorbis exustus

After acclimating the snails for 48 to 96 hours, the snails were taken out and the water adhering to the snail was removed and, the foot cleaned with tissue paper. The heart of the snail was located and punctured, and the haemolymph was directly collected from the heart in heparinised capillary tube. In this way about 0.04 ml, 0.1 ml and 0.15 ml haemolymph could be obtained from each snail of shell size 7 ± 1, 11 ± 1, and 15 ± 1 mm, respectively.

2.2.4.2 Lymnaea acuminata f. rufescens

The acclimatized snails were taken out, and the water adhering to the snail was removed, and the foot cleaned with tissue paper. The haemolymph was collected from the sinus with a heparinized capillary tube. Haemolymph was also collected by touching the foot with the tip of a micropipette. As a result the snail was forced to retract deeply into its shell and extruded haemolymph. In this manner about 0.05, 0.10 and 0.2 ml haemolymph

could be obtained from each snail of shell size 13 ± 1, 18 ± 1 mm, and 21 ± 1 mm, respectively.

2.2.5 Total Number of Haemocytes

The total number of haemocytes in the blood was counted using a haemocytometer. After collecting the haemolymph as mentioned earlier, the first two drops of haemolymph were expelled while the third drop was discharged on to a haemocytometer. The cells were allowed to settle for 1 to 2 minutes and the counts were taken by the WBC method using Carl-Zeiss microscope, and objective 10 X. The cells in the corner four squares were counted and the number of cells per cubic millimeter was calculated and expressed as haemocytes per cubic millimeter. To avoid counting the same cells twice and yet not to miss the cells that touch the outside boundary of the square, cells that touched the left and upper boundary lines were counted and those that touched the right and lower boundaries were ignored as recommended by Coulombe (1970). The number of snails used from each size group of each snail species is as follows: I. exustus, N=15 for the large size group, and N=20 for each of the remaining two size groups, L. acuminata f. rufescens, N=15 for the large size groups, and N=20 for each of the remaining two size groups.

2.2.6 Factors Influencing the Haemocyte Number

In order to study the effect of various abiotic and biotic factors on total haemocyte counts, the snails selected were infection

free, and of the intermediate size group. The various abiotic factors selected were temperature, pH, and heavy metal toxicity, and the biotic factor was snail-conditioned water.

2.2.6.1 Temperature

In the experiment designed to study the effect of temperature on total haemocyte counts, four temperature constants, i.e., 20^{o}, 25^{o}, 35^{o}, and $40^{o}C$, were selected and for each temperature constant 40 snails of each species were used. The B.O.D. incubator was set for the desired temperature. Four 500 ml capacity beakers filled with dechlorinated and well aerated water were placed in the incubator at specific temperature constant, and when the water reached the desired temperature constant 10 snails were put in each beaker and haemolymph samples collected at 2, 6, 12 and 24 hr post-exposure to find the total haemocyte counts. The same method was followed for other temperature constants also. Ten snails of each specimens kept in each of the four 500 ml capacity beaker filled with dechlorinated and aerated water at room temperature ($29\pm1^{o}C$) served as the controls. Collection of haemolymph samples from snails of the control group was identifically carried out at these time periods and total cell counts recorded.

2.2.6.2 pH

In order to study the effect of pH on total haemocyte counts, 40 infection-free specimens of I. exustus were selected and 10 were

transferred to each of the four 500 ml capacity beaker containing aerated, dechlorinated tap water of pH 6.25. The experiment was carreid out at other pHs also, i.e., 6.75, 7.75 and 8.25 using the same number of specimens. In the case of L. acuminata f. rufescens only 20 specimens were used at each pH level. The pH of the water was adjusted to the desired level with phosphate buffer. An equal number of specimens of both the snail species reared in stored, dechlorinated, and aerated tap water and maintained at pH 7.25, the pH of the water from the paddy fields, served as the controls. Haemolymph samples were collected from snails, individually, of the control and the experimental groups at 2, 6, 12 and 24 hr post-exposure and total haemocyte counts recorded.

2.2.6.3 Snail-conditioned water

In order to investigate the effect of snail-conditioned water on haemocyte number, 100 snails of each species not used previously were kept individually in the wells of standard multicell containers filled with aerated, dechlorinated water for each time period of 2, 6, 12 and 24 hr. At the end of each time period, the snails were removed, and the snail conditioned water of each time period was collected in 250 ml beakers. A fresh group of 10 infection-free snails of each species was transferred into the beaker containing 2 hr snail-conditioned water. Haemolymph samples from each snail were collected at 2 hr and total counts recorded.

Similarly, 3 groups each of 10 infection-free snails of each species were maintained for 6, 12, and 24 hr in snail-conditioned water of these time periods and total haemocytes counts performed at 6, 12, and 24 hr respectively. During this period, the water was well aerated. An equal number of snails of each species kept in well aerated, dechlorinated water served as the controls, and the total haemocyte counts of control snails were also carried out at 2, 6, 12 and 24 hours.

2.2.6.4 Copper toxicity

The LC_{50} value and the sublethal concentrations of copper selected for the experiments are reported in detail in chapter 4.

Snails of the two species selected to study the effect of sublethal concentrations of copper were acclimated for 24 to 48 hrs in well aerated, dechlorinated tap water. Infection-free snails of intermediate size groups were selected for the experiments. After acclimatization, 3 groups of 50 snails each for each species, were exposed to three sublethal concentration of copper, 0.010, 0.015, and 0.020 ppm respectively, keeping the ratio of 1 animal to 25 ml of solution constant. They served as the experimentals. Fifty snails of each species maintained in aerated, dechlorinated tap water served as the control. Water in all containers was changed after 24 hours, and the copper concentrations in the experimental tanks were maintained. During

copper exposure the snails were not fed, but care was taken to ensure that crowding and oxygen availability did not act as limiting factors.

The total haemocyte counts were taken for short term period of 2, 6, 12, 24 and 48 hr post-exposure. At each time period, 10 snails from each experimental group and ten from the control group were taken out, haemolymph collected, and counts recorded as mentioned earlier in sections 2.2.4 and 2.2.5.

2.2.7 Estimation of Packed Cell Volume

The Packed Cell Volume (PCV) was determined by the microhaematocrit method (Coulombe, 1970). The haemolymph was drawn into formalin-rinsed heparinised capillary tube and one end of the tube was sealed with sealing wax. The tubes were then centrifuged in a microhaematocrit centrifuge for 5 minutes at 11500 rpm. After centrifugation, the tubes were removed and the column of packed cells was measured as the percentage of whole haemolymph using the scale provided in the microhaematocrit centrifuge (N = 15 for each group).

2.2.8 Estimation of Haemoglobin

The haemoglobin content of *I. exustus* was determined by Cyanomethemoglobin method described by Ortho Diagnostic System (1986). A sample of 0.02 ml haemolymph was taken in a test tube and 5 ml of Aculute reagent (modified Drabkin reagent) was added into it and shaken well. The potassium ferricyanide present in

the reagent converts the haemoglobin iron from ferrous to ferric state to form methaemoglobin which combines with potassium cyanide of Aculute reagent forming stable Cyanomethaemoglobin which was read spectrophotometrically at 540 nm. The standard graph was prepared from the haemoglobin standard supplied by Ortho Diagnostic Systems and the results expressed as g/100 ml ($\underline{N}$ = 15 for each group).

2.2.9 Computation and Presentation of Data

The experimental results are expressed graphically as well as in tabular form. The data were statistically analysed by students's '$\underline{t}$' test (Croxton et al., 1975) to manifest the variation in comparison with the controls. The variations were represented at three significance levels, viz. $\underline{P} < 0.05$, 0.01, 0.001. All the computations were carried out using a personal computer (Casio fx - 730P).

2.3 RESULTS

2.3.1 Total Haemocyte Number

2.3.1.1 *Indoplanorbis exustus* (Table 1A)

Statistical analysis of the data on total haemocyte counts revealed that the average values of haemocyte number in the three size groups showed significant variations. The total haemocyte count in 15 ± 1 mm size group was significantly higher than the counts in the other two size groups ($P < 0.001$). The total haemocyte count in 7 ± 1 mm size group was significantly lower than the counts in 11 ± 1 mm size group ($P < 0.001$).

2.3.1.2 *Lymnaea acuminata* f. *rufescens* (Table 1B)

Statistical analysis of the data revealed significant difference in total haemocyte number among snails of all the three size groups. The total haemocyte count in 13 ± 1 mm size group was significantly lower than the count in 21 ± 1 mm size group ($P < 0.001$). When the count in 18 ± 1 mm group was compared with that in 21 ± 1 mm size group the latter showed significantly higher value ($P < 0.001$).

2.3.2 Factors Influencing Haemocyte Number

2.3.2.1 Temperature

2.3.2.1.1 *Indoplanorbis exustus* (Table 2A ; Figure 1)

In *I. exustus*, when the total haemocyte count in snails exposed to 20°C was compared with the values in the controls, the

Table 1A. Total Haemocytes/mm^3 in the three size groups of *Indoplanorbis exustus*

Size group	7±1 mm	11±1 mm	15±1 mm
N	20	20	15
Mean value	386.00	737.00	1071.00
± SD	120.54	156.98	250.21
Range	200.00-590.00	500.00-1050.00	660.00-1400.00

Table 1B. Total Haemocytes/mm^3 in the three size groups of *Lymnaea acuminata* f. *rufescens*

Size group	13±1 mm	18±1 mm	21±1 mm
N	20	20	15
Mean value	866.00	1026.00	1712.00
± SD	299.00	357.00	539.65
Range	332.00-1200.00	390.00-1795.00	965.00-2950.00

counts in 20°C exposed snails were found to be significantly higher at 2 ($P < 0.01$), 6 ($P < 0.01$), 12 ($P < 0.001$) and 24 hr ($P < 0.01$). When the total haemocytes in 25°C exposed snails were compared with the control values, the haemocyte count in the experimentals were found to be significantly higher at 2 ($P < 0.01$), 6 ($P < 0.01$) and 12 hr ($P < 0.05$). Snails exposed to 35°C and 40°C did not show any significant change in the total number of haemocytes when compared with their corresponding controls at any time period.

2.3.2.1.2 Lymnaea acuminata f. rufescens (Table 2B ; Figure 2)

In L. acuminata f. rufescens, when the mean values of total haemocytes/mm^3 of 20° exposed snails were compared with the values of control, the values of 20°C exposed snails were found to be significantly higher at 2 ($P < 0.01$), 6 ($P < 0.05$), 12 ($P < 0.01$), and 24 hr ($P < 0.05$). Similar was the case, when snails were exposed to 25°C. The mean values of total haemocyte number were significantly higher than the controls at 2 ($P < 0.05$), 6 ($P < 0.01$), 12 ($P < 0.05$), and 24 hr ($P < 0.001$). Snails exposed to 35°C showed significantly higher haemocyte number than their controls at 2 ($P < 0.01$), 6 ($P < 0.01$), and 12 hr ($P < 0.01$). The total haemocyte counts in 40°C exposed snails when compared with those of the controls showed significantly higher value only at 2 hr ($P < 0.01$). At 6 and 12 hr there was no significant variation while at 24 hr a significant drop in the total haemocyte count was observed ($P < 0.001$).

Table 2A. Total number of circulating haemocytes/mm^3 in Indoplanorbis exustus at different temperature at 2, 6, 12 and 24 hr post-exposure (size group 11±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs
Control	N	10	10	10	10
	Mean value	818.00	900.00	948.00	1029.00
	± SD	207.13	246.89	240.03	219.56
30°C	Range	500.00-1210.00	730.00-1540.00	570.00-1410.00	680.00-1240.00
	N	10	10	10	10
	Mean value	1574.00**	1608.00**	1754.00 ***	1588.00 **
	± SD	582.03	620.87	455.20	429.87
20°C	Range	750.00-2610.00	860.00-2690.00	1060.00-2430.00	390.00-2190.00
	N	10	10	10	10
	Mean value	1241.00**	1415.00**	1222.00 *	1194.00
	± SD	275.90	459.08	282.95	311.77
25°C	Range	740.00-1740.00	910.00-2530.00	710.00-1670.00	740.00-1770.00
	N	10	10	10	10
	Mean value	721.00	1084.00	879.00	1085.00
	± SD	209.62	408.25	242.20	524.45
35°C	Range	480.00-1110.00	710.00-1990.00	530.00-1260.00	550.00-1930.00
	N	10	10	10	10
	Mean value	619.00	1096.00	677.00	969.00
	± SD	243.92	458.72	348.64	326.06
40°C	Range	340.00-1140.00	460.00-1960.00	350.00-1500.00	520.00-1520.00

Significance Level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

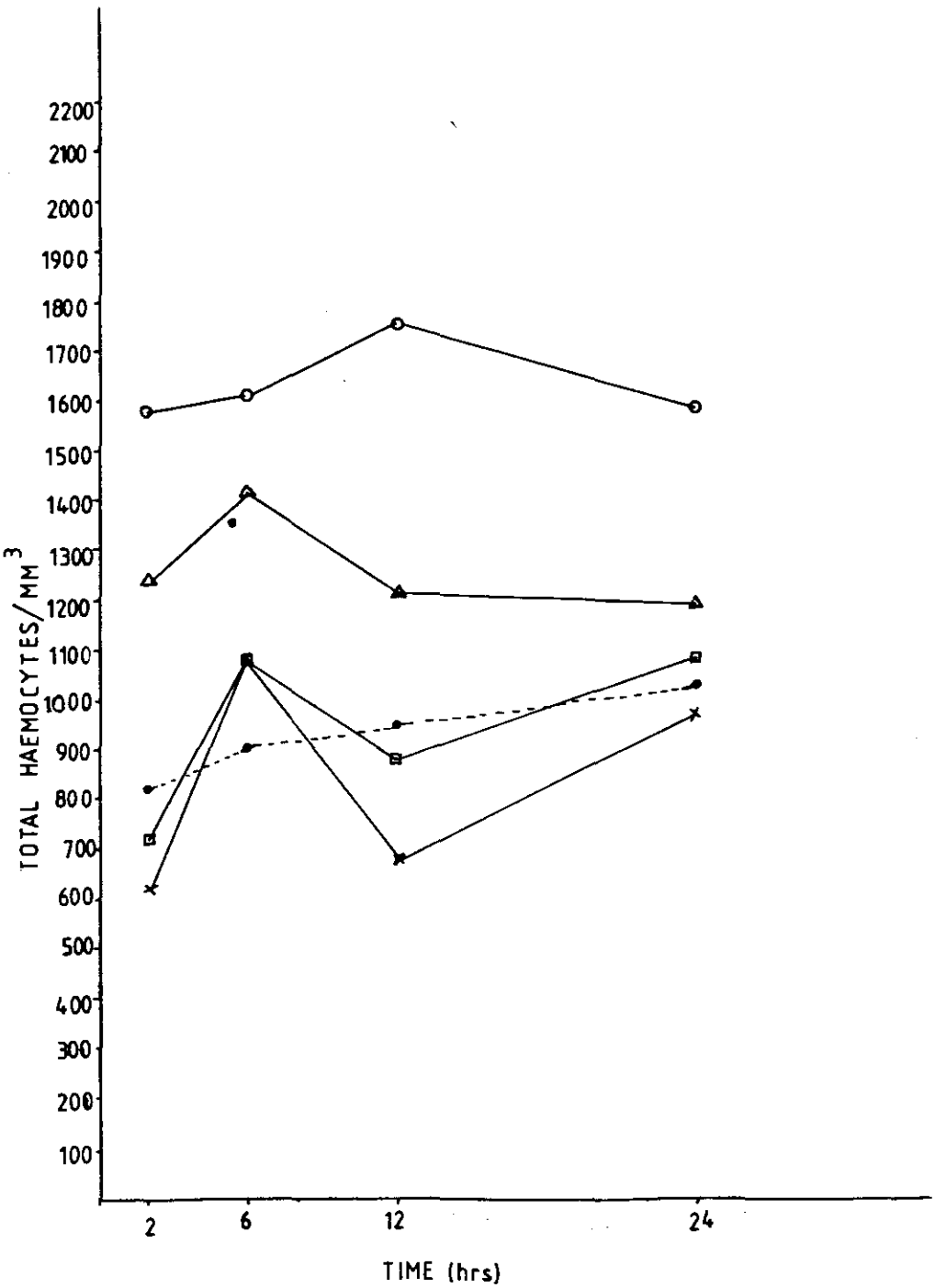

Figure 1. Total Haemocytes/mm^3 in I. exustus *exposed to varying temperatures. Control (-•-), 20°C (⊙), 25°C (△), 35°C (⊡), and 40°C (×).*

Table 2B. Total number of circulating haemocytes/mm^3 in Lymnaea acuminata f. rufescens at different temperatures at 2, 6, 12 and 24 hr post-exposure (size group 18±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs
	N	10	10	10	10
Control	Mean value	944.00	963.00	936.00	926.00
	± SD	464.27	356.30	333.83	396.02
30°C	Range	520.00-1795.00	573.00-1420.00	560.00-1465.00	492.00-1580.00
	N	10	10	10	10
Control	Mean value	2386.00**	1409.00*	1483.00**	1687.00*
	± SD	1191.70	354.29	424.87	883.66
20°C	Range	1160.00-3717.00	777.00-1820.00	970.00-2120.00	830.00-3270.00
	N	10	10	10	10
Control	Mean value	1703.00*	1802.00**	1445.00*	2464.00***
	± SD	695.50	702.00	445.50	699.00
25°C	Range	725.00-2840.00	820.00-2640.00	935.00-2440.00	1370.00-3270.00
	N	10	10	10	10
Control	Mean value	1679.00**	2101.00**	1637.00**	1367.00
	± SD	543.00	807.20	534.00	435.00
35°C	Range	1010.00-2860.00	1020.00-3610.00	940.00-2520.00	820.00-2170.00
	N	10	10	10	10
Control	Mean value	1710.00**	1281.00	880.00	341.00***
	± SD	491.25	678.00	363.10	167.82
40°C	Range	1030.00-2390.00	600.00-2500.00	440.00-1340.00	160.00-680.00

Significance Level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

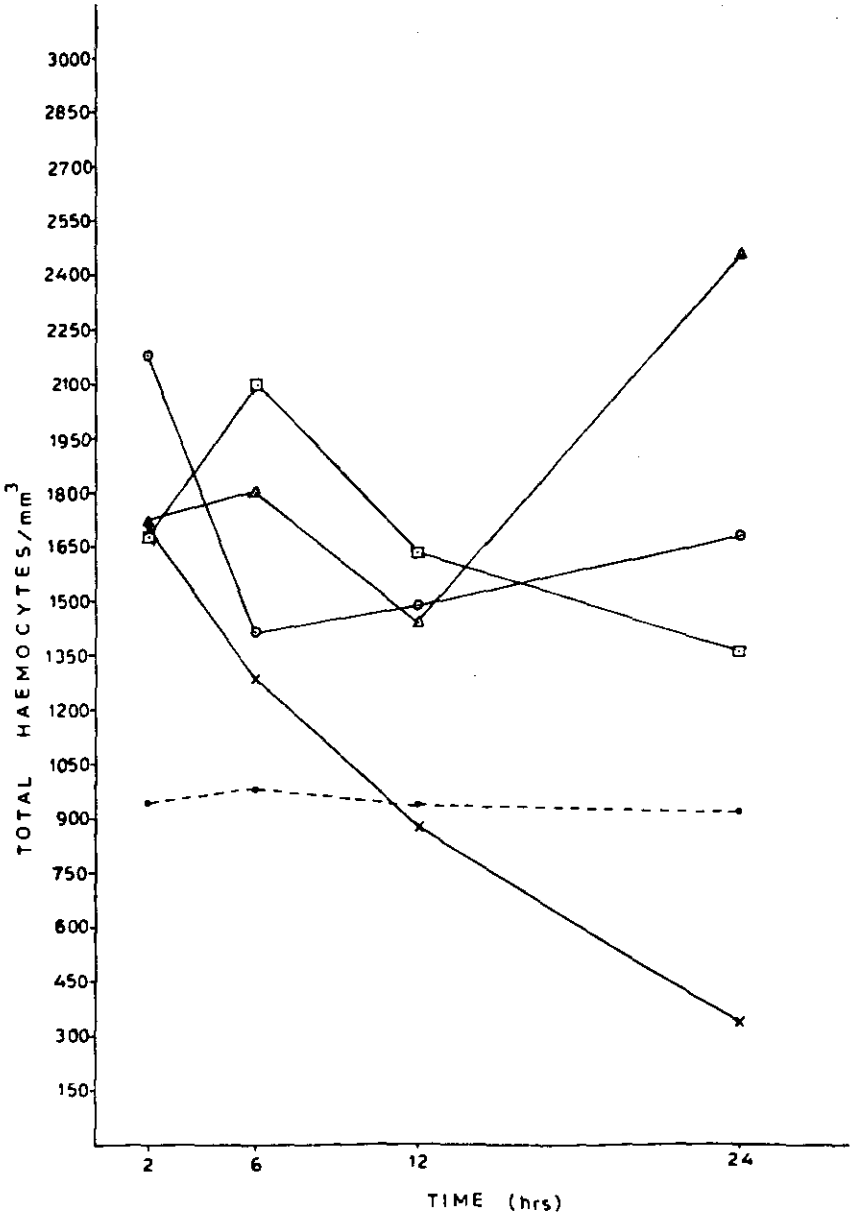

Figure 2. Total Haemocytes/mm^3 in <u>*L. acuminata*</u> *f.* <u>*rufescens*</u> *exposed to varying temperatures. Control (—•—), 20°C (⊙), 25°C (△), 35°C (⊡), and 40°C (×).*

2.3.2.2 pH

2.3.2.2.1 Indoplanorbis exustus (Table 3A ; Figure 3)

In I. exustus, statistically significant change in total haemocyte counts was observed only at 2 hr post-exposure in all pH levels. The mean values of total haemocyte number were found to be significantly lower at pH 6.25 ($P < 0.01$), 6.75 ($P < 0.05$), 7.25 ($P < 0.01$), and 8.25 ($P < 0.05$) than the corresponding values in the controls. At 6 hr exposure the only significant change observed was at pH 8.25; where the haemocyte number was significantly higher than the control ($P < 0.05$). At other time periods no significant variation in total count was observed at any pH level.

2.3.2.2.2 Lymnaea acuminata f. rufescens (Table 3B ; Figure 4)

In L. acuminata f. rufescens, the haemocyte number at pH 6.25 when compared with the controls, showed significantly higher value at 2 ($P < 0.001$) and 6 hr ($P < 0.05$) post-exposure. At 12 hr post-exposure there was no significant change while at 24 hr a significant decrease ($P < 0.05$) in the number of haemocytes was observed. The snails exposed to pH 6.75 did not show any significant change in the number of haemocytes at any time period. The number of haemocytes was found to be significantly higher in snails exposed to pH 7.75 than in the controls at 2 ($P < 0.01$) and 6 hr ($P < 0.01$) post-exposure. When compared with the controls, a significant fall in haemocyte number was observed at 24 hr post-exposure in snails exposed to pH 8.25 ($P < 0.001$).

Table 3A. Total number of circulating haemocytes/mm^3 in Indoplanorbis exustus at different pH levels at 2, 6, 12 and 24 hr post-exposure (size group : 11±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs
Control pH 7.25	N	10	10	10	10
	Mean value	908.00	789.00	737.00	762.00
	± SD	300.65	364.91	264.15	126.82
	Range	490.00-1340.00	350.00-1310.00	440.00-1290.00	630.00-950.00
pH 6.25	N	10	10	10	10
	Mean value	540.00**	763.00	749.00	947.00
	± SD	191.02	292.31	259.93	337.93
	Range	350.00-880.00	490.00-1350.00	460.00-1310.00	550.00-1520.00
pH 6.75	N	10	10	10	10
	Mean value	597.00*	799.00	596.00	783.00
	± SD	148.10	282.58	137.53	222.96
	Range	440.00-840.00	410.00-1130.00	390.00-800.00	490.00-1260.00
pH 7.75	N	10	10	10	10
	Mean value	573.00**	836.00	679.00	862.00
	± SD	153.19	190.77	273.92	307.49
	Range	430.00-980.00	680.00-1300.00	460.00-1330.00	430.00-1480.00
pH 8.25	N	10	10	10	10
	Mean value	680.00*	1076.00*	835.00	942.00
	± SD	164.86	124.91	229.89	241.60
	Range	520.00-920.00	900.00-1290.00	490.00-1240.00	620.00-1380.00

Significance Level : * $P < 0.05$ ** $P < 0.01$

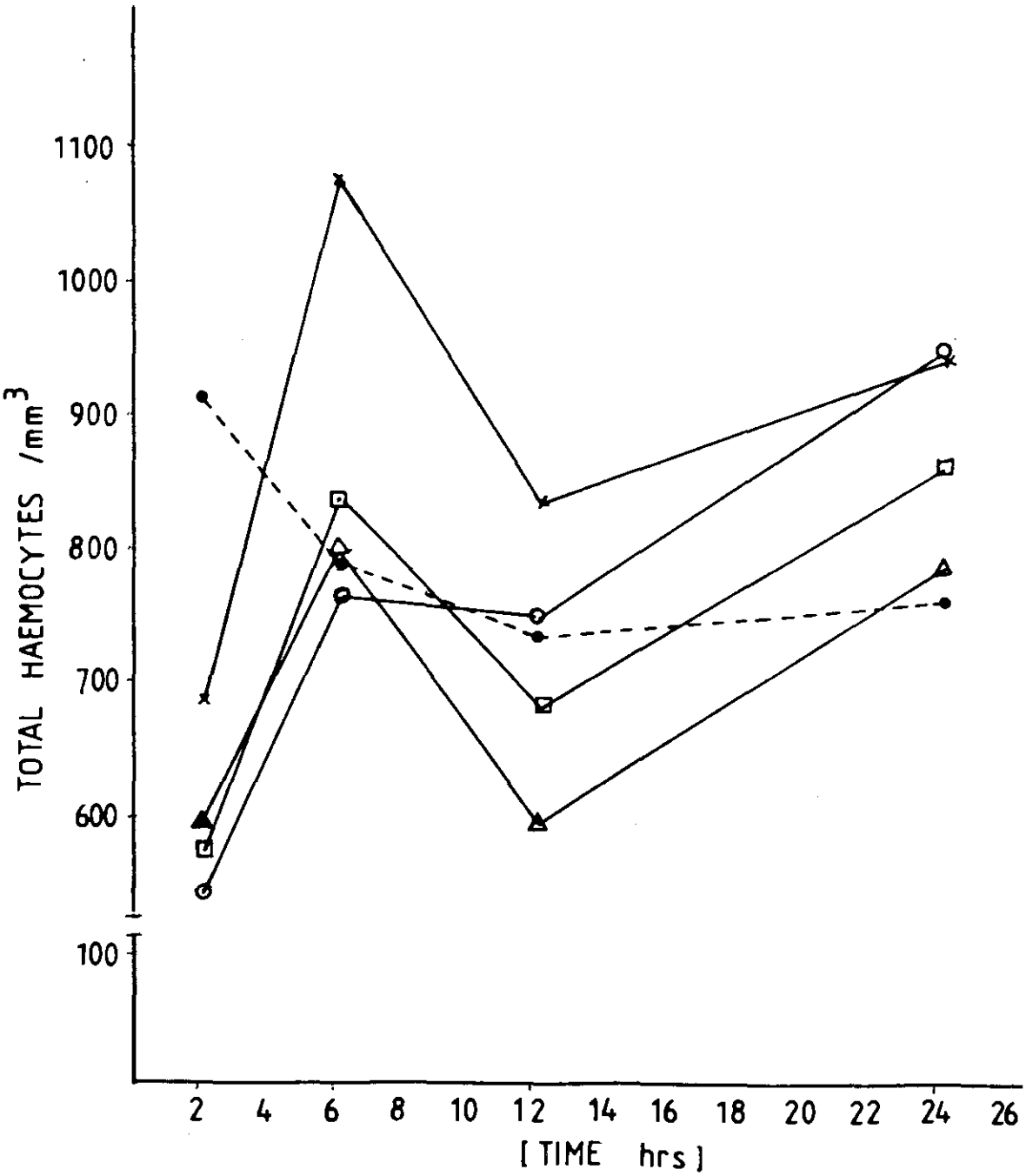

Figure 3. Total Haemocytes/mm^3 in I. exustus *exposed to varying pH. Control (—•—), pH 6.25 (⊙), pH 6.75 (△), pH 7.75 (⊡), and pH 8.25 (×).*

at different pH levels at 2, 6, 12 and 24 hr post exposure

	Hours	2 hrs	6 hrs	12 hrs	24 hrs
Control pH 7.25	N	5	5	5	5
	Mean value	1161.00	1097.00	1183.00	1114.00
	± SD	149.21	142.28	129.45	119.02
	Range	965.00-1322.00	905.00-1260.00	990.00-1340.00	960.00-1260.00
pH 6.25	N	5	5	5	5
	Mean value	1713.00***	1520.00*	1312.00	922.00*
	± SD	126.37	233.34	155.14	110.31
	Range	1520.00-1850.00	1250.00-1780.00	1170.00-1540.00	750.00-1050.00
pH 6.75	N	5	5	5	5
	Mean value	1202.00	1260.00	1276.00	1080.00
	± SD	105.98	51.47	85.02	106.15
	Range	1065.00-1360.00	1190.00-1300.00	1180.00-1370.00	937.00-1190.00
pH 7.75	N	5**	5	5	5
	Mean value	1524.00	1516.00**	1300.00	1210.00
	± SD	85.54	105.02	76.40	111.18
	Range	1400.00-1600.00	1420.00-1690.00	1235.00-1430.00	1075.00-1325.00
pH 8.25	N	5	5	5	5
	Mean value	942.00	1112.00	1203.00	780.00***
	± SD	86.71	87.00	108.08	57.00
	Range	850.00-1080.00	1000.00-1200.00	1095.00-1380.00	700.00-850.00

Significance Level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

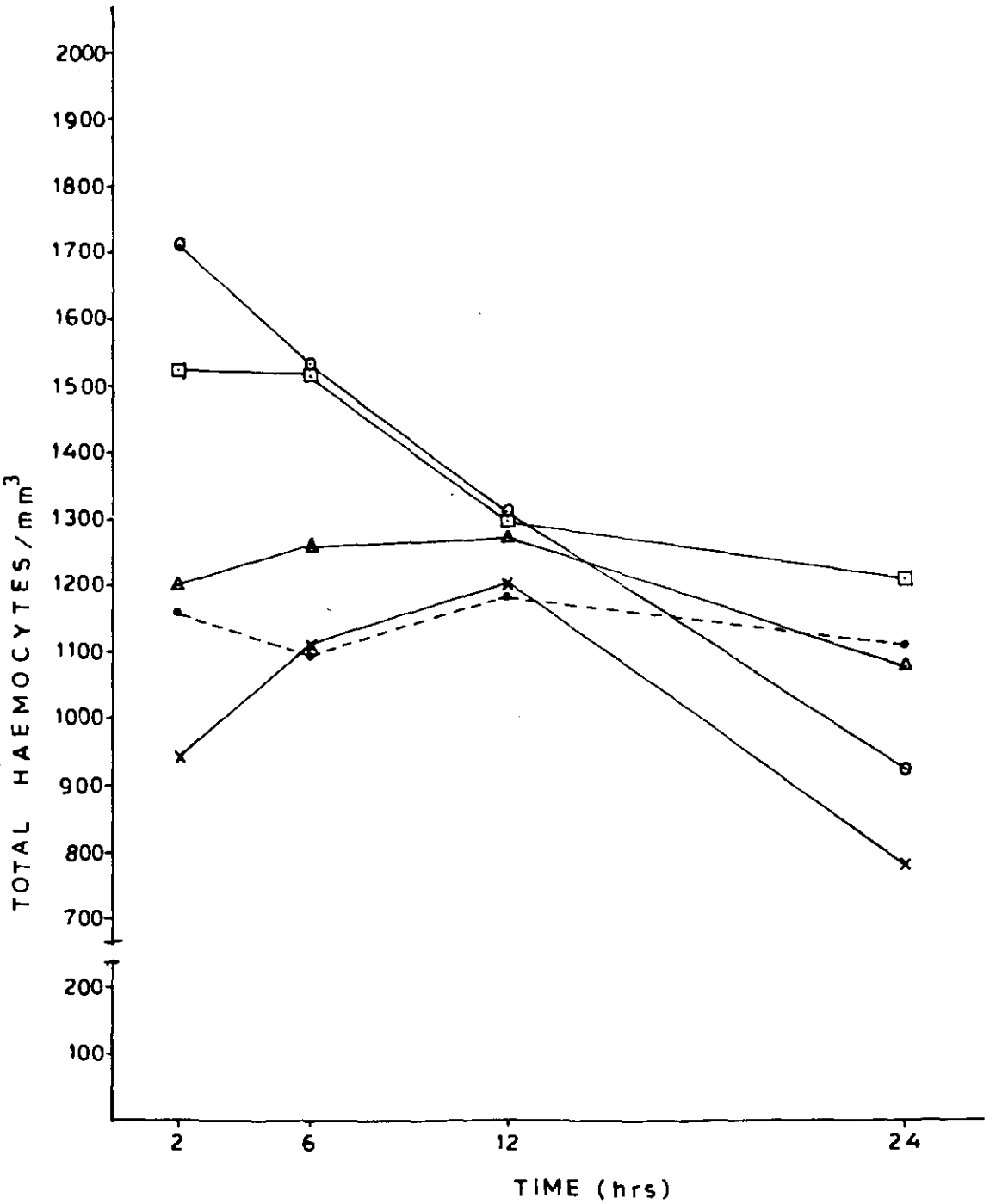

Figure 4. Total Haemocytes/mm^3 in <u>*L. acuminata f. rufescens*</u> *exposed to varying pH. Control (-•-), pH 6.25 (⊙), pH 6.75 (△), pH 7.75 (⊡), and pH 8.25 (×).*

2.3.2.3 Snail-conditioned water

2.3.2.3.1 Indoplanorbis exustus (Table 4A)

From the results, it was observed that there was no significant variation in the number of haemocytes in I. exustus exposed to snail-conditioned water at any time period.

2.3.2.3.2 Lymnaea acuminata f. rufescens (Table 4B)

There was no statistically significant variation in the number of haemocytes in L. acuminata f. rufescens exposed to snail conditioned water at any time period.

2.3.2.4 Copper toxicity

2.3.2.4.1 Indoplanorbis exustus (Table 5A ; Figure 5)

In I. exustus, when the number of haemocytes/mm^3 in 0.010 ppm copper-dosed snails was compared with that of the controls, the former showed a significantly higher values than the controls at 6 ($P < 0.001$), 12 ($P < 0.001$), 24 ($P < 0.001$), and 48 hr ($P < 0.01$). Similarly, 0.015 ppm dosed snails also showed significantly higher values at 2 ($P < 0.05$), 6, 12, 24 ($P < 0.001$), and 48 hr ($P < 0.05$) than the control values. The mean values of haemocyte number in 0.020 ppm dosed snails were significantly higher at 6 ($P < 0.001$), 12 ($P < 0.01$), 24 ($P < 0.01$), and 48 hr ($P < 0.001$) post-exposure than the control values.

When the counts of 0.010 ppm dosed snails were compared with those of 0.015 ppm and 0.020 ppm dosed snails, 0.010 ppm dosed snails

Table 4A. Total number of circulating haemocytes/mm^3 in Indoplanorbis exustus exposed to snail conditioned water (N = 10 for each time period)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs
Control	Mean	1055.00	1105.00	1027.00	964.00
	± SD	280.92	200.84	368.90	302.30
	Range	600.00-1290.00	680.00-1400.00	610.00-1410.00	570.00-1400.00
Snail-conditioned water	Mean	1272.00	1139.00	1324.00	1229.00
	± SD	449.24	358.03	248.56	440.46
	Range	630.00-1870.00	720.00-1940.00	840.00-1620.00	600.00-1860.00

Table 4B. Total number of circulating haemocytes/mm^3 in Lymnaea acuminata f. rufescens exposed to snail conditioned water (N = 10 for each time period)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs
Control	Mean	944.00	963.00	936.00	926.00
	± SD	464.27	356.30	333.83	396.02
	Range	520.00-1795.00	573.00-1420.00	560.00-1465.00	492.00-1580.00
Snail-conditioned water	Mean	963.00	960.00	1090.00	958.00
	± SD	175.12	259.94	229.06	161.71
	Range	560.00-1260.00	560.00-1420.00	740.00-1490.00	760.00-1320.00

Table 5A. Total haemocytes/mm³ in [illegible] of copper (size group 11±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	10	10	10	10	10
	Mean value	901.00	814.00	820.00	930.00	993.00
	± SD	348.88	222.62	154.99	242.12	271.17
	Range	560.00-1640.00	530.00-1210.00	590.00-1040.00	710.00-1540.00	570.00-1410.00
0.010 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	926.00	2004.00***	1405.00***	1555.00***	1862.00**
	± SD	242.90	378.39	384.54	351.54	734.22
	Range	640.00-1450.00	1330.00-2550.00	950.00-2210.00	1200.00-2170.00	780.00-2940.00
0.015 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	1392.00*	1870.00***	1501.00***	2071.00***	1692.00*
	± SD	393.12	418.40	374.92	614.98	697.07
	Range	770.00-1920.00	1280.00-2410.00	1000.00-2330.00	1490.00-3630.00	820.00-2670.00
0.020 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	1238.00	1851.00***	1521.00**	1508.00**	2151.00***
	± SD	348.66	738.57	649.98	396.11	557.90
	Range	810.00-1830.00	970.00-3300.00	750.00-2820.00	1080.00-2310.00	1160.00-3200.00

Significance Level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

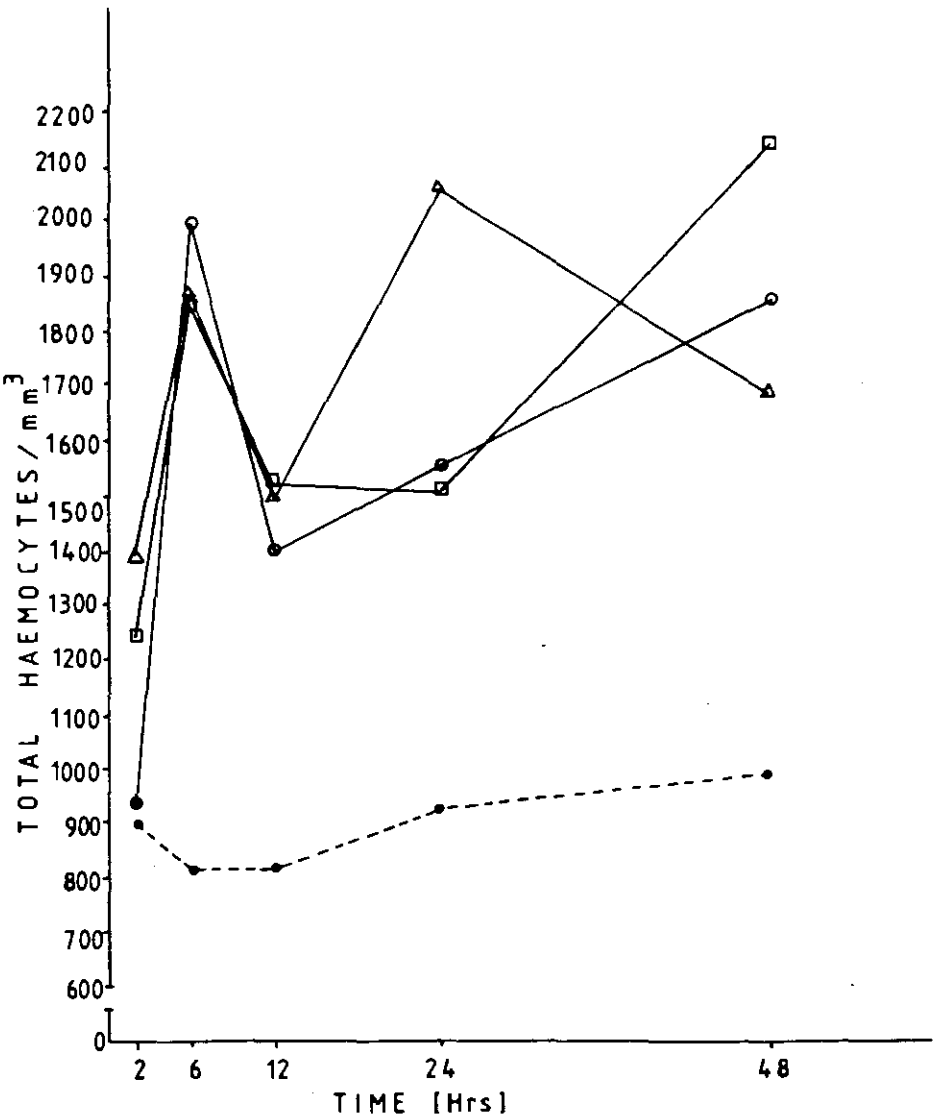

Figure 5. Total Haemocytes/mm^3 in I. exustus *dosed with three sublethal concentrations of Copper. Control (-•-), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).*

at 2 hr showed significantly lower ($\underline{P} < 0.01$ and $\underline{P} < 0.05$) values than the values of 0.015 ppm and 0.020 ppm dosed snails, respectively. At 24 hr post-exposure, 0.010 ppm dosed snails showed significantly lower value than the value of 0.015 ppm dosed snails, and 0.015 ppm dosed snails showed significantly higher value ($\underline{P} < 0.05$) than that of 0.020 ppm dosed snails.

2.3.2.4.2 Lymnaea acuminata f. rufescens (Table 5B ; Figure 6)

In L. acuminata f. rufescens when the total counts of 0.010 ppm copper dosed snails were compared with those of the controls, the former showed significantly higher value at 2, 6, 12 and 24 hr post-exposure ($\underline{P} < 0.001$); similarly 0.015 and 0.020 ppm copper dosed snails also showed significantly higher values than the controls at all the time periods studied i.e., 2, 6, 12, 24 and 48 hr post-exposure ($\underline{P} < 0.001$)

When the counts of 0.010 ppm copper dosed snails were compared with those of 0.015 ppm dosed snails, significantly higher values in 0.010 ppm were observed at 6 ($\underline{P} < 0.01$), 12 and 24 hr post-exposure ($\underline{P} < 0.001$) while a significant decline was observed at 48 hr post-exposure ($\underline{P} < 0.01$). Similarly 0.010 ppm dosed snails showed significantly higher values than the values of 0.020 ppm dosed ones at 2 ($\underline{P} < 0.05$), 6 ($\underline{P} < 0.05$), 12 ($\underline{P} < 0.001$), and 24 hr ($\underline{P} < 0.001$) post-exposure but significantly lower value at 48 hr post-exposure ($\underline{P} < 0.001$). When the counts of 0.015 ppm and 0.020 ppm copper-dosed snails were compared, 0.015 ppm dosed snails showed significantly lower values at 24 ($\underline{P} < 0.01$), and 48 hr ($\underline{P} < 0.05$) post-exposure.

Table 5B. Total haemocytes/mm^3 in Lymnaea acuminata f. rufescens dosed with three concentrations of copper (size group 18±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	10	10	10	10	10
	Mean value	1084.00	1234.00	1221.00	1144.00	1166.00
	± SD	207.77	322.10	299.30	227.41	336.12
	Range	840.00-1465.00	960.00-1770.00	782.00-1580.00	855.00-1620.00	810.00-1870.00
0.010 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	3422.00***	3288.00***	3587.00***	3951.00***	1118.00
	± SD	705.90	924.46	712.71	590.28	415.31
	Range	2550.00-4380.00	1400.00-4250.00	2360.00-5060.00	2730.00-4570.00	700.00-2040.00
0.015 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	2972.00***	2130.00***	2098.00***	2327.00***	2407.00***
	± SD	659.94	297.73	621.08	222.46	1174.98
	Range	1860.00-3860.00	1710.00-2780.00	1120.00-3190.00	2130.00-2780.00	1080.00-4480.00
0.020 ppm of Cu^{2+} dosed	N	10	10	10	10	10
	Mean value	2731.00***	2453.00***	2197.00***	2974.00***	3414.00***
	± SD	576.14	595.27	589.95	453.14	514.82
	Range	1630.00-3570.00	1180.00-3090.00	1270.00-3120.00	1990.00-3760.00	2720.00-4330.00

Significance Level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

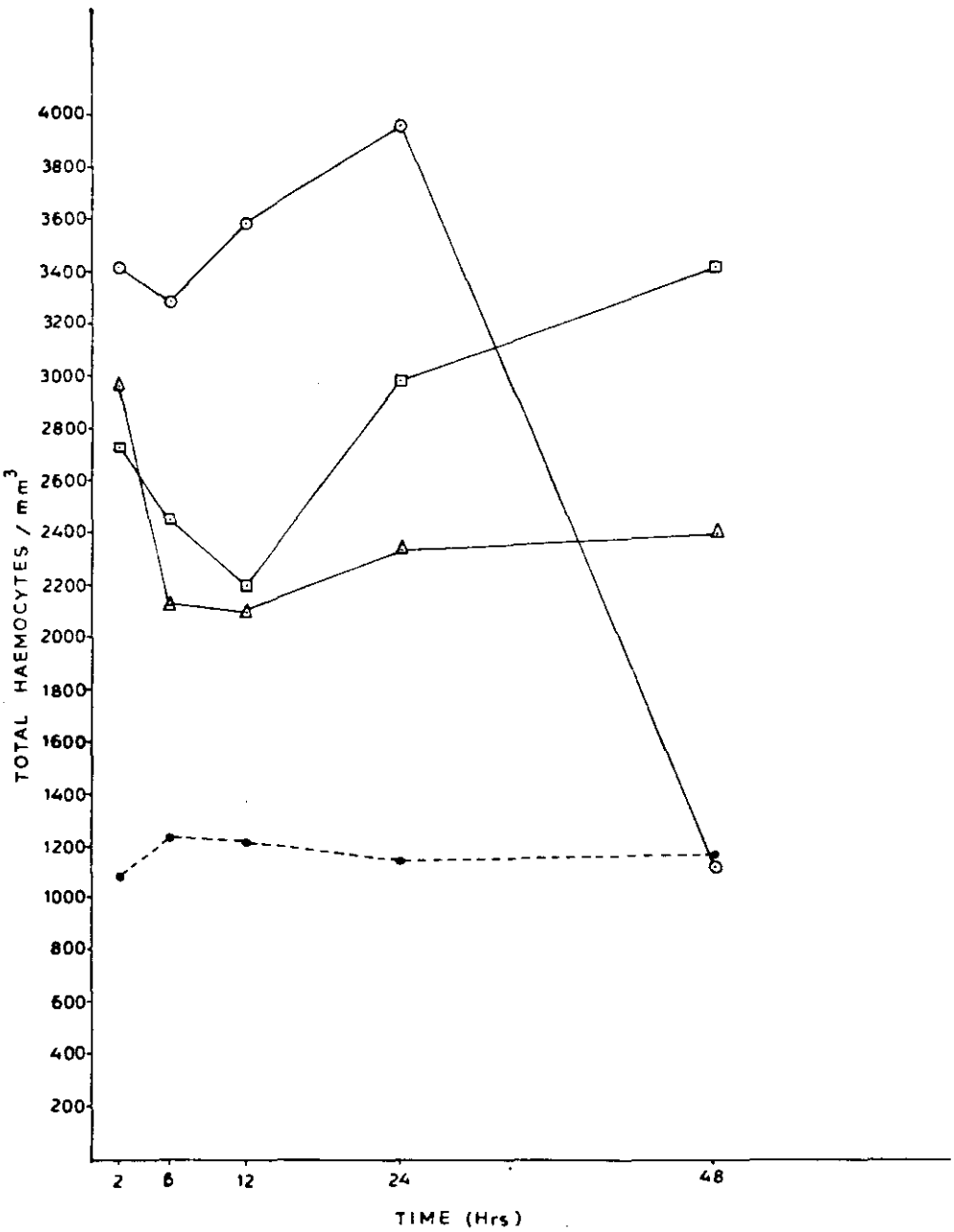

Figure 6. Total Haemocytes/mm^3 in <u>*L. acuminata f. rufescens*</u> *dosed with three sublethal concentrations of Copper. Control (—•—), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).*

2.2.3.3. Packed Cell Volume (PCV)

The results on packed cell volume of I. exustus and L. acuminata f. rufescens showed much lower values which cannot be detected in the microhaematocrit scale. The values obtained in both the snail species were below 1%.

2.3.4 Haemoglobin (Table 6)

Table 6gives the mean values, standard deviation, and range of haemoglobin concentration (g/100 ml) in the three size groups of I. exustus. The highest haemoglobin concentration was observed in the intermediate size group which was significantly higher than the concentrations in snails of the other two size groups ($P < 0.001$, and $P < 0.05$).

Table 6. Total Haemoglobin concentration (g/100 ml) in three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	15	15
Mean	0.768	1.248	1.088
± SD	0.146	0.119	0.201
Range	0.533-1.000	1.000-1.500	0.766-1.467

2.4 DISCUSSION

In the present study both in I. exustus and L. acuminata f. rufescens significant variations were found in the mean values of total haemocyte counts among the three size groups studied. The results indicate that small snails possess fewer circulating haemocytes than the larger ones. Comparable results have been reported in B. glabrata (Stumpf and Gilbertson, 1978), and L. stagnalis (Sminia, 1981; Dikkeboom et al., 1984). Dikkeboom et al. (1984) reported that the number of circulating haemocytes in juvenile L. stagnalis was 3 to 4 times lower than that of adults. When the smallest and the largest size group snails of both species were compared, total haemocyte count was found to be 2-3 times higher in the largest. Similarly, the volume of haemolymph was also much lower in snails of the smallest size group. Stumpf and Gilbertson (1976) reported that in the two stains of the gastropod, B. glabrata, a logarithmic increase in cell number was observed with increasing shell size. However, they indicated that the association between shell size and haemocyte number does not necessarily suggest a cause and effect relationship, but attributed to a third factor, such as age. Thus, it may be concluded that shell size/age of the snail is a factor which influences the total number of circulating haemocytes in I. exustus, and L. acuminata f. rufescens.

Previous studies have indicated that juvenile snails are more susceptible to parasitic infection than adults (Mohandas, 1971;

Meuleman, 1980; Meuleman et al., 1982; Dikkeboom et al., 1984). The number of haemocytes is a factor which decreases or increases the defence mechanism. Earlier reports (Sminia, 1972; 1974; 1977) on L. stagnalis have shown that haemocytes are engaged in cellular defence mechanisms such as phagocytosis, and encapsulation. The phagocytic capacity of the circulating haemocytes in juvenile L. stagnalis is lower than that of adults (Dikkeboom et al., 1985b). This shows that in small snails the cellular line of defence mechanism is weak, and the fact that these snails have less volume of haemolymph indicates that their humoral defence mechanism is also weak. Thus, both in I. exustus and L. acuminata f. rufescens, the fewer number of circulating haemocytes, with qualitatively less development, and less volume of haemolymph in the smallest size group snails may be the two important factors that determine their susceptibility to larval trematode infection.

There are several abiotic and biotic factors which affect the total number of haemocytes. Various factors which affect the number of haemocytes in freshwater gastropods were reported by Muller (1956), Sminia (1972), Michelson and Dubois (1975), Stumpf and Gilbertson (1978, 1980), Jeong et al. (1980), Granath and Yoshino (1983), and Wolmarans and Yssel (1988).

Temperature is an important abiotic factor which influences the total haemocyte counts, and the influence of temperature was reported by Pauley and Krassner (1971); Davies and Partridge (1972),

and Stumpf and Gilbertson (1978). A temperature dependent rise in haemocyte number was reported in B. glabrata (Stumpf and Gilbertson, 1978), and P. vulgata (Davies and Partridge, 1972). Pauley and Krassner (1971) reported that in gastropods leukocytosis is stimulated by temperature changes. The results on the effect of temperature on total haemocyte count in I. exustus showed that temperatures lower than the room temperature have significant influence on the total cell counts. At higher temperatures, the snails did not show any significant variation in the number of circulating haemocytes. At 20 and 25°C, the haemocyte number was found to be elevated significantly except at 24 hr at 25°C. In P. vulgata, the concentrations of circulating blood cells varied from about 1 x 10^6 cells per ml at 5°C to 9 x 10^6 cells per ml at 25°C. Also, in B. glabrata and L. stagnalis (Sminia, 1981), the number of circulating haemocytes increased rapidly within a few hours when the temperature rises. At 35 and 40°C a non-significant general decrease in the number of haemocytes was observed. Feng (1965) reported that at higher temperature, the haemocyte number may be affected by heart rate. The drop in haemocyte number may be the result of a drop in heart rate at high temperatures, or due to cell death. The maximum cell count was observed at 20°C at all time periods studied, and the count was found to be related inversely to rise in temperature. Thus, it can be concluded that at low temperature haemocytosis takes place resulting in an increase in the number while at higher

temperatures heart-rate drops, or cell mortality takes place both leading to a drastic drop in the cell number.

In L. acuminata f. rufescens, the number of circulating haemocytes was found to increase at all time periods at 20, 25 and 35°C. Temperatures close to the room temperature were found to enhance the number of circulating haemocytes. At higher temperature, 40°C, mortality of snails begins after 12 hr post-exposure, and about 25% death was observed within 24 hours. After an initial elevation in the number at 40°C, a drastic drop in total count was observed at 24 hr post-exposure. The initial elevation may be an immediate response of snails to stress which may lead to haemocytosis. As a result of continuous stress at high temperature the heart rate is believed to have dropped leading to a significant decrease in total count; or there is cell death. The response to temperature stress in terms of total haemocyte number differed in the two snail species and this difference may be attributed to the species difference. It may be noted that this significant leucocytosis at low temperatures particularly in I. exustus, might be one of the reasons for the observed low infection rate in this snail species by Mohandas (1971). In specimens collected during cold months the larval trematode infection rate was low while in those collected during summer months, it was high. Since molluscan haemocytes are involved in defence mechanisms, it is presumed that snails attain same resistance during cold months when the total count is high.

The results regarding the effect of pH on the number of circulating haemocytes in I. exustus and L. acuminata f. rufescens showed that pH is a factor which might effect the physiology of the snails to a certain extent. In I. exustus, the number of circulating haemocytes was found to decrease in all the experimentals at 2 hr post-exposure. The observed decrease in the total counts at 2 hr post-exposure is attributed either to the sudden changes in the pH of the external media which might have affected the physiology of the snails leading to the transmigration of haemocytes across epithelial surface, or to cell mortality due to the sudden stress as reported by Pickwell and Steinert (1984). Later the total counts were normalised and it is presumed that there was mobilization of haemocytes from some other sources to compensate the loss. In several situations haemocytes are reported leaving the body by diapedesis after traversing the epithelial layer (Stauber, 1950; Brown and Brown, 1965; Ruddell et al., 1978; Miller and Feng, 1987). Sminia et al. (1983) reported that an interchangeable pool of haemocytes occurs in the haemolymph and connective tissue, and under certain conditions extrusion of haemocytes from the reservoir into the haemolymph occurs, thus creating a large population of circulating haemocytes. Thus, the significant increase in total cell count at 6 hr post-exposure after the initial decrease at pH 8.25 may be attributed to temporary haemopoiesis, through translocation of tissue haemocytes to haemolymph.

In L.acuminata f. rufescens, at the lowest pH, 6.25, significant increase in the total number of circulating haemocytes was observed at 2 and 6 hr post-exposure while a decrease was observed at 24 hr post-exposure. The significant increase may be attributed to the fact that under stress, the haemocytes migrate from the reservoir compartment to the haemolymph since an interchangeable pool of haemocytes occurs in the haemolymph and connective tissue. The same reason can be attributed to the increase in cell number at 2 and 6 hr post-exposure in those snails exposed to pH 7.75. During later time periods, the snails being under continuous stress, the changed pH might have affected the physiology which may lead to cell mortality resulting in decrease in the number of circulating haemocytes. This was reflected at 24 hr post-exposure in snails exposed to pH 6.25 and 8.25. No significant variation in the number of circulating haemocytes was observed at pH 6.75. This may be due to the fact that L. acuminata f. rufescens is usually found in waters of pH 6.80 to 7.50. A pH of 6.75 being close to the normal pH, did not produce any significant change in the physiological conditions of the animal, and hence a change in haemocyte number was not observed.

Both the species of snails exposed to snail-conditioned water did not show any significant change in the number of circulating haemocytes at any time period studied. Similar results were reported by Wolmarans and Yssel (1988) in B. glabrata kept for 2 hr in

snail-conditioned water. The results indicate that snail-conditioned water did not produce any physiological stress in the two species and it does not seem to be a factor influencing the total counts in I. exustus, and L. acuminata f. rufescens.

In copper-dosed I. exustus the total circulating haemocytes was significantly higher than the controls at all concentrations and at all time periods studied. An exception was noted at 2 hr post-exposure in 0.010 and 0.020 ppm copper dosed snails where the values were non-significantly higher. The increase in the number of circulating haemocytes at all concentrations may be due to transmigration of cells from the reservoir compartment to the haemolymph as a result of stress elicited by copper ions. Snails dosed with 0.010 ppm copper showed significant increase in the number of haemocytes at 6 hr post-exposure followed by a decrease at 12 and 24 hr post-exposure, and again an elevation at 48 hr post-exposure. In 0.015 ppm dosed snails, after initial elevation at 2 and 6 hr post-exposure, a decline was noted at 12 hr followed by an increase at 24 hr and finally a drop at 48 hr post-exposure. Similarly in 0.020 ppm dosed snails after 6 hr post-exposure, the number of cells decreased at 12 and 24 hr followed by an increase at 48 hr post-exposure. This cyclic increase and decrease among the experimentals may be explained as follows. In freshwater gastropods the majority of haemocytes are amoebocytes with granules in the cytoplasm (Sminia, 1972, 1981, 1983; Ottaviani, 1983;

Dikkeboom et al., 1984), and it was reported that although metal stress causes reduction in haemocyte counts, mature granulocytes are seldom affected. The finding of greater number of granulocytes in stressed and polluted molluscs justifies this (Feng et al., 1971; Ruddell and Rains, 1975; Pickwell and Steinert, 1984; Seiler and Morse, 1988). Since granulocytes play important roles in nutrient digestion and transport, excretion, and in internal defence, molluscs exposed to pollutants would need increased number of granulocytes to remove the overload of pollutants and pollutant-laden particulate material (Suresh and Mohandas, 1990b). This is reflected as the increase in the number of granulocytes. In several situations haemocytes have been reported leaving the body by diapedesis after traversing the epithelial layers and the decrease in cell number at certain time intervals may be due to the transmigration of copper-laden amoebocytes by diapedesis or due to cell death. The increase in cell number may also be due to mitosis of leukocytes or to the continuous haemopoiesis from the amoebocyte-producing organ as reported in B. glabrata (Jeong et al., 1983). In this connection it may be noted that these fluctuations in total counts reflected in fluctuations in the activity levels of acid and alkaline phosphatases also in copper-stressed I. exustus. In all the three sub-lethal concentrations, copper induced leucocytosis and in all the three concentrations, the activity of the enzymes was either normal or high, and at no time period the activity was found inhibited. In L. acuminata f. rufescens also there was significant

leucocytosis at all time periods in the experimentals except at 48 hr in 0.010 ppm dosed ones. Although the explanation given for I. exustus holds good for the fluctuations in the total counts of L. acuminata f. rufescens, it could not be correlated with enzyme activity levels.

The results on haemoglobin concentration in I. exustus showed size groupwise variation and snails of the intermediate size group showed more haemoglobin concentration than in snails of the other two size groups. Lee and Cheng (1972) reported that total protein and haemoglobin in B. glabrata of different sizes vary, and haemoglobin concentration gradually increases with growth of the snail. In general, they observed that the haemoglobin concentrations changed with alterations in the total protein concentrations. Lee and Cheng (1972) also reported that smaller snails having a shell diameter between 10 and 16 mm, have a tendency to include a higher percentage of haemoglobin than larger ones, as evidenced by the higher haemoglobin/total protein ratios. On the other hand, larger snails tend to include a greater concentration of total protein in their haemolymph. But in I. exustus although haemoglobin and protein concentrations of still larger size group (above 15 mm) could not be taken; protein and haemoglobin concentrations in snails of shell size 11 and 15 mm showed variation. Between these two size groups haemoglobin concentration did not increase with increase in size, but protein concentration increased, as indicated by Lee and Cheng (1972).

Thus, it is concluded that (a) shell size/age is a factor which influence the number of haemocytes in the two species studied, (b) significant leukocytosis in large size groups snails can be correlated with increased resistance in these snails, (c) abiotic factors such as temperature, and pH affect total cell count, (d) snail-conditioned water does not influence the total count, and (e) copper ions induce leukocytoses in both the species of snails.

C H A P T E R - III

INORGANIC AND ORGANIC CONSTITUENTS IN THE HAEMOLYMPH OF *INDOPLANORBIS EXUSTUS* AND *LYMNAEA ACUMINATA* F. *RUFESCENS*

3.1 INTRODUCTION

Freshwater molluscs are hyperosmotic, and maintain their body fluid and tissue osmolarities above those of the environment. All freshwater pulmonates have haemolymph osmolarities above those of the environment (Machin, 1975), so that an osmotic gradient is continually maintianed between their hyperosmotic body fluids and tissues and the dilute freshwater environment, from which they gain water by osmosis. Thus, in order to regulate body fluid and tissue concentrations at hypersosmotic concentrations freshwater pulmonates must constantly excrete excess water and actively regain lost ions against their respective osmotic and ionic gradients (Robertson, 1964; Prosser, 1973a,b; Machin, 1975). Most investigations on osmoregulatory physiology in freshwater pulmonates have been carried out on *L. stagnalis*, the largest of the common freshwater pulmonates in northern temperate regions (Robertson, 1964; Van Aardt, 1968; Greenaway, 1970; Machin, 1975). The body fluid osmolarity of basommatophoran pulmonates ranges from 124-150 mOsm/Kg H_2O which is less than that of terrestrial pulmonates (142-360 mOsm/Kg H_2O) (Machin, 1975), but higher than freshwater prosobranch snails (74-113 mOsm-Kg H_2O) (Van Aardt, 1968; Machin, 1975). Such decreases in body fluid osmolarity are adaptive in freshwater animals, reducing osmotic and ionic gradients between the tissues and freshwater, and

therefore decreasing water gain and solute loss rates (Prosser, 1973a,b).

In order to maintain hyperosmotic condition with the external medium, freshwater pulmonates excrete the body fluids of excess water by osmosis. Both freshwater pulmonates and prosobranchs produce relatively concentrated excretory fluids having 75% of haemolymph concentration (Robertson, 1964; Van Aardt, 1968). Thus, the excretory fluid represents a site of considerable solute loss which must be restored by active uptake of solutes from the environment. To overcome the continuous flux of solutes from the haemolymph through the excretory fluids, freshwater molluscs develop various osmoregulatory mechanisms and hence the study of ionic composition in the haemolymph has significant importance.

Compared to marine molluscs, the ionic concentrations and osmotic pressures are always much less in freshwater forms and some of the freshwater bivalves are among the most dilute animals known. In marine molluscs, the ionic regulation consists mainly of raising potassium and calcium ion concentrations, blood sodium and chloride ions remaining practically in equilibrium with the surrounding medium, but in freshwater species, the concentration of all the ions including sodium and chloride ions appears to be regulated (Schoffeniels and Gilles, 1972). Freshwater molluscs as well as terrestrial ones produce urine hypotonic to the blood (Little, 1965b, 1968, 1972). The reabsorption of ions inevitably decreases the amount of salt loss

from the body. Reabsorption of salts from urine appears to be the main system involved in the blood ionic regulation of marine and terrestrial species. However, in freshwater molluscs, this mechanism can only compensate part of the salt loss both through the urine and body walls. The loss of salts must thus be balanced in these species by an active uptake from the surrounding medium (Schoffeniels and Gilles, 1972). The blood of freshwater molluscs is much more concentrated in sodium and chloride ions than the environmental medium. Sodium and chloride are generally the most abundant ions, while the concentrations of potassium and magnesium are very low.

Active uptake of sodium, potassium and calcium by freshwater molluscs has been demonstrated. The uptake of chloride was first demonstrated in Anodonta and Lymnaea by Krogh (1939). He reported that in Anodonta, sodium is actively absorbed even in the absence of external chloride. This is supported and demonstrated in four families of freshwater bivalves (Dietz, 1979). The chloride pump exchanges chloride for bicarbonate and sodium uptake mechanism exchanges sodium for hydrogen ions in L. stagnalis (De With et al., 1980). This mechanism was also reported in Ligumia subrostrata and Carunculina texasensis (Dietz and Branton, 1979). In L. stagnalis sodium uptake is stimulated by loss of haemolymph and by sodium depletion, which leads to a fall in haemolymph volume as well as sodium concentration (Greenaway, 1970). De With et al. (1980) reported that a low internal pH appropriately stimulates exchanges of sodium

and hydrogen ions and inhibits exchange of chloride and bicarbonate ions in L. stagnalis.

Graves and Dietz (1980) reported diurnal fluctuation in sodium influx and in the concentration of sodium and chloride in the haemolymph of C. texasensis. Greenaway (1970) postulated hormonal control in ion regulation and it may be that sodium uptake is influenced by neurosecretion from the dark green cells (Swindale and Benjamin, 1976). Freshwater pulmonates maintain a continual net uptake of calcium carbonate, the major component of the shell. In freshwater, calcium is the major dissolved cation, its concentration ranging from 1-3 mg Ca/Kg H_2O to concentrations greater than 400 mg Ca/H_2O (Hunter and Lull, 1977). Majority of the freshwater basommatophoran snails are highly calciphilic and are more successful in waters of higher calcium concentrations (Young, 1975). Some environmental calcium concentration ranges reported for freshwater pulmonates are; 30-280 mg Ca/Kg H_2O for Lymnaea palustris (Hunter, 1972, 1975), and 6.9-93 mg/Kg H_2O for Biomphalaria pfeifferi (Harrison et al., 1970). Several studies have shown that freshwater pulmonates can absorb calcium required for shell growth directly from the external medium (Greenaway, 1971; Wilbur, 1972). However, a significant proportion of shell calcium may also be absorbed from ingested material when calcium is taken by across the digestive tract epithelium (Van der Borght and van Puymbroeck, 1966, Young, 1975).

When the external concentration is less than 0.5 mM, uptake of calcium is against the electrochemical gradient. Greenaway (1971) reported that the shell of freshwater pulmonates acts as a calcium buffer or store from which lost haemolymph or tissue calcium can be released during periods of calcium depletion caused by short term decreases in external calcium concentrations such as caused by flooding.

The calcium cells, a special type of connective tissue cell which is rich in calcium, are reported in gastropods by Sminia et al. (1977). The main function of these cells are in the synthesis and repair of shell and operculum and in the buffering of pH of body fluids. (Burton, 1970; Istin and Girard, 1970). In L. subrostrata, haemolymph calcium rises to anoxia, as much as eight fold. The calcium or bicarbonate may be raised at low salinities in freshwater and oligohaline bivalves (Bedford, 1973; Murphy and Dietz, 1976; Deaton, 1981). In the case of Rapana thomasiana taken out of water the calcium content and buffer value of haemolymph rise as in bivalves (Alyakrinskaya, 1972), while P. virens when made anaerobic, the haemolymph pH falls and lactate accumulates, but neither calcium or magnesium changes in concentration (Meenakshi, 1956). In L. stagnalis exposed to water equilibrated with 10% CO_2 in air, the concentration of calcium in the haemolymph rises in a few hours from about 3.6 to about 13 mM. At the same time, the number visible $CaCO_3$ containing cells in the connective tissue of the foot markedly falls (Sminia et al., 1977). In Planorbis corneus, the level of

calcium was found to be correlated with the level of potassium (Sorokina and Zelenskaya, 1967). It is not known whether there is hormonal control of calcium uptake, but there is evidence for a hormone in the ganglia of Helisoma that somehow raises haemolymph calcium (Khan and Saleuddin, 1979).

The normal ionic concentrations (mM) in the haemolymph of several gastropods are reported. Some of them are given below: Pila globosa, Na^+ = 54.7, K^+ = 4.9 and Ca^{2+} = 7.8 (Saxena, 1957); Viviparus viviparus, Na^+ = 34.0, K^+ = 1.2, Ca^{2+} = 5.7 and Cl^- = 31.0 (Little, 1965); P. corneus, Na^+ = 50.0 K^+ = 1.9, Ca^{2+} = 5.9 (Burton, 1968); and L. stagnalis, Na^+ = 55.3, K^+ = 1.7, Ca^{2+} = 3.5 and Cl^- = 36.2 (De With and Sminia, 1980). The effect of starvation on the ionic concentration of L. stagnalis was studied by De With et al. (1980). The changes in starvation are mostly complete in one day or even 6-8 hr in the case of sodium and potassium (De With, 1978). Some of the changes in starvation could follow from reduced metabolic production of CO_2. The lowered PCO_2 would raise the pH and so both stimulate Cl/HCO_3 exchange and inhibit Na/H exchange. The resulting depletion of bicarbonate ions would restore the pH towards the normal. The number of calcium cells containing concretions falls in starvation, and correlates more with haemolymph calcium than with the environmental calcium (De With and Sminia, 1980). De With and Sminia (1980) have also shown that concentrations of calcium and carbonate are lowered in starvation, but the relationship of this to the solubility of $CaCO_3$ is

controversial. The role of the shell here is unclear, but it helps maintain haemolymph calcium when calcium is lost in the medium (Greenaway, 1971).

Prasad et al. (1985) studied the effects of xiphidio cercarial infections on the ionic composition of the L. luteola. Sodium, potassium and calcium levels in foot mantle, digestive gland, and body fluid have been estimated. There is marked increase in sodium and potassium in body fluid during infection while no change is observed in the tissues. The most significant observation made is the increased calcium levels in body fluid with ion concentration decrease in tissues, especially mantle and digestive gland. However, such marked increase will have profound effect on the overall neuromuscular physiology of the snail. The increase in the calcium levels in the body fluids could be attributed to maintenance of pH of blood, since anaerobic conditions are reported to prevail in vivo in infected snails. The concomitant decrease in the calcium levels observed in the mantle and digestive gland may be attributed to the leakage of clacium into the body fluid.

The nitrogenous degradation products, ammonia, urea and uric acid, are generally transported with the body fluids to places where they are excreted or further processed. The amounts of nitrogenous degradation products produced are indicative of the activity of the protein, and nucleic acid metabolism of an animal so that any unusual stress imposed on the latter must make itself apparent in a change in the amounts of ammonia, urea and uric acid produced.

Rao and Narayanan (1976) analysed the nitrogenous compounds in the body fluid of four selected freshwater gastropods, L. luteola, I. exustus, P. globosa and V. bengalensis. Lymnaea and Indoplanorbis differ from Pila and Viviparus in their ratio of ammonia to urea. While it is 3.73 in Lymnaea, and 3.6 in Indoplanorbis, it is 7.36 in Pila and 6.6 in Viviparous. Lymnaea and Indoplanorbis are reported to be ureotelic while Pila and Viviparus ammonotelic. Urea retension is reported to be a reason for Lymnaea and Indoplanorbis to be the preferred host for larval trematodes. Reddy et al. (1974) reported the levels of ammonia, urea, and uric acid in the hepatopancreas, mantle, foot and body fluid of active, aestivating and revived P. globosa. The ammonia and urea levels decreased on aestivation, while the uric acid level increased several fold. On revival, the ammonia level increased in the body fluid but decreased in the foot, mantle and hepatopancreas, and the urea and uric acid levels increased to a considerable extent in all the tissues. A shift towards uric acid synthesis on aestivation and urea synthesis on revival through uricolysis has been suggested.

There are a few reports on the concentration of urea in the haemolymph. De Jorge et al. (1965) reported a high value of 30.5 mg/100 ml of urea for Strophocheilus oblongus, while Tramell and Campbell (1970) were unable to identify any urea in S. oblongus. Vasu and Giese (1966) found approximately 9 mg/100 ml of urea in Cryptochiton stelleri during the winter. In two individuals of

L. stagnalis, Friedl (1961) determined chromatographically, 0.46 and 0.61 mg/100 ml of urea, respectively. Becker and Schmale (1978) found that in normally fed B. glabrata, the urea concentration fluctuated between 0 and 1.05 mg/100 ml, and in 5 days starved snails it was found to be 5.12 mg/100 ml. Meyer and Becker (1980) reported the effects of exposure (to ammonium chloride solutions) on urea concentrations in the haemolymph, urea excretion rate, and an incorporation rate of $^{14}CO_2$ in urea in the digestive gland, in vitro. During these experiments significant increase in the urea concentration in the haemolymph was reported.

Comparison of NH_3-N concentrations in the body fluid of various molluscs showed that the NH_3-N concentration in the haemolymph of B. glabrata is very low, of about 0.1 mg/100 ml. Florkin and Houet (1938) also reported a low NH_3 value of 0.051-0.071 mg/100 ml in A. cygnea. The highest value was reported in terrestrial pulmonates, H. pomatia with 1.2 and Arion rufus with 1.4 mg/100 ml NH_3-N. Becker and Schmale (1978) reported that NH_3-N concentration in normally fed B. glabrata varied between 0.031 and 0.145 mg/100 ml, and that in 5 day starved snails rose to an average value of 0.111 mg/100 ml. The rise in the NH_3-N and urea concentrations may be either due to a decrease in the activity of the excretion process, caused by starvation which brings about an increased concentration of urea or the body protein decomposition during the period of starvation.

The energy metabolism in gastropods is generally carbohydrate based as reported in freshwater snails L. stagnalis (Veldhuijzen and Van Beek, 1976), and P. corneus (Emerson, 1967). Glucose is the main form of carbohydrate detected in the tissues and body fluid of most gastropods. Other sugars found in gastropods include galactose, fructose, xylose, ribose, fucose, mannose etc. Rao and Onnurappa (1979) identified fructose, xylose and ribose in the digestive gland, and glucose and galactose in both the digestive gland and body fluids of L. luteola. Renwrantz et al. (1976) reported glucose (216 µg/ml), galactose (24 µg/ml) fucose (8 µg/ml), and mannose (8 µg/ml) in the ultrafiltrate of the haemolymph of H. pomatia. Sugar derivatives such as N-acetylgalactosamine and N-acetyl glucosamine also occur but are usually incorporated into glycoproteins. The blood glucose levels reported in a number of of gastropods include, Ariolimar columbianis, 1.55 mM (Meenakshi and Scheer, 1968); L. stagnalis, 0.17 mM (Friedl, 1968, 1971); B. glabrata, 1.07-1.56 mM (Cheng and Lee, 1971); L. alte, 1.54 mM (Kulkarni, 1973); H. pomatia, 1.20 mM (Renwrantz et al., 1976), and B. glabrata, 0.62 mM (Liebsch et al., 1978). Scheerboom et al. (1978) reported blood glucose level of 0.61 - 1.11 mM in L. stagnalis maintained on Bemax, while lettuce fed snails showed 0.28 - 0.34 mM (Scheerboom and Hemminga, 1978). Becker (1972) reported intraindividual variability in glucose level in A. glabratus and reported that glucose concentrations varied between 0.06 and 0.96 mM over a period of 6 hr.

In gastropod tissue carbohydrate occurs mainly in two types of connective tissue cells, the granular cells and vesicular connective tissue cells. Sminia (1972) reported comparable results in L. stagnalis. The granules found in the granular cells are cysteine-rich glycoproteins, and these cells are dispersed throughout the body. The vesicular connective tissue cells are the major storage cell for glycogen with an important role in the nutrition of the tissues. The main storage carbohydrates in gastropods are glycogen and galactogen. Glycogen is mainly found throughout the tissues especially in the hepatopancreas, foot and mantle, and the concentrations are approximately 10-30% of the dry weight (Livingstone, 1982). Glycogen also occurs in the muscle tissues, and in P. virens glycogen is reported in triturative stomach muscles and the radular muscle (Suryanarayanan and Alexander, 1973). Rao et al. (1979) reported that the levels of glycogen depend on the extraction methodology, and found a variation of 1.4 to 2.6 times in L. luteola. Galactogen is restricted to the albumen gland portion of the female reproductive tract of adult pulmonates and to eggs (Goudsmit, 1972, 1973). It is a major nutritive reserve of the embryos and can also be used as an emergency food source in starvation, only after complete utilization of glycogen (Livingstone and de Zwaan, 1983). The occurrence of galactogen has been reported in the albumen glands of L. stagnalis, H. pomatia Catinella vermeta, Omalonyx felina, and Oxlyoma retusa (Rudolph, 1974).

Haemolymph glucose concentrations vary greatly with individual species, and may be influenced by handling, food quality and quantity, seasonal changes, parasitic infection, and indirectly by temperature (Veldhuijzen, 1975b), and photoperiod (Bohlken et al., 1978) through their effect on reproductive activity. The haemolymph glucose concentration is related to the quality of food assimilated (Meenakshi and Scheer, 1968; Friedl, 1971; Scheerboom 1978; Stanislawski and Becker, 1979) or quantity of the food. Scheerboom (1978) reported that snails fed with small lettuce rations have the same glucose concentrations as those of standard snails, and when assimilation was between 20 and 30 mg (dry weight), the glucose levels increased with increasing amount of food assimilated. The consumption of food itself is under the control of the haemolymph glucose concentration, and is inhibited by concentrations above 120 µg/ml (Scheerboom and Doderer, 1978). The increased food availability and intake result in accumulation of carbohydrate which results in higher blood sugar levels (Lambert and Dehnel, 1974; Scheerboom and van Elk, 1978).

The carbohydrate metabolism of many gastropods is seasonally variable. The carbohydrate levels are generally highest in summer and autumn, and lowest in winter (Marques and Pereira, 1970; Chatterjee and Ghose, 1973; McLachlan and Lombard, 1980). In H. Pomatia, glycogen is synthesized and stored in several tissues during autmn and subsequently catabolized during winter hibernation. In spring, the snails begin refeeding and accumulate galactogen

in the albumen gland. In summer the galactogen is transferred to the eggs and no more galactogen is then synthesised until the next egg laying season (Goudsmit, 1973, 1975). During aestivation, as a result of decreased oxygen consumption and Kreb's cycle oxidation, haemolymph body reserves, particularly carbohydrate, are utilized resulting in decreased level of carbohydrates (Heeg, 1977; Krupanidhi et al., 1978, Swami and Reddy, 1978; Horne, 1979). Starvation also results in reduced metabolic rate, and carbohydrate consumption is increased in starved gastropods (Emerson, 1967; Christie et al., 1974; Stanislawski and Becker, 1979). Changes in carbohydrate levels associated with circadian fluctuations were reported in the slug L. alte. The total carbohydrate of several tissues was the highest during the inactive light phase and lowest during the active dark phase (Kumar et al., 1981).

Parasitism is a factor which results in decreased tissue carbohydrate levels and blood glucose concentrations as a result of infection as reported in B. alexandrina (Mohamed and Ishak, 1981), and B. glabrata (Cheng and Lee, 1971, Christie et al., 1974) infected with Schistosoma mansoni; I. exustus infected with Cercariae indicae (Vaidya, 1979), and in L. luteola (Krishna and Simha, 1977) infected with xiphidio, amphistome or furcocercus cercariae. However, Manohar and Rao (1976) reported no change in carbohydrate and sugar levels in L. luteola. Ishak et al. (1975), reported that S. mansoni infected B. alexandrina and Bulinus truncatus showed lowered capacity

for Kreb's cycle oxidations, lowered cytochrome oxidase activity, lowered gluconeogenesis, increased lactate production, and depletion in glycogen reserves. The depletion in glycogen is due to the shift from an aerobic to anaerobic metabolism. Similarly, it must be partly responsible for decreased gluconeogenesis.

Hormonal regulation for carbohydrate metabolism is reported in molluscs and several studies indicate the existence of mechanisms for regulating blood glucose levels and for counteracting hypo and hyperglycemia (see Joosse and Geraerts, 1983). In L. stagnalis, although fixed maximum blood glucose concentrations are not apparent, a minimum level is maintained in starved animals (Veldhuijzen, 1975a). Insulin-like activity and homologues of pancreatic β-cells in the digestive tract have been identified in several gastropods, H. aspersa (Goddard et al., 1964), H. pomatia (Ammon et al., 1967), and Buccinum undatum (Boquist et al., 1971; Davidson et al., 1971).

The serum proteins in molluscs are reported to be engaged in maintaining the osmolarity, transport of ions, immune mechanisms, and homoeostasis. Proteins have also been implicated to perform detoxification of metal ions (Simkiss and Mason, 1983), and recognition of self and non-self materials (Cheng, 1986). The concentration of total free-aminoacids in freshwater gastropods is low in the tissues (4-10 mM), and haemolymph (0.3-1.5 mM) (Simpson et al., 1959; Gilbertson and Schmid, 1975; Reddy and

Swami, 1978; Stanislawski et al., 1979). Almost all aminoacids are reported in gastropod haemolymph, the important being alanine, glycine, glutamine, aspartate, serine, and threonine. The aminoacid concentrations in the tissues as well as extracellular fluid compartments of molluscs vary with diet, season, temperature, desiccation, anaerobiosis, osmotic pressure, pollution, parasitism, and during reproduction and developmental stages. Respiratory pigments, haemocyanin and haemoglobin, are the other major proteins found in molluscan haemolymph. Haemoglobin is reported in I. exustus and in B. glabrata, while haemocyanin in P. globosa and L. stagnalis. Haemoglobin has also been reported in bivalves (see Patel and Patel, 1964). The primary function of these proteins is to increase the absorption coefficient of oxygen in circulating fluids, thus augmenting the oxygen content of the blood (Ghiretti and Ghiretti-Magaldi, 1972).

Variations in haemolymph protein concentration of B. glabrata have been described by several authors (Gilbertson,et al.1967; Lee and Cheng, 1972; Becker and Hirtbach, 1975). Lee and Cheng (1972) described a decline in the total protein concentrations in the haemolymph after 2 days of starvation, while Becker and Hirtbach (1975) reported a decline after 7 days of starvation. Reddy and Naidu (1978) studied protein concentration in the blood of active, aestivated, and one month starved P. globosa and reported greater orientation towards protein metabolism during starvation. Although

there was a general decrease in total protein content in the blood upon aestivation and starvation, the decrease upon starvation was significantly higher (Reddy and Naidu, 1978). Haemolymph protein concentration and free aminoacid levels in B. glabrata were found to change during infection (Dusanic and Lewert, 1963; Gilbertson et al., 1967; Lee and Cheng, 1972). In B. glabrata infected with S. mansoni, the protein concentration had declined to one third that of uninfected snails after 70 day post-exposure (Gress and Cheng, 1973), but B. glabrata challenged with bacteria showed an elevation in the serum protein (Cheng et al., 1978a). The increase in protein concentration was attributed to hyper-synthesis of some yet unidentified host humoral protein fraction.

Although knowledge of lipids in molluscs has increased considerably, there is still little insight as to how molluscs manage to achieve and to maintain a characteristic composition of lipids, despite the often quite different lipid composition of the diet (Voogt, 1983). In gastropods, lipids are mainly stored in gonads, digestive gland, and midgut gland (Giese, 1966; Lumbert and Dehnel, 1974; Streit, 1978). Holland et al. (1975), reported high lipid contents in the veliger larvae of several Littorina species.

Information on the mechanism of transport of lipid in the haemolymph of molluscs is scarce. Giese (1969) reported that the lipid level in the visceral mass of gastropods decreases, while in the ovary it increases (just before spawning), and increases

again afterwards. These changes suggest mobilization of the nutrients and transport to gonads especially to the ovary. The mechanism of transport and composition of lipid in C. stelleri were reported by Allen (1977). Gilbert and Chino (1974) determined the rate of entry and exit of lipid into plasma lipid compartments by invitro experiments using prelabelled plasma or the reverse. The ingested lipid is transported in the form of free fatty acids and triglycerides in blood.

The metabolism in carnivorous prosobranchs is reported to be lipid oriented (Stickle and Duerr, 1970). In Melania scabra (Muley, 1975), a reciprocal relationship was observed between the synthesis of lipids and utilization of proteins, and vice versa. Chatterjee and Ghose (1973) reported the utilization of lipid during breeding in V. bengalensis and Acrostoma variabile. Streit (1978) reported that in Ancylus fluviatilis lipid storage is larger in summer than in winter, and during starvation lipid is used first followed by carbohydrates. In B. africanus, the main reserve seems to be protein, but during aestivation lipid is metabolised over carbohydrates, whereas under starvation, the opposite takes place (Heeg, 1977). Allen (1977) reported that there was no sex-wise difference in the plasma lipid concentration. Parasitism is an important factor which influences lipid concentration to a certain extent. Lipase activity is elevated under exposure to Bacillus megaterium indicating increased lipid hydrolysis during bacterial

stress (Cheng and Yoshino,1976a). Babu et al. (1981) reported rapid depletion during cercarial infection in L. luteola.

In this chapter results of a study on the various inorganic - sodium, potassium, calcium, chloride, and ammonia - and organic - urea, glycogen, total carbohydrate, total protein, and lipids - constituents in the haemolymph of the three size groups of I. exustus and L. acuminata f. rufescens are reported.

3.2 MATERIALS AND METHODS

Methods of collection of snails, acclimatisation, selection of size groups, and method of collection of haemolymph were the same as described in section 2.2.

The number of snails employed for studying the various haemolymph constituents in the three size groups each of the two species of snails is given below. In the case of I. exustus, 15 snails each from 7 ± 1 mm, and 15 ± 1 mm, and 20 from 11 ± 1 mm size group were employed to study the haemolymph sodium, potassium and calcium. To study the chloride contents 25 snails from 7 ± 1 mm, 30 from 11 ± 1 mm, and 20 from 15 ± 1 mm size groups were employed. Groups of 25 snails from 7 ± 1 mm and 11 ± 1 mm, each and 20 from 15 ± 1 mm were used to study the total carbohydrate present in the haemolymph. To study other parameters, viz. urea, ammonia, glycogen, total protein, and total lipid, 15 snails from each size group were employed.

In the case of L. acuminata f. rufescens, 12 snails from each size group were selected for studying the various haemolymph constituents except chloride for which 15 snails from each size group were employed.

3.2.1 Estimation of Sodium, Potassium and Calcium

Sodium, potassium and calcium levels were estimated by the Flame photometric method (Robinson and Ovenston, 1951., Oser, 1965). For this, 0.2 ml of haemolymph was added to 2.0 ml of conc. nitric acid and kept over a sand bath for 15 minutes. The acid digest was then made upto 10 ml in a 10 ml standard flask with deionized distilled water. Sodium, potassium and calcium were quantitatively estimated using a Flame Photometer (Elico, Type-22). Standard solutions of sodium, potassium, and calcium were prepared using AnalaR grade chemicals. The concentrations of sodium, potassium and calcium in the samples were calculated from the standard graphs, and expressed as μ equivalents/ml of haemolymph.

3.2.2 Estimation of Chloride

Chloride content in the haemolymph was estimated using a Chloride Meter (ELICO CHLORIDE METER MODEL EE - 34) with its associated standards and reagents. Chloride Meter offers simple, rapid and highly accurate chloride estimation without the need of calibration curve. After standardisation with standard NaCl solution (100 mEq per litre), 0.1 ml of haemolymph was added to the reagent

mixture (acid buffer and gelatin solution), titrated, and the reading was directly measured and expressed as milliequivalents/litre.

3.2.3 Estimation of Ammonia

Phenol-hypochlorite method, as described by Grasshoff and Johannsen (1972) was employed to determine the haemolymph ammonia. For this, 0.2 ml of haemolymph was added to 2.0 ml of 80% ethanol and centrifuged at 5,000 rpm for 5 minutes. The supernatant was taken in a clean test tube and made up to 5 ml. To this, 0.2 ml of phenol solution was added followed by 0.2 ml of sodium nitroprusside solution and shaken well. Then 0.5 ml of oxidising solution was added and mixed well. The test tubes were capped tightly and kept in dark for 2 hrs, and later the readings were measured at 640 nm. The concentration of ammonia in the haemolymph was expressed as NH_3-N in mg/100 ml, and calculated from a standard graph using NH_4Cl as standard.

3.2.4 Estimation of Urea

Diacetyl-monoxime method as described by Natelson (1972) was employed to estimate the haemolymph urea. For this, 0.1 ml of haemolymph was added to 3.3 ml of distilled water followed by 0.3 ml of 10% sodium tungstate and 0.3 ml of 2/3 N H_2SO_4. The sample was then centrifuged at 3,000 rpm for 10 minutes and 1 ml of supernatant was taken in a clean test tube. To this, 1 ml distilled water was added followed by 0.4 ml of diacetyl monoxime and 1.6 ml

of sulphuric acid-phosphoric acid mixture, and kept in a boiling water bath for 30 minutes, then cooled and read spectrophotometrically at 480 nm. The concentration of urea in mg/100 ml was found out from a standard graph prepared with urea standard.

3.2.5 Estimation of Glycogen

Glycogen in the haemolymph was determined following the method of Montgomery (1957). The method is as follows: 0.1 ml haemolymph was pipetted into a clean test tube containing 1.0 ml of 10% Trichloroacetic acid to deproteinize the haemolymph. It was then centrifuged for 10 minutes at 2,500 rpm, and the supernatant was decanted into another test tube. For determination of glycogen, 1.2 ml of 95% ethyl alcohol was added to 1.0 ml of the supernatant, kept undisturbed for overnight in a refrigerator, then centrifuged at 2,500 rpm for 15 minutes. The supernatant was gently decanted. The precipitate was dissolved in 2.0 ml distilled water, and 0.1 ml of 80% phenol was added and mixed well. To this 5.0 ml of conc. sulphuric acid was added forcefully with a blowout pippette for thorough mixing, and kept for 30 minutes at room temperature. After, cooling, the optical density was measured at 490 nm, and the glycogen present in the haemolymph was measured from a standard graph using glucose as the standard. The glycogen present in the haemolymph was expressed as µg/ml (in glucose equivalents).

3.2.6 Estimation of Total Carbohydrate

The total carbohydrate present in the haemolymph was determined following the method of Dubois et al. (1956). For this, 0.1 ml of haemolymph was pipetted into a clean test tube containing 0.1 ml of 80% phenol. To this, 1.9 ml of distilled water was added followed by 5.0 ml of conc. H_2SO_4. The samples were mixed well and kept at room temperature for 30 minutes. After cooling, the optical density was measured at 490 nm. Total carbohydrate present in the haemolymph was measured from a standard curve prepared with glucose standard, and the results were expressed as µg glucose equivalents per ml.

3.2.7 Estimation of Total Protein

Protein was estimated employing Lowry's method (Lowry et al., 1951). For this, 0.1 ml haemolymph was deproteinized in 1.0 ml of 10% Trichloroacetic acid and centrifuged at 2,500 rpm for 10 minutes. The supernatant was drained and 1.0 ml of 0.1 N NaOH was added to the precipitate. From this, 0.2 ml of extract was pipetted into another test tube and made upto 1.0 ml with distilled water. To this, 5.0 ml of alkaline copper reagent was added and mixed well. After 10 minutes, 0.5 ml of Folin's phenol reagent was added and shaken well. After 45 minutes the optical density was measured at 500 nm and the protein concentration was found out from a standard curve employing bovine serum albumin as the standard. The results were expressed as mg protein/ml of haemolymph.

3.2.8 Estimation of Lipid

Sulphophosphovanillin method as described by Barnes and Blackstock (1973) was employed to estimate lipid present in the haemolymph. For this, 0.2 ml haemolymph was taken in a clean test tube, and 1.0 ml of methanol was added followed by 2.0 ml of chloroform and 2.0 ml of methanol-chloroform mixture (1:2). This mixture was shaken well, and 0.2 ml of 0.9% NaCl was added to it. The mixture was poured into a separating funnel, mixed well and allowed to stand for 30 minutes. The lower phase was then separated into a clean test tube and dried in a vacuum desiccator over silica gel. Then, 0.5 ml conc. H_2SO_4 was added to it and mixed well. The test tube was plugged with non-absorbant cotton and placed in a boiling water bath for 10 minutes. After cooling, 5.0 ml of phosphovanillin reagent was added to 0.2 ml of the acid digest, mixed well and allowed to stand for 30 minutes. The developed colour was measured spectrophotometrically at 520 nm. Cholesterol was used for preparing the calibration curve, and the result expressed as µg/ml (in cholesterol equivalents).

3.2.9 Computation and Presentation of Data

The experimental results are represented in Tabular form and the data analysed statistically by two tailed student's 't' test to manifest the variation among different size groups. The variations were reported at three significant levels, viz. $P < 0.05$, 0.01, and 0.001 . All the computations were carried out using a personal computer (Casio Fx-730P). All spectrophotometry readings were done using HITACHI Model UV-Vis spectrophotometer U-2000.

3.3 RESULTS

3.3.1 Sodium

3.3.1.1 Indoplanorbis exustus (Table 7A)

Statistical analysis of the data revealed that the average value of 15 ± 1 mm size group was significantly lower than the average values of the two other size groups ($P < 0.001$), while the values in 7 ± 1 mm and 11 ± 1 mm size groups, between them, showed no significant variation.

3.3.1.2 Lymnaea acuminata f. rufescens (Table 7B)

Statistical analysis of the data revealed that the average values of 21 ± 1 mm size group was significantly lower than the values in the two other size groups ($P < 0.001$). There was no significant variation in values between 13 ± 1 mm and 18 ± 1 mm size groups.

3.3.2 Potassium

3.3.2.1 Indoplanorbis exustus (Table 8A)

Statistical analysis of the data revealed that there was significant variation among the average values of haemolymph potassium in the three size groups. The average value in the 15 ± 1 mm size group was significantly lower than the values in 11 ± 1 mm ($P < 0.001$) and 7 ± 1 mm ($P < 0.001$). When the value in the size group 7 ± 1 mm was compared with that of 11 ± 1 mm, the former showed significantly higher value than the latter ($P < 0.05$).

Table 7A. Haemolymph Sodium (μ equivalents/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	20	15
Mean value	42.45	42.12	30.95
± SD	2.44	5.74	3.47
Range	39.13 - 47.82	33.04 - 53.91	23.47 - 35.65

Table 7B. Haemolymph Sodium (μ equivalents/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	38.54	37.41	30.84
± SD	3.92	3.49	3.52
Range	31.30 - 45.20	31.30 - 45.20	25.20 - 35.60

Table 8A. Haemolymph Potassium (μ equivalents/ml) in the three size groups of *Indoplanorbis exustus*

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	20	15
Mean value	3.24	2.61	1.47
± SD	0.66	0.84	0.47
Range	2.56 - 3.84	1.02 - 4.10	1.02 - 2.56

Table 8B. Haemolymph Potassium (μ equivalents/ml) in the three size groups of *Lymnaea acuminata* f. *rufescens*

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	1.96	1.62	1.28
± SD	0.66	0.53	0.27
Range	1.02 - 3.07	1.02 - 2.05	1.02 - 1.54

3.3.2.2 Lymnaea acuminata f. rufescens (Table 8B)

Statistical analysis of the data revealed significant variation in values betwen size groups 13 ± 1 mm and 21 ± 1 mm. The average value of potassium was significantly higher in 13 ± 1 mm than in 21 ± 1 mm ($P < 0.01$).

3.3.3 Calcium

3.3.3.1 Indoplanorbis exustus (Table 9A)

When the datas were statistically analysed significant variations in calcium level among the three size groups were noted. The average value of calcium in the size group 11 ± 1 mm was significantly lower than that in 7 ± 1 mm ($P < 0.001$), and 15 ± 1 mm size group ($P < 0.01$), while 7 ± 1 mm size group snails showed significantly higher haemolymph calcium than in 15 ± 1 mm size group ($P < 0.01$).

3.3.3.2 Lymnaea acuminata f. rufescens (Table 9B)

When the values in the three size groups were compared, 21 ± 1 mm size group showed significantly higher values than the other two size groups ($P < 0.001$). There was no significant variation in values between 13 ± 1 mm and 18 ± 1 mm size groups.

3.3.4 Chloride

Statistical analysis of the data revealed that there was no significant variation in chloride levels in the three size groups of I. exustus (Table 10A), and L. acuminata f. rufescens (Table 10B).

Table 9A. Haemolymph Calcium (μ equivalents/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	20	15
Mean value	29.66	16.90	22.40
± SD	5.89	3.97	5.92
Range	22.50 - 40.00	10.00 - 26.00	8.00 - 29.00

Table 9B. Haemolymph Calcium (μ equivalents/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	11.41	10.08	16.25
± SD	3.75	2.06	2.22
Range	9.00 - 14.00	7.00 - 14.00	13.00 - 18.00

Table 10A. Haemolymph Chloride (μ equivalents/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	25	30	20
Mean value	38.92	38.13	36.12
± SD	4.63	6.32	5.38
Range	30.00 - 50.00	26.00 - 54.00	28.00 - 52.00

Table 10B. Haemolymph Chloride (μ equivalents/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	15	15	15
Mean value	43.60	42.26	43.93
± SD	6.06	3.82	4.02
Range	33.00 - 59.00	38.00 - 52.00	38.00 - 51.00

3.3.5 Ammonia

Statistical analysis of the data revealed that there was no significant variation in ammonia levels in the three size groups of I. exustus (Table 11A), and L. acuminata f. rufescens (Table 11B).

3.3.6 Urea

3.3.6.1 Indoplanorbis exustus (Table 12A)

Statistical analysis of the data revealed that the average values in 15 ± 1 mm size group was significantly higher than the average values in the two other size groups ($P < 0.01$). There was no significant variation in haemolymph urea values among 7 ± 1 mm, and 11 ± 1 mm size groups.

3.3.6.2 Lymnaea acuminata f. rufescens (Table 12B)

When data were statistically analysed, significant variation in values was found only between size groups 13 ± 1 mm and 21 ± 1 mm. The haemolymph urea value in the size group 21 ± 1 mm was found to be significantly higher than the value in 13 ± 1 mm size group ($P < 0.05$).

3.3.7 Glycogen

3.3.7.1 Indoplanorbis exustus (Table 13A)

There was significant variation in haemolymph glycogen levels between two size groups. The average value of glycogen in 15 ± 1 mm size group was significantly higher than the values in

Table 11A. Haemolymph Ammonia (mg/100 ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	15	15
Mean value	1.52	1.35	1.33
± SD	0.49	0.57	0.29
Range	0.44 - 2.23	0.63 - 2.77	0.63 - 1.93

Table 11B. Haemolymph Ammonia (mg/100 ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	0.39	0.39	0.39
± SD	0.12	0.13	0.11
Range	0.25 - 0.55	0.19 - 0.62	0.22 - 0.55

Table 12A. Haemolymph Urea (mg/100 ml) in the three size groups of *Indoplanorbis exustus*

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	15	15
Mean value	2.50	2.61	3.47
± SD	0.62	0.55	0.87
Range	1.61 - 3.83	1.61 - 3.83	1.81 - 4.84

Table 12B. Haemolymph Urea (mg/100 ml) in the three size groups of *Lymnaea acuminata* f. *rufescens*

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	6.36	7.75	9.28
± SD	4.05	2.17	1.89
Range	2.62 - 13.33	4.84 - 10.90	5.64 - 12.52

Table 13A. Haemolymph Glycogen (µg glucose/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	15	15
Mean value	41.86	53.56	117.55
± SD	13.56	17.79	33.09
Range	20.10 - 73.04	25.85 - 81.36	71.80 - 169.14

Table 13B. Haemolymph Glycogen (µg glucose/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	69.20	47.61	118.27
± SD	20.12	16.48	39.92
Range	34.40 - 112.00	26.00 - 77.00	77.50 - 198.20

the other two groups ($P < 0.001$). There was no significant variation in values among size groups 7 ± 1 mm and 11 ± 1 mm.

3.3.7.2 Lymnaea acuminata f. rufescens (Table 13B)

There was significant variation in haemolymph glycogen levels among the three size groups. The mean value of glycogen in 21 ± 1 mm size group was significantly higher than the values in the other two groups ($P < 0.001$). When the value in 13 ± 1 mm size group was compared with the value in 18 ± 1 mm size group, the latter size group showed a significantly lower glycogen level ($P < 0.001$).

3.3.8 Total Carbohydrate

3.3.8.1 Indoplanorbis exustus (Table 14A)

Statistical analysis of the data revealed that there was size related significant elevation in total carbohydrate level in the haemolymph of the three size groups. The average value in 15 ± 1 mm size group was significantly higher than those in the other two groups ($P < 0.001$). When the values in 7 ± 1 mm size group and 11 ± 1 mm size group were compared, the latter showed significantly higher value ($P < 0.001$).

3.3.8.2 Lymnaea acuminata f. rufescens (Table 14B)

The total carbohydrate content in the haemolymph was found to be size dependent. The average value in 21 ± 1 mm size group was significantly higher than the values in the other two groups

Table 14A. Haemolymph Total Carbohydrate (µg glucose/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	25	25	20
Mean value	939.26	2128.92	3720.82
± SD	358.31	1337.25	910.09
Range	334.00 - 1497.00	766.60 - 4988.00	1453.50 - 4843.00

Table 14B. Haemolymph Total Carbohydrate (µg glucose/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	347.66	410.08	496.00
± SD	77.01	70.98	77.41
Range	214.40 - 457.10	285.00 - 507.30	300.20 - 561.20

($P < 0.001$). When the value in 13 ± 1 mm size group was compared with that in 18 ± 1 mm, the latter showed significantly higher value ($P < 0.05$).

3.3.9 Total Protein

3.3.9.1 Indoplanorbis exustus (Table 15A)

There was significant variation in haemolymph protein values among the three size groups, and it was size dependent. The average value in 15 ± 1 mm size group was significantly higher than those in 11 ± 1 mm ($P < 0.05$), and 7 ± 1 mm ($P < 0.001$). When the value in 7 ± 1 mm size group was compared with that in 11 ± 1 mm, the latter showed significantly higher protein level ($P < 0.01$).

3.3.9.2 Lymnaea acuminata f. rufescens (Table 15B)

Statistical analysis of the data revealed size related increase in the level of haemolymph protein among the three size groups. The mean protein value in 21 ± 1 mm size group was significantly higher than those in 18 ± 1 mm ($P < 0.05$), and 13 ± 1 mm size group ($P < 0.001$). Similarly, there was significantly higher level of protein in 18 ± 1 mm size group than in 13 ± 1 mm ($P < 0.01$).

3.3.10 Total lipids

3.3.10.1 Indoplanorbis exustus (Table 16A)

The mean lipid value in 15 ± 1 mm size group was significantly lower than those in the other two size groups ($P < 0.001$). There was no significant variation in the mean values between 7 ± 1 mm and 11 ± 1 mm size groups.

Table 15A. Haemolymph Protein (mg /ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	15	15
Mean value	12.92	15.71	18.32
± SD	2.62	2.71	2.38
Range	8.65 - 16.04	12.31 - 20.44	15.29 - 23.48

Table 15B. Haemolymph Protein (mg/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	3.54	4.73	5.78
± SD	0.76	1.31	1.29
Range	2.56 - 4.93	2.52 - 6.31	3.51 - 7.61

Table 16A. Haemolymph Lipid (µg cholesterol/ml) in the three size groups of *Indoplanorbis exustus*

Size group	7±1 mm	11±1 mm	15±1 mm
N	15	15	15
Mean value	413.60	371.18	82.86
± SD	97.06	64.14	18.66
Range	245.20 - 534.80	234.20 - 475.00	53.09 - 109.39

Table 16B. Haemolymph Lipid (µg cholesterol/ml) in the three size groups of *Lymnaea acuminata* f. *rufescens*

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	12
Mean value	116.43	112.49	94.73
± SD	34.50	24.79	22.50
Range	60.30 - 168.50	68.60 - 157.30	47.40 - 128.30

3.3.10.2 Lymnaea acuminata f. rufescens (Table 16B)

The mean lipid value in 21 ± 1 mm size group was significantly lower than the values in the other two groups ($P < 0.05$). There was no significant variation in the mean values between 13 ± 1 mm and 18 ± 1 mm size groups.

3.4 DISCUSSION

The results on various inorganic and organic constituents in the haemolymph of snails of the three size groups of both I. exustus and L. acuminata f. rufescens showed an almost size dependent variation in constituents except in the case of chloride and ammonia. No significant variations in the concentration of chloride and ammonia were noticed in both the snail species. The concentrations of urea, carbohydrate, glycogen, and protein were found to increase, with increase in size/age.

Analysis of haemolymph ionic concentrations in the three size groups of I. exustus and L. acuminata f. rufescens showed interesting relationships. In both the species, the concentrations of Na^+ and K^+ ions showed progressive decrease with increase in size/age of the snails. A significant decrease in both sodium and potassium concentrations was observed in the large size group of both the species. Besides the correlation between concentrations of these two ions and snail size, an intimate relationship between Na^+ and K^+ ions also was evident. The concentrations were found to go together, high or low, in different size groups of both the species,

indicating a coordinated control mechanisms for both ions. Burton (1965) and Prasad et al., (1985) reported a comparable behaviour of Na^+ and K^+ ions in many non-marine molluscs. The significant fall in ionic composition seen in the large size group snails could be due to increase in dilution of haemolymph, or due to the higher concentrations of free amino acids and urea in the haemolymph in the large size group snails of both the species. Involvement of nitrogenous compounds, especially free amino acids, in maintenance of body water content and osmolarity of gastropods has been reported by Gilles (1974, 1979), and Shylaja and Alexander (1975). The concentration of chloride ions in the three size groups of L. acuminata f. rufescens and I. exustus was found to be more or less stable irrespective of size difference. The Cl^- concentration was not found altered by the size dependent changes noticed in Na^+ and K^+ concentrations, suggesting some independent mechanism for maintenance of Cl^- level in the haemolymph of gastropods. A separate Cl^- regulatory path way was reported in L. stagnalis (De With et al., 1980), L. subrostrata ,and in C. texasensis (Dietz and Branton, 1979), and it appears P. virens has also an independent path way for Cl^- regulation.

Majority of the freshwater pulmonates are considered to be highly calciphilic and are reported to be more successful in waters of higher calcium concentration (Young, 1975). In the haemolymph of gastropods, besides the occurrence of Ca^{2+} as free ions, calcium in higher concentrations was reported in calcium cells (Sminia et al., 1977). Analysis of haemolymph calcium in the three size

groups of I. exustus and L. acuminata f. rufescens failed to provide a definite pattern of variation. In L. acuminata f. rufescens, the large size group snails showed the highest concentration while in I. exustus, the highest concentration of calcium occurred in the small size group. Since Ca^{2+} ions are involved in a multitude of important functions besides osmoregulatory function, shell growth etc., the difference seen in the haemolymph concentration of Ca^{2+} in L. acuminata f. rufescens and I. exustus can be considered only as due to species difference. It may be noted that all species do not respond similarly since many factors other than calcium concentration of the medium affect deposition rate. Although a correlation between the levels of Ca^{2+} and K^{+} was suggested by Sorokina and Zelenskaya (1967) in P. corneus it was not found in the three size groups of snails studied.

Ammonia and urea are the major nitrogenous products released by aquatic pulmonates. L. stagnalis and I. exustus are reported to be ureotelic, and they produce more urea than ammonia during excretion (Rao and Narayanan, 1976). In molluscs, the metabolic activities are directly reflected in the haemolymph, and hence the higher tissue concentration of urea on comparison with ammonia can be expected to reflect in the haemolymph also. The results showed that the concentration of urea in the haemolymph of both I. exustus and L. acuminata f. rufescens was higher than that of ammonia. Similarly high levels of urea (De Jorge et al., 1965; Vasu and Giese,

1966) as well as low level of ammonia (Florkin and Houet, 1938; Rao and Narayanan, 1976) were reported in molluscs. Analysis of ammonia concentration in the three size groups of both the snail species showed a moderately constant level irrespective of size difference. The maintenance of the constant level of ammonia may be due to the fact that higher concentrations of ammonia has a toxic effect in tissues. Excess ammonia whenever produced can be directly released into the surrounding medium through the epithelium by diffusion (Campbell, 1973; Campbell and Bishop, 1970). When the two snail species were compared, the haemolymph NH_3-N concentration in I. exustus was found to be comparatively higher than that of L. acuminata f. rufescens, in all the size groups. Haemolymph urea concentration in both I. exustus and L. acuminata f. rufescens was found to be size dependent, and to increase steadily with increase in shell size. Since urea concentration in haemolymph is a direct indicator of the rate of protein catabolism in snails, the rate of catabolism of protein in larger size group is presumed to be more than that in the small size group. The significantly higher haemolymph protein concentrations in large size groups snails indicate that more protein is available for catabolism.

Analysis of organic constituents showed considerable variations in total carbohydrate and glycogen levels in the three size groups of both I. exustus and L. acuminata f. rufescens. In both the species a general trend for increase in the concentrations of both total

carbohydrate and glycogen was observed in relation to the size/age of the animals. In both species, the largest size group showed highly significant increase in glycogen and carbohydrate levels than the other two size groups. Thus, it may be considered that age/shell size is a factor which to a certain extent determines the level of both glycogen and total carbohydrate in I. exustus and L. acuminata f. rufescens. In molluscs, many reasons were suggested for the variation in haemolymph carbohydrate levels. Joosse and Geraerts (1983) have reported that there exist high levels of tolerance in gastropods to fluctuations in glucose levels. The decrease in carbohydrate levels was attributed to lower food consumption and also to the removal of glucose from the haemolymph by Scheerboom and Doderer (1978). Cheng and Lee (1971) reported wide variations in haemolymph glucose concentrations related to food consumption. They reported high glucose concentrations in actively feeding snails; but in L. stagnalis, Veldhuijzen (1975a) reported a rather constant glucose concentration during feeding, poor diet or starvation for 15 days. The present analysis is in agreement with the observation of Gabbot et al. (1979) who indicated that there exists no homoeostatic mechanisms for the control of both blood sugar levels in bivalves and gastropods. In M. edulis, the glycogen synthetase activity was found to be related to glucose levels as a result of feeding. The increased levels in the concentration of both carbohydrate and glycogen in relation to shell/size may be attributed to the increased feeding

rate with increase in size. High haemolymph glucose level repress locomotion and food consumption, probably by nervous pathways, and when the level is higher than the limit, glucose itself may activate endocrine centers to stimulate its removal from the haemolymph (see Joosse and Geraerts, 1983). When the haemolymph carbohydrate levels in both the snail species were compared. I. exustus showed a considerably high value than L. acuminata f. rufescens. Haemolymph carbohydrates are divided into low molecular weight carbohydrates and glycogen (Thompson, 1977). In M. edulis low molecular weight carbohydrates constitute 70-90% of the plasma carbohydrate. In the present study, the low molecular weight carbohydrate in the haemolymph constituted 75-90%, and above 95%, in L. acuminata f. rufescens and I. exustus, respectively. In both the species, a size dependent increase in the total number of haemocytes as well as in haemolymph carbohydrate levels was observed (Table 1 A and B). Similar results were reported by Thompson (1977) in M. edulis, where a correlation between the haemolymph glycogen level and haemocyte number was seen.

The results on protein concentration in the three size groups of both I. exustus and L. acuminata f. rufescens showed an increasing trend in relation to size/age. From the significant variation in protein concentration among the three size groups of both the snail species it can be assumed that age/size of the snail is a factor which to a certain extent determines the protein concentration. Similar results were observed in B. glabrata (Lee and Cheng, 1972) where

larger snails tend to include a greater concentration of total protein in their haemolymph. Various reasons are suggested for variation in haemolymph protein content. They include different diet, season, temperature, infection, environmental stress, reproduction and developmental changes (Bishop et al., 1983). Among the various factors, those related to reproduction and developmental stages are more applicable to the variations cited in the present study. In both the snail species studied a size/age dependent increase in carbohydrate and protein levels along with decreased lipid concentration was observed. Active mobilization of both carbohydrate and protein from different compartments is indicative of high metabolic activity. In I. exustus and L. acuminata f. rufescens the pronounced increase in both carbohydrate and protein as observed in the large size groups (adults) along with a decline in lipid concentration strongly suggests variations related to reproductive cycle. Similar variations in protein concentration in relation to reproductive cycles were also reported in C. virginica (Fretter and Graham, 1962) and in M. edulis (Thompson, 1977). As the snails increase in age, growth of soft body parts is well coordinated with that of the shell. For shell growth calcium is transported, and in many cases transport of calcium through cells is accompanied by the presence of calcium-binding proteins (see Joosse and Geraerts, 1983). The increase in protein concentration might also be due to the presence of calcium-binding proteins.

In addition to reproduction and development, the concentration of respiratory pigments play an important role in determining the concentration of haemolymph protein. Lee and Cheng (1972) reported that haemoglobin concentration changes with alterations in the total protein concentration. An increase in protein concentration in relation to haemoglobin concentration was reported in B. glabrata. Thus in I. exustus, the increased concentration in haemolymph protein may be related to the concentration of haemoglobin (see Table 6). In molluscs, Muley (1975) suggested a reciprocal relationship between the synthesis of lipids and utilization of protein and vice versa. Same results were observed in both I. exustus and L. acuminata f. rufescens. In both species, along with an increase in haemolymph protein, a progressive decrease in lipid content was observed in the three size groups.

The results on haemolymph lipid concentration in both the snail species showed an inverse relation with respect to the shell size/age. A drastic drop in lipid content was observed in the large size group in both the snail species. Lawrence and Giese (1969), and Webbes (1970) reported that in molluscs, the haemolymph concentration of lipids is indicative of reproductive and developmental stages of the animals. In molluscs, the storage organs for lipids are mantle, foot and digestive gland, and during reproductive phase lipids from these organs are transported to the gonads through haemolymph. The high haemolymph lipid level in the small size group snails is an indication

of the large scale movement of lipid to gonads prior to gametogenesis or the individuals of this size group are relying on carbohydrate metabolism (and hence significant drop in total carbohydrate value) and lipid is sparingly used. The significant drop in lipid level in the large size group of both the snail species may be attributed to the constant expenditure of lipids during gametogenesis. Similar results were observed in Arion empiricorum (Catalan et al., 1977) Semperula maculata (Nanaware and Varute, 1976).

C H A P T E R - IV

EFFECTS OF COPPER ON THE ACTIVITY PATTERN OF ACID AND ALKALINE PHOSPHATASES IN THE HAEMOLYMPH OF *INDOPLANORBIS EXUSTUS* AND *LYMNAEA ACUMINATA* F. *RUFESCENS*

4.1 INTRODUCTION

Many heavy metals occur naturally in aquatic environments, and in trace amounts are essential to the normal metabolism of aquatic organisms. In addition to the non-essential trace metals, such as lead, cadmium, mercury, arsenic, and others, the essential metals such as copper, zinc, iron, and cobalt have important biochemical functions in the organism. They form either an electron donor system or function as ligands in complex enzymatic compounds. The concentrations of essential trace elements are generally higher in the organism than in water. If there is too great an abundance of essential heavy metals in the organism, the metal content in the organism can be regulated by homoeostatic control mechanisms. If the heavy metal concentration in water or food is too high, the homoeostatic mechanisms cease to function, and the essential heavy metals act in an either acutely or chronically toxic manner, thus in the event of a resulting extended bioaccumulation of heavy metals the organism may be damaged (Prosi, 1983).

Copper is an important trace element and is a constituent of many animals and is essential for normal growth and development. It is found to be a necessary component of haemocyanin, several enzymes like oxidases, tyrosinases, and in other important molecules such as

cytochrome oxidases. Copper is found to be toxic to aquatic animals when its concentration exceeds tolerable limits. Regulation of the concentration of the ionic form of copper present in the cells of lamellibranchs would be particularly important because it is an essential trace element and always present in the cells (O'Dell, 1976). When the concentration rises above the normal, physiological levels, copper is found to be highly toxic (Sunda and Guillard, 1976; Engel and Sunda, 1979).

George et al. (1978) have reported that Ostrea edulis and Crassostrea virginica exposed to high concentrations of copper may accumulate copper in the granular amoebocytes, which are able to store it by compartmentation process within membrane limited vesicles. In Mytilus galloprovincialis, copper is accumulated in the digestive gland (Viarengo et al., 1981b). Copper is known to occur intracellularly as granules and within membrane bound vesicles resembling lysosomes (Coombs and George, 1978), and bound to soluble low molecular weight, metallothionein-like proteins (Howard and Nickless, 1977a), or to smaller organic molecules (Coombs, 1974; Howard and Nickless, 1977b, 1978).

Copper is found to be lethal to a number of gastropods and pelecypods. Among heavy metals copper was found to be more toxic to Biomphalaria glabrata (Bellavere and Gobri, 1981) and Lymnaea acuminata (Khangarot, 1982) than chromium and cadmium. It is known that even minute concentrations of copper are lethal to snails particularly in

waters of low alkalinity, turbidity and organic content (Cheng and Sullivan, 1974). When the fresh water snail Lymnaea was exposed to 0.25 ppm of copper, 100% mortality occured within 24 hrs (Batte et al., 1951). In the case of B. glabrata, it is reported that as little as 0.07 ppm of copper as copper sulphate in deionized water effects 100% mortality within 72 hrs (Malek and Cheng, 1974).

The LC_{50} value of copper in a number of molluscs were reported. Thus in L. luteola, copper sulphate at 2 mg/l is found to be most toxic based on concentration duration product (Babu and Rao, 1985). LC_{50} stage is reached by 6 hr. Khangarot (1982) reported that the LC_{50} value for copper in L. acuminata was 0.034 mg/litre. In Meretrex casta (Kumaraguru et al., 1980) and Villorita cyprinoides var. cochinensis (Lakshmanan, 1982) the LC_{50} values reported were 0.057 ppm and 1.21 ppm, respectively. Viarengo et al. (1981b) has reported that copper accumulated in the digestive gland cells of metal exposed mussels, M. galloprovincialis is rapidly detoxified and eliminated from the cells. This is achieved either by increasing the synthesis of copper thioneins of high heavy metal affinity (Viarengo et al., 1980, 1981a, and 1984) or by the accumulation of the metal in lysosome and residual bodies, which are subsequently eliminated by exocytosis (Viarengo et al., 1981b).

Studies by Sullivan and Cheng (1976) have revealed that B. glabrata is relatively insensitive to internal accumulation of copper. This evidence tends to support the hypothesis advanced earlier

by Sullivan and Cheng (1975) that copper acts externally on sensitive epithelia, rather than internally. The effect of copper on the behaviour pattern of M. casta was also studied by Kumaragura et al. (1980). They reported that in animals subjected to a concentration of 0.5 ppm for a period of one week, an abnormal liquid pouch was noted on the mantle. In general, all the animals examined in all the experimental concentrations were sluggish with a lot of mucus coagulation on the gills, mantle, and gills had turned to yellowish green in colour. The metal accumulation in the exposed animals was nearly a hundred times of that found in controls.

The effects of copper on various physiological aspects of molluscs have also been reported. Yager and Harry (1964) reported that the permeability of the surface epithelial membrane is disrupted and as a result uptake of copper was lowered in snails exposed to toxic levels of copper. In B. glabrata, Von Brand et al. (1949) found that exposure to toxic concentrations of cupric ions causes lowering of rate of oxygen consumption. This decline is due to rapid retraction of the snail into its shell upon exposure to copper and consequently to a decrease in surface area available for uptake of oxygen rather than to a direct effect of copper on oxygen consumption of the snail. A decreased rate of oxygen consumption in L. luteola during copper treatment was also reported by Babu and Rao (1985). Cheng and Sullivan (1973) studied the effects of copper on the heart rate of B. glabrata and reported that toxic concentrations of copper have a measurable inhibitory effect on the heart rate of B. glabrata.

The cidal effect of copper sulphate on L. luteola was studied in relation to lipid metabolism (Reddy and Rao, 1983). They reported that as a result of detoxification, alterations were observed in the levels of glycerol, phospholipids, glycerides, sterols, sterol esters, and free fatty acids in foot, mantle, and digestive gland. Formation of copper complexes with biological material has been reported by several investigators (Eichhorm, 1973) and copper complex formation is said to be a storage or detoxifying mechanism. Copper forms copper complex with long chain fatty acids (Iwayama, 1959). The increased fatty acid level through increased lipase activity in copper treated L. luteola could be for complexing of copper for storage or detoxification (Reddy and Rao, 1987).

It is also known that many toxic substances or their metabolites can cause cell injury by reacting primarily with biological membranes, and among the changes are included those in the content or activity of enzymes or other membrane components (Moore, 1985).

Heavy metals are found to inhibit, stimulate, or influence the rate of action of enzymes by activation, inactivation, uncoupling reactions or mechanisms yet to be defined (see Suresh and Mohandas, 1990a). Iordăchesw et al. (1978) have reported that copper strongly activates acid proteases purified from the hepatopancreas of M. galloprovincialis. An increase in the number of lysosomes was reported by Lindquist (1968), and changes in the activities of lysosomal enzymes have been demonstrated histochemically and biochemically. Lethal concentrations of copper have an inhibitory

effect on respiratory enzymes, and on enzymes related to excretion of metals (Hubschmann, 1967). Babu and Rao (1985) have reported that copper inhibits the activity of cytochrome oxidase and activates peroxidase in L. luteola. Copper sulphate was found to inhibit the oxidation of exogenous phenylene diamine by tissue homogenates of B. alaxandrina (Ishak et al., 1970).

Molluscs in general, have low enzyme levels and information on their specific roles is sparse. Enzymes by themselves are not present in the haemolymph, unless they belong either to the haemocytes or leak from intracellular confines of the damaged tissues. Hence serum enzyme levels are of diagnostic value (Jyothirmayi and Rao, 1987). They have added that changes in haemolymph enzyme activity profiles as a result of exposure to pollutants, parasitism or challenge with bacteria, can be of significant diagnostic value. Lysosomes are the important store house of about three dozen hydrolytic enzymes which are found to sequester many anthropogenic substances, and play an important role in their bioaccumulation (Dingle and Fell, 1969). Lysosomes are also involved in physiological activities, such as intracellular digestion, storage, excretion, resorption, cell proliferation, immune mechanism, and in the control of the cellular economy (Rosenbaum and Ditzion, 1963; Deduve and Wattiaux, 1966; Sumner, 1969; Owen, 1972; Moore et al., 1978a,b; Cheng, 1983). Many heavy metals like zinc, iron, cadmium, uranium etc. have been demonstrated to be lysosomal inclusions in many cells of bivalves (Lowe and Moore, 1979; George, 1983). The molluscan granulaocytes

are the major sites of lysosomal enzyme synthesis (Cheng, 1983), and under normal conditions these acid hydrolases are restricted to within lipoprotein lysosomal membrane in latent phase. The enzymes are released from lysosomes into the surrounding cytoplasm, when the granulocytes were challenged with either abiotic or biotic factors (Moore et al., 1979; Cheng, 1980). Lysosomal enzymes are released into the serum also by degranulation (Cheng et al., 1975, 1977; Cheng and Yoshino, 1976a, b; Foley and Cheng, 1977; Mohandas et al., 1985).

Lysosomes and cell membranes are the first targets of pollutants because the lysosomes are concerned with the disintegration of foreign materials and the cell membrane is the first barrier to a xenobiotic encounter. In the haemolymph, the enzyme activity is determined by its synthesis, its release into the haemolymph compartment, and its final loss. Many xenobiotics induce alterations in the bounding membrane of the lysosomes leading to destabilisation (Moore and Lowe, 1985). The destablisation causes release of hydrolytic enzymes from the lysosomal compartment into cytosol (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 association between lysosomal hydrolases and the lysosomal membrane (Koenig, 1969; Verity, 1973) results in most of the enzyme activity being normally latent, if for any reason the lysosomal membranes are rendered unstable, free

hydrolases may be released into the cytoplasm with resultant autolytic cell damage (Miller and Wolfe, 1968; Koenig, 1969). A cell rich in one enzyme is often poor in another, enzyme masking may account for some of this variation but enzymatic heterogeneity is pronounced. It is clear that at least two lysosomal enzymes are present simultaneously in a single cell (Huffman and Tripp, 1982).

Acid and alkaline phosphatases are groups of enzymes that hydrolyse phosphomonoesters in a relatively non-specific manner with optimal activity in the acidic and alkaline regions, respectively. Alkaline phosphatase (E C 3.1.3.1) differs form acid phosphatase (E C 3.1.3.2) in subcellular distribution. Alkaline phosphatase activity in rat mesenteric arteries was highly concentrated in the plasma membrane (Kwan, 1983) whereas acid phosphatase activity with the lysosomes (Wolinsky et al., 1974; Wantanabe et al., 1981). Gupta et al. (1975) and Verma et al. (1980) reported that acid phosphatase is a hydrolytic enzyme which takes part in the dissolution of dead cells and as such is a good indicator of stress in biological systems.

ACP and ALP are the enzymes concerned with biosynthesis of fibrous protein (Johnson and McMinn, 1958), mucopolysaccharides (Kroon, 1952), or they may serve as a regulator of intra cellular phosphate concentration (Gutman, 1959). They play an active part in the dissolution of the body's cells, and are also believed to be involved in permeability processes and associated with nucleic acid synthesis (Cox et al., 1967). Stimulation or inhibition

of these enzymes will thus result in disturbances in metabolism. ACP is associated with lysosomes and as such has been designated as one of a marker enzymes for the population of subcellular vacuoles (Deduve et al., 1966).

The presence of acid and alkaline phosphatases was reported in a large number of molluscs. Lysosomes are found in the granulocytes of different species of molluscs (Cheng, 1975; Yoshino and Cheng, 1976), and these organelles are associated with intracellular degradation (Cheng and Cali, 1974; Cheng et al., 1974). Cheng and Rodrick (1975) have reported the presence of β– glucuronidase, acid phosphatase, alkaline phosphatase, lipase, aminopeptidase, and lysozyme in the cellular and serum components of C. virginica and Mercenaria mercenaria, and Rodrick and Cheng (1974) in B. glabrata haemolymph. Lysosomal enzymes were reported in the haemocytes of gastropods including B. glabrata (Jeong and Heyneman, 1976; Cheng et al., 1977) and L. stagnalis (Sminia, 1972). Acid phosphatase has been found to be naturally present in the haemocytes and haemolymph of Patella vulgata (Cooper willis, 1979), and in the bivalves Sunetta scripta and Villorita cyprinoides var. cochinensis (Suresh and Mohandas, 1990a).

Phosphatases are metal requiring enzymes, traces of added cations might display or affect the metal containing moiety and modify enzyme activity. The phosphatases recognized by Robinson (1923) proved to be amongst the first enzymes of diagnostic importance.

Martiland and Robinson (1926) demonstrated the presence of phosphatase in the plasma, and increased levels were found to occur in a variety of bone and liver diseases. The influence of metal ions on the activity pattern of acid and alkaline phosphatases was reported in a large number of molluscs. Thus, Koeing (1963), Norseth (1968), and Roesijadi (1980) reported increased lysosomal activities in organisms exposed to sublethal concentrations of metal ions. The lysosomes play a fundamental role in the homoeostasis of copper as well as in the detoxification of the metals in the digestive gland cells of copper exposed mussels (Viarengo et al., 1985). Heavy metals inhibit the activities of several enzymes (Dixon and Webb, 1967). Yoshino et al. (1966) reported that Hg^{2+} inhibits enzymes of Kreb's cycle. Webb (1966) and Jackim et al. (1970) reported that heavy metals usually inhibit enzymatic and metabolic process in vitro. Jackim et al. (1970) found out in their in vitro alkaline phosphatase study that berrylium and cadmium were the strong inhibitors of this enzyme, with copper, lead, mercury and silver producing progressively less inhibition. Lead, copper, silver, mercury, and cadmium added directly to the enzyme preparations inhibited acid phosphatase activity in decreasing order. Except for silver, in vivo exposure to all heavy metal salts inhibited liver acid phosphatase. In Mytilus edulis, concentration dependent labilisation of lysosomes was observed on exposure to copper (Harrison and Berger, 1982). There was no significant reduction in lysosomal integrity when the concentration of copper was low, but there was a significant reduction in lysosomal

latency when the concentration was the highest. Chandy and Patel (1985) have reported that at lower concentrations the metal ions were engulfed into lysosomes and subsequently transformed into biologically inactive forms. 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 lysosomes of haemocytes and eventually removed. This transformation and inactivation of metal ions, are probably carried out by the acid hydrolases released from lysosomes. Suresh and Mohandas (1990a) reported increased ACP activity in copper-stressed bivalves in the early time periods, and indicated that it may be due to destabilization of the lysosomal membrane caused by metal ions and the consequent release of the enzyme into the haemolymph or due to hypersynthesis of acid phosphatase which is subsequently released into the haemolymph.

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 destabilization caused by metal ions in higher concentration will result in lesser enzyme turn over.

Regarding the effect of pesticides on ACP activity, Nagarathnamma (1982) reported that the intracellular digestion of damaged material by lysosomes could be prompted by methyl parathion damage to subcellular structures. The higher the concentration of the pesticide, the greater will be the tissue damage and it is

reflected as increase in acid phosphatase activity. The following reasons are reported for the elevation of acid phosphatase in the hepatopancreas due to pesticide exposure: (i) the toxicant might induce proliferation of smooth endoplasmic reticulum in hepatopancreas that leads to more production and liberation of microsomal enzymes resulting in an increased level of acid phosphatases (as observed by Hart and Fouts, 1965), (ii) degradation and necrosis induced in hepatopancreas by toxicants may cause release of acid phosphatase (confirmed by the observations of Onikieno, 1963), (iii) peroxidation of the lysosomal membrane leading to membrane breakdown or increase in permeability of lysosomal membrane or both by the toxicants results in liberation of acid phosphatase thereby causing increased level (Novikoff, 1961), and (iv) ACP may increase due to increased uptake of certain metabolites and ions since these enzymes are reported to be involved in this process (Simkiss, 1964).

There are also reports which indicate that pesticides can inhibit ACP activity. Dalela et al. (1980) studied the effect of phenol and pentachlorophenol on the hepatic acid and alkaline phosphatase and reported significant inhibition. The inhibitory effects of phenol and dinitrophenol on acid and alkaline phosphatases were also observed by Verma et al. (1980). Yap et al. (1975), and Desaih (1978) after PCP exposure pointed out that uncoupling of oxidative phosphorylation is the main cause for inhibition of phosphatases. Uncoupling of oxidative phosphorylation was also pointed

out by Dalela et al. (1980) and Verma et al. (1980) for the inhibition of alkaline and acid phosphatases.

It is reported that the levels of several lysosomal enzymes are initially elevated in the haemocytes of B. glabrata when challenged in vitro or in vivo with bacteria. These hydrolases are subsequently released into serum (Cheng and Yoshino, 1976a; Cheng et al., 1977, 1978a). Cheng et al. (1978b) reported the same effect when B. glabrata was challenged in vivo with trematode miracidia. Cheng and Butler (1979) reported that there was an elevation in the levels of acid phosphatase activity in both the haemocytes and serum fractions of B. glabrata that had been challenged with heat killed Bacillus megaterium. The ACP activity in haemocytes of L. stagnalis increased after in vitro challenge with bacteria (Sminia, 1972). According to Michelson and Dubois (1973) the concentration of the enzyme increased in snails infected with Schistosoma mansoni. Targett (1962) and Gilbertson et al. (1967) reported an increase in the activity of alkaline phosphatase in S. mansoni infected snails with respect to diminished concentration of haemolymph phenyl alanine. The challenge with bacteria results in the hyper-synthesis of lysosomal enzymes within haemocytes and subsequently these enzymes are released into the serum through degranulation (Cheng, 1975; Cheng et al., 1975; Foley and Cheng, 1977; Mohandas et al., 1985).

In this chapter the activity pattern of ACP and ALP in three size groups of I. exustus and L. acuminata f. rufescens; and in one

size group each of copper-stressed snails of the two snail species is reported. Copper was chosen as the pollutant because copper is the active ingredient in all the molluscicides now in use.

4.2 MATERIALS AND METHODS

Methods of collection of snails, rearing, acclimatisation, selection of size groups, collection of haemolymph, and the computation of the data are described in section 2.2.

4.2.1 Toxicity Studies

4.2.1.1 The toxicant

The toxicant selected was copper (Cupric sulphate supplied by Glaxo laboratories, Bombay), which is commonly used as a molluscicide. 100 ppm of $CuSO_4$ solution was prepared in double distilled water and added to the test media to get the desired concentrations. The various concentrations of the toxicant are expressed in ppm.

4.2.1.2 Letal toxicity of copper

Lethal toxicity studies provide information about the relative lethality of a toxicant. This test is designed to determine the highest concentration of a pollutant that is sufficient to affect some percentage, usually 50% of a limited number of organisms.

4.2.1.2.1 Indoplanorbis exustus

In order to find out the 96 hr LC_{50} value for copper in I. exustus, laboratory conditioned infection-free snails of the size

group 11 ± 1 mm in shell height were selected. The snails were exposed to logarithmic series of concentrations of copper and the exposure was carried out in glass troughs. Ten animals were used for each test concentration of the toxicant, and during experiments the glass vessels were covered with perpex sheet. The experiments were carried out at room temperature (30 ± 1.5°C), and the snails were fed with Lemna sp. during the tests. Triplicates and controls were run for all the experiments. The test media were replenished totally every 24 hrs. The snails were observed every 12 hour and the cumulative mortality was recorded. The 96 hr LC_{50} value was calculated using Probit Analysis (Finney, 1971).

4.2.1.2.2 Lymnaea acuminata f. rufescens

Infection free snails of the size group 18 ± 1 mm in shell height were selected to study the 96 hr LC_{50} values. The methodology used was the same as described in section 4.2.1.2.1.

4.2.1.3 Sublethal toxicity studies

The objective of the toxicity studies was to find out the concentrations of the toxicants capable of inducing abnormal responses on the activities of the organisms. The activity pattern of the enzymes in haemolymph under different sublethal concentrations of copper was studied. The concentrations of copper employed for the sublethal toxicity studies were derived from the 96 hr LC_{50} value. The duration of these studies extended up to 48 hrs, and the activities of the enzymes were measured at 2, 6, 12, 24 and 48 hrs post-exposure

with copper. Snails were not fed during these experiments. Controls were run simultaneously.

4.2.2 Enzyme Analysis

The level of activity of enzymes in the haemolymph of three size groups of snails as well as under different sublethal concentration of copper was studied. To study the enzyme activity in three different size groups (7 ± 1 mm, 11 ± 1, and 15 ± 1 mm) 12 samples from each size group were selected in the case of I. exustus, while in the case of L. acuminata f. rufescens, 12 samples each from 13 ± 1 mm and 18 ± 1 mm size groups, and 6 from 21 ± 1 mm size group were selected (one sample is the haemolymph pooled from five snails). To study the enzyme activity pattern in both species of snails exposed to three sub-lethal concentrations of copper, the intermediate size group was chosen (I. exustus 11 ± 1 mm; and L. acuminata f. rufescens 18 ± 1 mm). For each concentration eight samples were used as experimentals (one sample is the haemolymph pooled from five snails), and five as controls (one sample is the haemolymph pooled from five snails).

4.2.2.1 Assay of Acid phosphatase activity

4.2.2.1.1 Indoplanorbis exustus

Acid phosphatase activity in the haemolymph of I. exustus was determined by employing the method described in Sigma Technical Bulletin No.104 with slight modifications (Anon, 1963). To 1.0 ml of (0.1 M) frozen citrate buffer of pH 5.2, 0.1 ml of the haemolymph

was added. To this buffer-enzyme mixture, 0.1 ml of substrate, containing 2.0 mg of p-Nitrophenyl phosphate sodium salt (Merck) in 0.1 ml of distilled water was added and incubated for one hour at 37 ± 0.05°C. After one hour incubation, the reaction was stopped by adding 2.0 ml of 0.25 N NaOH. p-Nitrophenyl phosphate was hydrolysed to p-Nitrophenol by the enzyme during the incubation period. The yellow colour developed in the alkaline medium was read spectrophotometrically at 410 nm. The enzyme activity was expressed as μ moles p-Nitrophenol liberated/minute/ml.

4.2.2.1.2 Lymnaea acuminata f. rufescens

The procedure described in section 4.2.2.1.1 was followed to estimate the acid phosphatase activity in L. acuminata f. rufescens, except that the pH of the citrate buffer used here was 4.2.

4.2.2.2 Assay of Alkaline phosphatase activity

4.2.2.2.1 Indoplanorbis exustus

Alkaline phosphatase activity in the haemolymph of I. exustus was determined following the method described in Sigma Technical Bulletin No.104 with slight modifications (Anon, 1963). To study the enzyme activity, 0.05 M glycine-NaOH buffer of pH 8.8 was used. The incubation temperature was 37 ± 0.05°C. To 1.0 ml of the frozen buffer, 0.05 ml of haemolymph was added. To this buffer enzyme mixture, 0.1 ml of substrate (2.0 mg of p-Nitrophenyl phosphate sodium salt (Merck) in 0.1 ml distilled water) was added and incubated in a water bath for one hour at 37 ± 0.05°C. After incubation for

one hour, the reaction was stopped by adding 10 ml of 0.05 N NaOH. The yellow colour developed was read at 410 nm. The enzyme activity was expressed as μ moles p-Nitrophenol liberated/minute/ml.

4.2.2.2.2 Lymnaea acuminata f. rufescens

The procedure described in section 4.2.2.2.1 was adopted to estimate the activity of alkaline phosphatase. The optimum pH of the enzyme activity was found to be 9.2.

4.3 RESULTS

4.3.1 Toxicity Studies

4.3.1.1 Lethal toxicity of copper

Lethal toxicity of copper for I. exustus and L. acuminata f. rufescens was studied and the results for the two species were as follows: The 96 hr LC_{50} for copper was computed and calculated, and for I. exustus it was found to be 0.036 ppm, and for L. acuminata f. rufescens it was found to be 0.030 ppm.

4.3.1.2 Sublethal toxicity of copper

Based on the LC_{50} value, three sublethal concentrations of copper were selected for both the snails species to study the activity pattern of enzymes i.e., 0.020, 0.015 and 0.010 ppm of copper.

4.3.2 Enzyme Analysis

4.3.2.1 Acid phosphatase activity in three size groups

4.3.2.1.1 Indoplanorbis exustus (Table 17A)

No statistically significant variation was observed in the

activity pattern of acid phosphatase among the three size groups of snails.

4.3.2.1.2 Lymnaea acuminata f. rufescens (Table 17B)

A significant decrease in acid phosphatase activity was observed in 18 ± 1 mm size group when compared with the activity in 21 ± 1 mm ($P < 0.05$). No significant variation in activity was observed in 13 ± 1 mm group when compared with the activities in the other two size groups.

4.3.2.2 Alkaline phosphatase activity in three size groups

4.3.2.2.1 Indoplanorbis exustus (Table 18A)

The mean value of alkaline phosphatase activity in 15 ± 1 mm size group was significantly lower than the values in 11 ± 1 mm and 7 ± 1 mm size groups ($P < 0.001$). There was no significant variation in enzyme activity values between 7 ± 1 mm and 11 ± 1 mm size groups snails.

4.3.2.2.2 Lymnaea acuminata f. rufescens (Table 18B)

The results showed significant variations in the activity pattern of alkaline phosphatase in the three size groups studied. The mean value of enzyme activity in 21 ± 1 mm size group was significantly higher than the values in 18 ± 1 mm ($P < 0.001$), and 13 ± 1 mm size groups ($P < 0.01$). But there was no significant variation in activity between 13 ± 1 mm and 18 ± 1 mm size group snails.

Table 17A. Haemolymph Acid Phosphatase activity(U/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	12	12	12
Mean value	0.0196	0.0256	0.0223
± SD	0.0085	0.0070	0.0073
Range	0.0080 - 0.0336	0.0153 - 0.0343	0.0127 - 0.0378

Table 17B. Haemolymph Acid Phosphatase activity (U/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	6
Mean value	0.0313	0.0260	0.0331
± SD	0.0073	0.0063	0.0017
Range	0.0191 - 0.0465	0.0165 - 0.0374	0.0306 - 0.0349

Table 18A. Haemolymph Alkaline Phosphatase activity (U/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	12	12	12
Mean value	0.6892	0.5476	0.1481
± SD	0.1799	0.2024	0.0563
Range	0.3860 - 1.0180	0.2530 - 0.8870	0.0450 - 0.2580

Table 18B. Haemolymph Alkaline Phosphatase activity (U/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	6
Mean value	0.6814	0.5980	0.9420
± SD	0.1460	0.1384	0.1972
Range	0.4050 - 0.9250	0.3730 - 0.8390	0.6020 - 1.1390

4.3.2.3 Sublethal effects of copper on the activity pattern of Acid phosphatase

4.3.2.3.1 Indoplanorbis exustus (Table 19A ; Fig.7)

The snails dosed with 0.010 ppm of copper did not show any statistically significant variation in acid phosphatase activity at 2, 6, 12, 24 and 48 hr post-exposure with respect to the control values. The 0.015 ppm copper dosed snails showed significantly higher value at 12 hr ($P < 0.05$) than the control value. No significant change in activity with respect to the control was observed at 2, 6, 24 and 48 hr post-exposure. In snails dosed with 0.020 ppm of copper, significantly higher acid phosphatase activity was observed at 12 ($P < 0.05$), 24 ($P < 0.001$), and 48 hr ($P < 0.01$) post-exposure with respect to the controls.

At 2 hr post-exposure, the enzyme activity was found to be maximum in 0.015 ppm copper dosed snails and it was significantly higher than those dosed with 0.010 ppm of copper ($P < 0.01$).

At 6 hr post-exposure, the activity was found to be significantly lower in those exposed to 0.020 ppm than in those exposed to 0.010 ppm ($P < 0.05$) and 0.015 ppm ($P < 0.01$) of copper.

A dose dependent increase in the level of acid phosphatase activity was observed at 12 hr post-exposure in snails dosed with the three sublethal concentrations of copper. When the activity in snails dosed with the three sublethal concentrations of copper was compared, the acid phosphatase activity was found to be significantly

Table 19: Haemolymph acid phosphatase activity (U/ml) in [illegible] with three concentrations of copper (size group - 11±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	0.0268	0.0266	0.0258	0.0251	0.0295
ntrol	± SD	0.0041	0.0048	0.0051	0.0061	0.0041
	Range	0.0229-0.0320	0.0212-0.0343	0.0209-0.0323	0.0163-0.0323	0.0246-0.0343
	N	8	8	8	8	8
010 ppm of $^{2+}$	Mean value	0.0237	0.0328	0.0296	0.0268	0.0300
	± SD	0.0057	0.0112	0.0045	0.0042	0.0125
sed	Range	0.0160-0.0302	0.0187-0.0457	0.0246-0.0376	0.0219-0.03570	0.0148-0.0448
	N	8	8	8	8	8
015 ppm of $^{2+}$	Mean value	0.0418	0.0363	0.0347*	0.0319	0.0274
	± SD	0.0148	0.0118	0.0065	0.0059	0.0085
sed	Range	0.0181-0.0666	0.0179-0.0503	0.0268-0.0445	0.0247-0.0448	0.0143-0.0434
	N	8	8	8	8	8
020 ppm of $^{2+}$	Mean value	0.0362	0.0231	0.0502*	0.0706***	0.0643**
	± SD	0.0159	0.0065	0.0198	0.0179	0.0209
sed	Range	0.0167-0.0591	0.0179-0.0344	0.0204-0.0785	0.0499-0.1065	0.0438-0.0926

gnificance level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

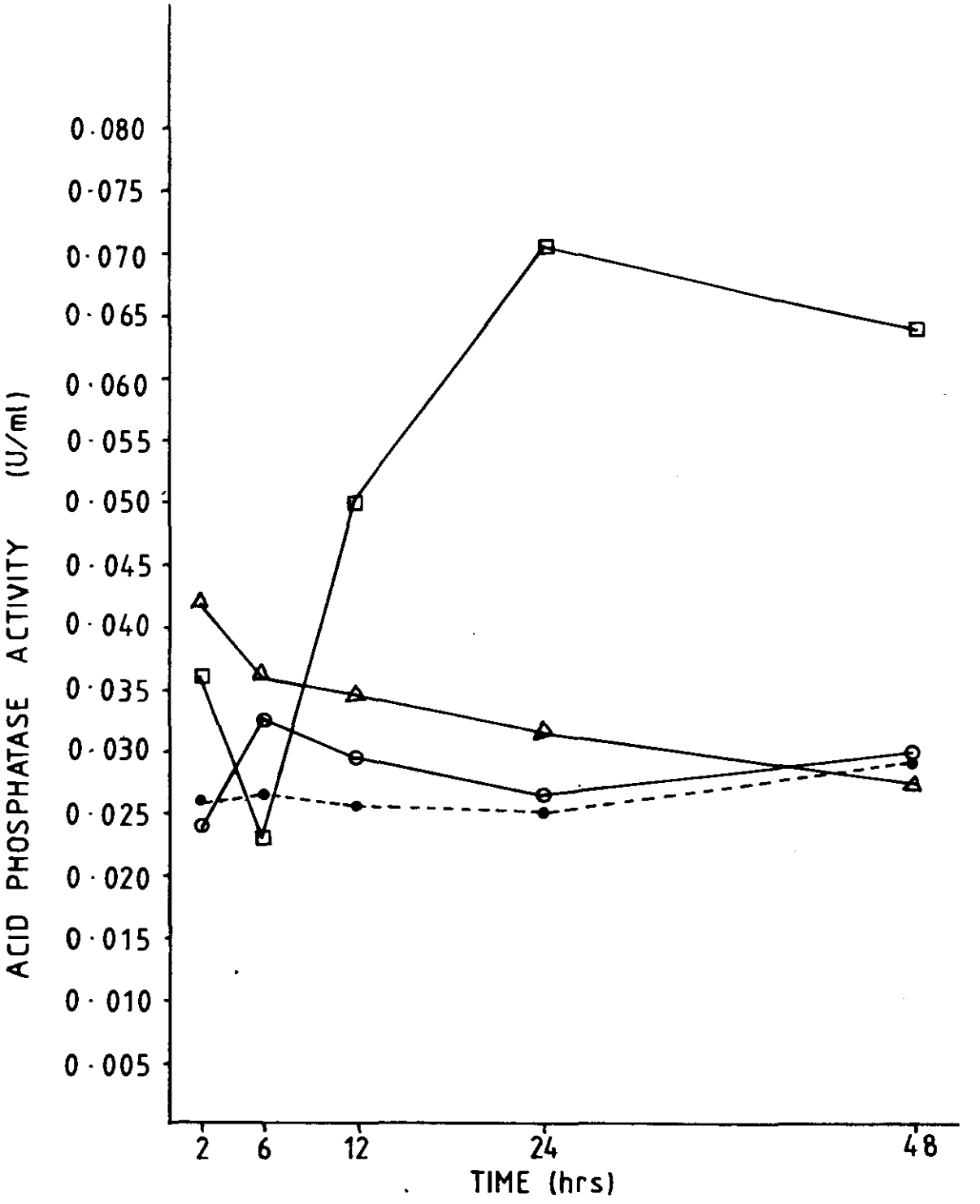

Figure 7. Haemolymph Acid Phosphatase Activity (u/ml) in I. exustus dosed with three sublethal concentrations of Copper. Control (-•-), 0.01 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).

higher in those exposed to 0.020 ppm than in those exposed to 0.015 ppm ($\underline{P} < 0.05$) and 0.010 ppm ($\underline{P} < 0.01$).

At 24 hr post-exposure also a dose dependent increase in the acid phosphatase activity was observed. When the activity in snails exposed to the three sublethal concentrations was compared, the activity was found to be maximum in 0.020 ppm copper dosed snails, and it was significantly higher than in those exposed to 0.010 ppm ($\underline{P} < 0.001$) and 0.015 ppm ($\underline{P} < 0.001$).

At 48 hr post-exposure, almost dose dependent increase in enzyme activity was observed. The maximum activity was observed in 0.020 ppm copper dosed snails, and it was significantly higher than in those exposed to 0.010 ppm ($\underline{P} < 0.001$) and 0.015 ppm ($\underline{P} < 0.001$).

4.3.2.3.2 Lymnaea acuminata f. rufescens (Table 19B ; Fig.8)

Snails dosed with 0.010 ppm of copper showed statistically significant variation at 2, 6, 24 and 48 hr exposure. At 2 ($\underline{P} < 0.05$) and 6 hr ($\underline{P} < 0.05$) the enzyme activity was significantly higher than the controls. At 12 hr post-exposure, there was no significant change but at 24 hr of post-exposure, the activity significantly decreased ($\underline{P} < 0.05$) when compared with the control. Finally at 48 hr, again a significant increase ($\underline{P} < 0.001$) in the activity was observed. Snails dosed with 0.015 ppm of copper also showed significant variations at 2, 6, 24 and 48 hr post-exposure. The mean values of acid phosphatase activity at 2 ($\underline{P} < 0.01$) and 6 hr ($\underline{P} < 0.05$) were significantly higher than their respective controls. At 12 hr

Table 19B. Haemolymph Acid Phosphatase Activity (U/ml) in *Lymnaea acuminata* f. *fluescens* dosed with three concentrations of copper (size group - 18±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	0.0258	0.0262	0.0280	0.0295	0.0267
	± SD	0.0050	0.0073	0.0069	0.0016	0.0032
	Range	0.0184-0.0309	0.0185-0.0374	0.0161-0.0332	0.0275-0.0316	0.0213-0.0296
0.010 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0343*	0.0389*	0.0318	0.0241*	0.0451***
	± SD	0.0054	0.0019	0.0041	0.0037	0.0037
	Range	0.0271-0.0417	0.0354-0.0415	0.0259-0.0382	0.0177-0.0281	0.0387-0.0496
0.015 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0369**	0.0462*	0.0315	0.0227**	0.0168**
	± SD	0.0049	0.0152	0.0086	0.0031	0.0056
	Range	0.0281-0.0428	0.0182-0.0685	0.0202-0.0465	0.0196-0.0286	0.0082-0.0242
0.020 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0407*	0.0379*	0.0376*	0.0253*	0.0272
	± SD	0.0114	0.0074	0.0037	0.0030	0.0033
	Range	0.0264-0.0586	0.0272-0.0502	0.0307-0.0431	0.0213-0.0295	0.0211-0.0313

Significance level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

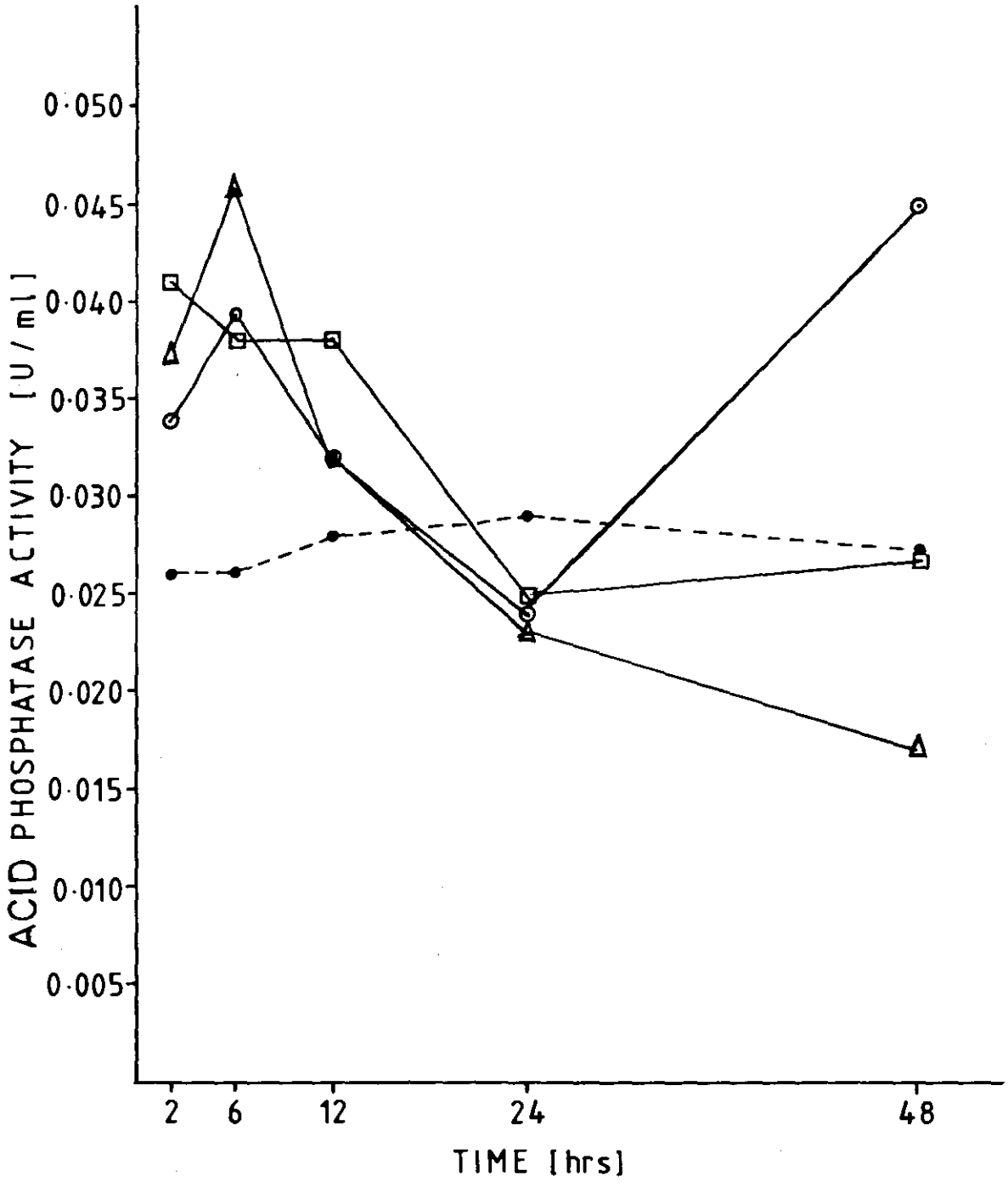

Figure 8. Haemolymph Acid Phosphatase Activity (u/ml) in L. acuminata f. rufescens dosed with three sublethal concentrations of Copper. Control (-•-), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).

post-exposure no significant variation was observed. But at 24 and 48 hr post-exposure, the enzyme activity was significantly lower ($\underline{P} < 0.01$) than the controls. The enzyme activity of 0.020 ppm exposed snails showed significant increase at 2 ($\underline{P} < 0.05$) 6 ($\underline{P} < 0.05$), and 12 hr ($\underline{P} < 0.05$) but decrease at 24 hr ($\underline{P} < 0.05$) when compared with their controls. At 48 hr post-exposure, there was no significant variation in the activity pattern.

At 2 and 6 hr post-exposure when the activity levels in snails exposed to the three sublethal concentrations of copper were compared, no significant variation was observed.

At 12 hr post-exposure the activity of acid phosphatase was found to be maximum at 0.020 ppm when compared with the activities in 0.015 ppm ($\underline{P} < 0.05$) and 0.010 ppm ($\underline{P} < 0.05$) dosed ones.

At 24 hr there was significant decrease in activity in 0.015 ppm ($\underline{P} < 0.05$) dosed snails when compared to those dosed with 0.020 ppm copper.

At 48 hr post-exposure, the acid phosphatase activity was elevated significantly in 0.010 ppm copper dosed snails and it was found to be the maximum among the three concentrations. The activity at 0.010 ppm concentration was significantly higher than the activity at 0.015 ppm ($\underline{P} < 0.001$) and 0.020 ppm ($\underline{P} < 0.001$). In 0.015 ppm dosed snails, the level of acid phosphatase activity was significantly lower than the activities in 0.010 ppm and ($\underline{P} < 0.001$) and 0.020 ppm ($\underline{P} < 0.001$) dosed snails.

4.3.2.4 Sublethal effects of copper on the activity pattern of Alkaline phosphatase

4.3.2.4.1 Indoplanorbis exustus (Table 20A ; Fig.9)

Snails dosed with 0.010 ppm of copper showed significant variations in enzyme activity at 6 and 12 hr post-exposure with respect to the control values. The alkaline phosphatase activity at 6 and 12 hr post-exposure was significantly higher than their controls ($P < 0.05$). At 24 and 48 hr post-exposure, although the activity was lower, it was not significant. In snails dosed with 0.015 ppm of copper significant increase in the enzyme activity was observed at 2 ($P < 0.05$), 24 ($P < 0.01$) and 48 hr ($P < 0.01$) post-exposure with respect to the controls. Although there was an elevation in alkaline phosphatase activity at 6 and 12 hr post-exposure, the elevation was not statistically significant. 0.020 ppm copper dosed snails showed significantly high enzyme activity at 2 ($P < 0.01$), 6 ($P < 0.001$), 12 ($P < 0.001$)24 and 48 hr ($P < 0.001$) post-exposure when compared with respective controls.

At 2, 6, 12, 24 and 48 hr post-exposure, the alkaline phosphatase activity was found to be elevated towards the highest concentration when compared among the experimentals. The maximum enzyme activity at 2 and 6 hr among the experimentals was observed in those dosed with 0.020 ppm of copper, and it was significantly higher than those dosed with 0.010 ppm of copper ($P < 0.05$).

Table 20A. Haemolymph Alkaline Phosphatase Activity (U/ml) in *Indoplanorbis exustus* dosed with three concentrations of copper (size group 11±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	0.5504	0.5780	0.5082	0.5346	0.5562
	± SD	0.1730	0.2343	0.1711	0.1954	0.1972
	Range	0.3530-0.7350	0.2530-0.8850	0.3580-0.7930	0.3380-0.7930	0.2880-0.8160
0.010 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.8541	0.8500*	0.9250*	0.4532	0.4710
	± SD	0.2862	0.1892	0.3282	0.1751	0.0962
	Range	0.6260-1.5090	0.5960-1.1670	0.5040-1.4300	0.3070-0.8460	0.2870-0.5850
0.015 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.9897*	0.9247	0.9492	1.0320**	1.0290**
	± SD	0.2828	0.3307	0.4639	0.2812	0.2647
	Range	0.5610-1.3880	0.4920-1.3940	0.3850-1.7970	0.7530-1.3970	0.4990-1.2870
0.020 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	1.1741**	1.1240**	1.4080***	1.4318***	1.1880***
	± SD	0.2853	0.3042	0.1293	0.3164	0.2077
	Range	0.9270-1.5570	0.6130-1.6090	1.2540-1.5670	1.1000-1.7590	0.9900-1.5610

Significance level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

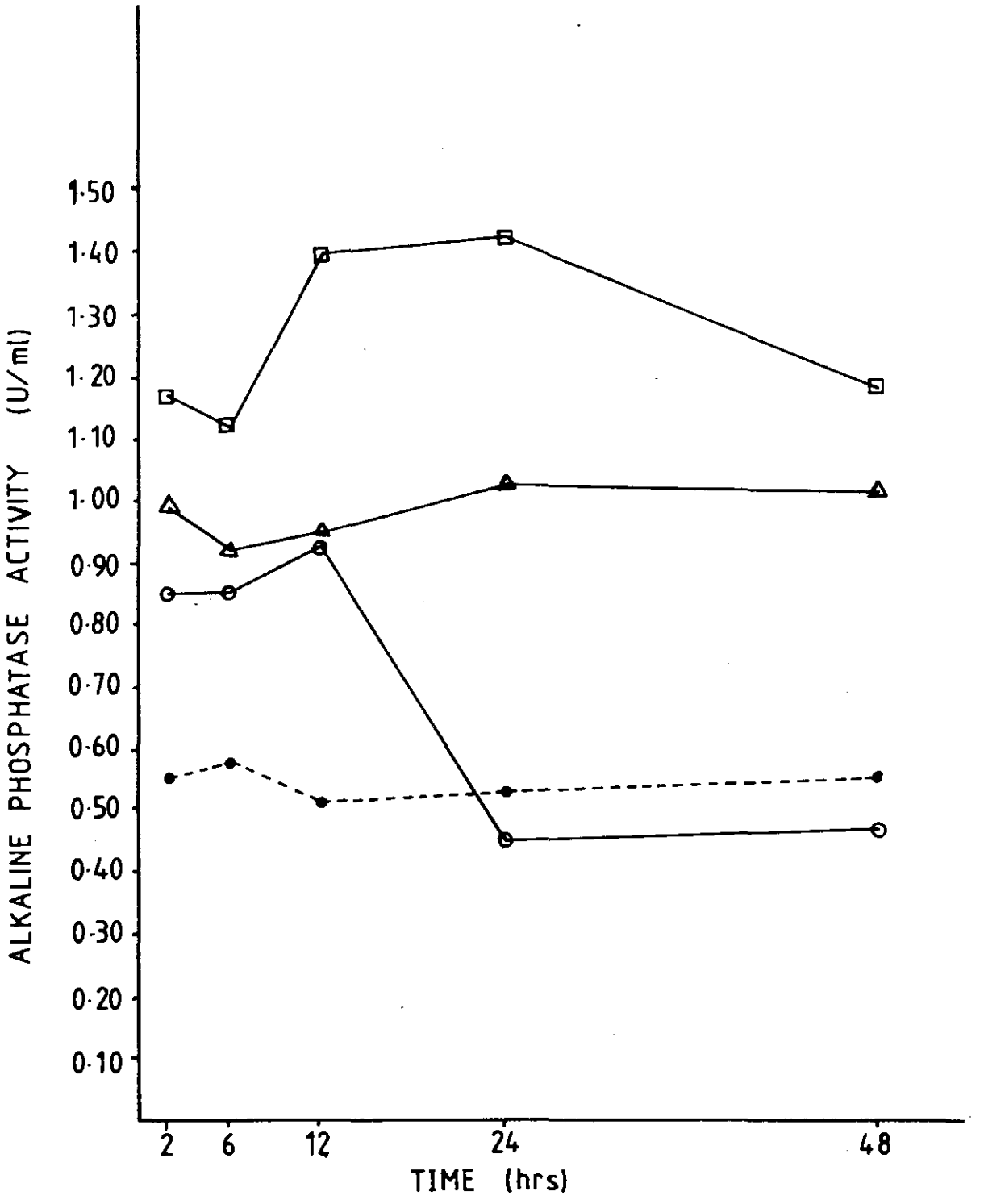

Figure 9. Haemolymph Alkaline Phosphatase Activity (u/ml) in I. exustus dosed with three sublethal concentrations of Copper. Control (---), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).

At 12 hr, the activity in 0.020 ppm copper dosed snails was significantly higher than the activities in 0.010 ppm ($\underline{P} < 0.001$), and 0.015 ppm copper dosed ($\underline{P} < 0.01$) snails.

At 24 hr, the activity was found to be maximum in those snails dosed with 0.020 ppm copper and it was significantly higher than in 0.010 ppm ($\underline{P} < 0.001$) and 0.015 copper dosed ($\underline{P} < 0.01$) snails.

At 48 hr, the elevation at 0.020 ppm was found to be significantly higher than those in 0.010 ppm ($\underline{P} < 0.001$) and 0.015 ppm ($\underline{P} < 0.001$).

4.3.2.4.2 Lymnaea acuminata f. rufescens (Table 20B ; Fig.10)

Snails dosed with 0.010 ppm of copper showed significant variations at 12, 24 and 48 hr post-exposure. The levels of alkaline phosphatase activity at 12 ($\underline{P} < 0.05$), and 24 hr exposure ($\underline{P} < 0.01$) were significantly lower and at 48 hr significantly higher than the ($\underline{P} < 0.05$) controls. When the enzyme activity in snails dosed with 0.015 ppm of copper was compared with that of the control, significant variation on the higher side was observed only at 6 hr exposure ($\underline{P} < 0.05$). No statistically significant variation in the alkaline phosphatase activity was observed between 0.020 ppm copper dosed snails and the controls.

At 2 hr post-exposure, the alkaline phosphatase activity in those dosed with 0.015 ppm of copper was significantly higher than those dosed with 0.010 ppm ($\underline{P} < 0.05$) and 0.020 ppm ($\underline{P} < 0.05$) of copper.

Table 20B. Haemolymph Alkaline Phosphatase Activity (U/ml) in Gammarus pulex f. rufescens dosed with three concentrations of copper (size group 18±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	0.5968	0.6138	0.6584	0.6718	0.5602
	± SD	0.2379	0.1211	0.2092	0.1050	0.1110
	Range	0.3690-0.8660	0.4450-0.7660	0.4230-0.8790	0.5810-0.8370	0.3730-0.6690
0.010 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.6510	0.5747	0.4725*	0.4777**	0.7101**
	± SD	0.1020	0.1103	0.0624	0.0904	0.0835
	Range	0.4550-0.7750	0.4070-0.7610	0.3690-0.5410	0.3650-0.6090	0.5860-0.8260
0.015 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.7317	0.7516*	0.6625	0.6555	0.5950
	± SD	0.0941	0.0769	0.1844	0.0942	0.1397
	Range	0.6110-0.9120	0.6340-0.8740	0.4940-1.0490	0.4770-0.8150	0.3390-0.8180
0.020 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.5614	0.5193	0.5510	0.5411	0.6287
	± SD	0.1960	0.1825	0.0797	0.1118	0.0879
	Range	0.1630-0.7560	0.1560-0.7470	0.4350-0.6420	0.3710-0.6560	0.4950-0.7600

Significance level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

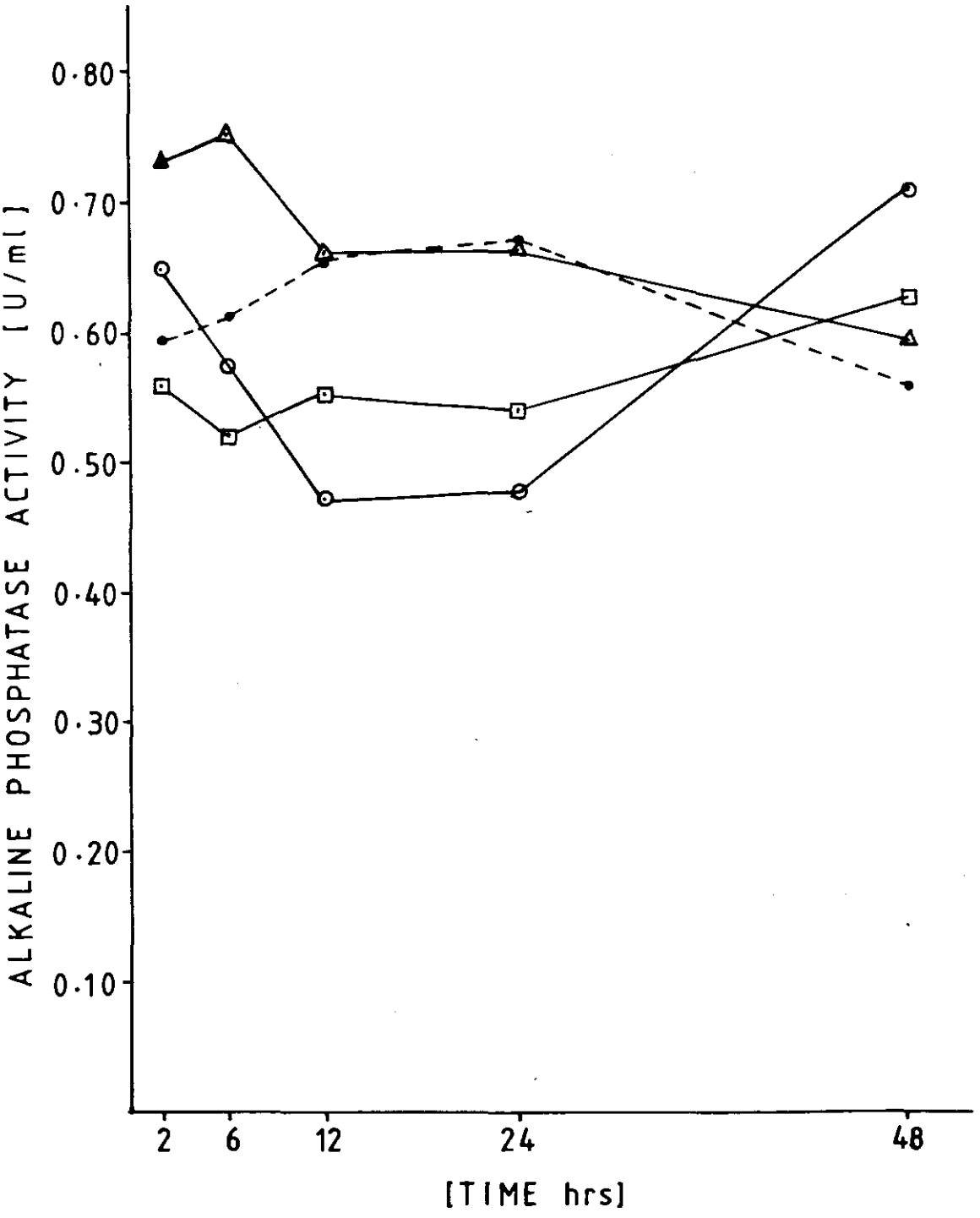

Figure 10. Haemolymph Alkaline Phosphatase Activity (u/ml) in L. acuminata f. rufescens dosed with three sublethal concentrations of Copper. Control (---), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).

At 6 hr post-exposure, the level of alkaline phosphatase activity in 0.015 ppm was significantly higher than that of 0.010 ppm ($\underline{P} < 0.001$) and 0.020 ppm dosed snails ($\underline{P} < 0.001$).

At 12 hr post-exposure of snails with copper, the enzyme activity in 0.010 ppm dosed snails was significantly lower than that in 0.015 ppm ($\underline{P} < 0.01$) and 0.020 ($\underline{P} < 0.05$).

At 24 hr post-exposure, the alkaline phosphatase activity in 0.015 ppm exposed snails was significantly higher than the activity in 0.010 ($\underline{P} < 0.001$) and 0.020 ppm ($\underline{P} < 0.01$) dosed snails.

At 48 hr exposure the enzyme activity was found to be significantly higher in 0.010 ppm copper dosed snails than in 0.015 ($\underline{P} < 0.05$) and 0.020 ppm ($\underline{P} < 0.05$) dosed snails.

4.4 DISCUSSION

In the present study, the mean values of haemolymph acid phosphatase activity did not show any significant difference among the three size groups of the snail, I. exustus. This shows that under normal conditions, the acid phosphatase activity did not change significantly among the size groups and it can be concluded that regarding I. exustus, age need not be a factor which determines the activity of the acid phosphatase. But in the case of L. acuminata f. rufescens, the ACP activity showed significant variation between the intermediate and large size groups. The ACP activity was found to be the maximum in the largest size group, while the activity in

small and intermediate group showed the same pattern. This may be attributed to the fact that in L. acuminata f. rufescens, age/shell size can be a factor which, to a certain extent, has some influence on the activity pattern of acid phosphatase, and obviously the species difference is very evident.

When the activity patterns of acid phosphatase in I. exustus dosed with three sublethal concentrations of copper were compared, significant elevation was observed at the highest concentration, i.e. 0.020 ppm. At the lowest concentration, 0.010 ppm, Cu^{2+} does not seem to have significant influence on the enzyme activity. The enzyme activity at 0.015 ppm was found to be higher but not significant except at 12 hr post-exposure, than the control at almost all time periods, and the activity was found to be linearly lowering towards 48 hr. At 0.020 ppm, after an initial elevation a linear significant increase was observed at 12, 24 and 48 hr post-exposure. These results indicate that the metal ion does not produce detectable response on the activity pattern of acid phosphatase at early time periods.

At 0.020 ppm, the significant increase in enzyme activity at later time periods may be interpreted as the result of the release of the enzyme into the haemolymph compartments from lysosomes. This release may also be due to hypersynthesis of the enzyme. Hypersynthesis of lysosomal enzymes under such circumstances has been indicated (Suresh and Mohandas, 1990a). The lysosomal activity may also be increased by the cytotoxicity of copper ions through increased

turnover of organelles and the subsequent release of the lysosomal enzymes. This is supported and explained on the basis of the studies by Koenig (1963), Norseth (1961), and Roesijadi (1980). The presence of xenobiotics induces changes in the lysosomal membrane and these induced alterations may lead to destabilisation of the membrane (Moore and Lowe, 1985). As a result of destabilisation, the hydrolytic enzymes from the lysosomes are released into cytosol (Moore, 1976; Baccino, 1978).

At 0.015 ppm, the initial non-significant elevation reached significant level at 12 hr post-exposure. At early time periods, it is interpreted, that the enzyme normally present in the system was sufficient enough to detoxify the metal ions. Since the snails were still under exposure, at 12 hr post-exposure there was hypersynthesis; and hence significantly higher values. This high enzyme level reached at 12 hr post-exposure was sufficient enough to detoxify the metal ions at later time periods, and hence no significant variation in the levels of activity was observed at 24 and 48 hr post-exposure.

Chandy and Patel (1985) have indicated that at lower concentrations, the metal ions that have entered the system are engulfed into lysosomes and subsequently transformed into biologically inactive forms. The lowest concentration used, 0.010 ppm, did not produce any significant response on the activity pattern of the enzyme, indicating that at this concentration the metal ions do not

cause destabilization or the normally available enzyme level is enough to detoxify the metal ions.

The results on the activity pattern of acid phosphatase in L. acuminata f. rufescens dosed with the three sublethal concentrations of copper showed an increase in enzyme activity at all three concentrations in the early time periods i.e., at 2, 6 and 12 hr post-exposure. The activity was found to decrease at all the three concentrations at 24 hr exposure. At the highest concentration, 0.020 ppm, the activity was normal at 48 hr, while at 0.015 ppm there was drastic decline, and at 0.010 ppm an increase in the activity.

The activity level of acid phosphatase was found to elevate towards the highest concentration at 2 hr post-exposure indicating concentration dependence. At higher concentrations, the activity was also found to be linearly declining after the immediate elevation. At early time periods, the significant elevation in enzyme activity at all concentrations may be attributed to the fact that the toxicant had caused hypersynthesis of this enzyme. At 0.010 ppm concentrations this elevated level of enzyme was sufficient enough to inactivate the metal ions even at 12 hr post-exposure and hence no significant variation in enzyme activity at this time period. But by 24 hr post-exposure, this level was not sufficient and there was no hypersynthesis at this time period, and hence the activity was significantly less. But as the snails were still under exposure, it is believed that there was hypersynthesis subsequently and hence

the enzyme activity was significantly higher at 48 hr post-exposure. At 0.015 ppm concentration, there was no hypersynthesis even at this time period and hence the enzyme activity was significantly lower both at 24 and 48 hr post-exposure. In the case of snails exposed to 0.020 ppm concentration, the high level of enzyme present in the haemolymph at early time periods as a result of hypersynthesis, was not sufficient to detoxify the metal ions at 24 hr post-exposure, and hence at this time period the activity level was significantly lower. But it is assumed that after this time periods the hypersynthesis just started again but did not reach significantly high level and this is reflected at 48 hr post-exposure when the activity level was normal. The diminution in enzyme activity is attributed to the fact that the enzyme conjugates with the metal ions. The binding affinity of heavy metal cation and protein is generally intense (Hilmy et al., 1981). The metal ions may also cause injury to the mitochondrial system which markedly block the action of enzymes. Simon (1953) reported that concentrations higher than those needed to prevent oxidative phosphorylation may injure the mitochondrial systems so markedly as to block the action of enzymes. The uncoupling of oxidative phosphorylation is not the only mechanism but other processes, such as oxidation accompanied by phosphorylation also inhibit enzyme activity. Based on the explanation given above, it may be argued that at low and high concentrations there was hypersynthesis of enzyme after 24 hr post-exposure but not in snails exposed to 0.015 ppm concentration. In this context it may be noted

that (i) cytochemically and functionally the lysosomes form a heterogenous population, (ii) lysosomes even in single cell types are quite variable in their enzymatic constitution, not all of the lysosomes in each cell include acid phosphatase, (iii) the chemical cycle occurring is non-synchronised, and (iv) molluscan species have different functional subpopulations performing different functions including enzyme activity (See, Mohandas, 1985). The difference in activity pattern of the enzyme in the two snail species clearly speaks of species differences.

When the mean activity levels of alkaline phosphatase were compared, the maximum activity was observed in the smallest size groups in I. exustus and the activity was minimum in the largest size group. In the case of L. acuminata f. rufescens, a totally different pattern was observed. The activity was found to be maximum in the largest size group and minimum in the intermediate size group. The difference in the enzyme activity pattern in different size groups may be interpreted to mean that the size group/age of the snail is a factor which, to a certain extent, determines the alkaline phosphatase activity. The difference in the activity pattern in the two snail species studied may be due to the species difference.

When dosed with the three sublethal concentrations of copper, the ALP activity in I. exustus was found elevated at certain time periods at 0.010 and 0.015 ppm, and at all time periods at 0.020 ppm. While Huffman and Tripp (1982) and Cheng and Rodrick (1975) consider

alkaline phosphatase as a lysosomal enzyme, Harts and Fouts (1965) and Reddy et al., (1984) treat this as plasma enzyme. According to the latter, copper induces proliferation of smooth endoplasmic reticulum resulting in the production and liberation of more of this enzyme. Considering that ALP is a lysosomal enzyme, the following explanation is given for the enzyme activity pattern.

At 0.010 ppm, the elevated enzyme level at 6 and 12 hr post-exposure as a result of hypersynthesis of this enzyme was sufficient enough to inactivate the metal ions and hence no significant variation in enzyme activity was noticed at late time periods, since the concentration was comparatively less. At 0.015 ppm, the initial significant elevation at 2 hr post exposure and subsequent non-significant decline at 6 and 12 hr post-exposure in enzyme activity are also attributed to the above reason. However, as the animals were under exposure beyond these time periods, to inactivate the metal ions at still higher concentration more enzyme was required which was hypersynthesized and released into the serum resulting in significant increase in enzyme activity at 24 and 48 hr post-exposure. In the case of snails exposed to 0.020 ppm of copper there was hypersynthesis of this enzyme to inactivate the metal from early time periods and this is reflected in the significantly high enzyme activity at all time periods.

The ALP activity in L. acuminata f. rufescens showed significant variations at low concentrations. At 0.10 ppm the enzyme activity

was found to decrease significantly at 12 and 24 hr post-exposure and increase significantly at 48 hr post-exposure. At 0.015 ppm of copper exposure significant elevation in activity was observed at 6 hr post-exposure. Although a decrease in activity pattern was noticed in those dosed with 0.020 ppm of copper, there was no significant variation in the values. At 0.010 ppm concentration, the decline in enzyme activity at 12 and 24 hr post-exposure may be attributed to the fact the toxicant has conjugated with enzyme resulting in the diminution in activity as suggested by Hilmy et al. (1981). In other words the metal ions were inactivated. Since the snails were still exposed, after 24 hrs there was hypersynthesis which is reflected as significantly high enzyme activity at 48 hr. In the case of snails exposed to 0.015 ppm concentration, this hypersynthesis had taken place at 6 hr post-exposure, and the high enzyme level was sufficient enough to detoxify the metal ions in the remaining time periods. From the Table 20B it is obvious that the activity is declining steadily from 6 hr onwards. At 0.020 ppm concentration, it is assumed that hypersynthesis has taken place before 2 hrs, and the enzyme level was sufficient enough to detoxify the metal ions for the remaining time periods. The value, as seen in the Table 20B at 48 hr post-exposure is the highest for any time period at 0.020 ppm concentration, and it indicates that at this time period there was again hypersynthesis to inactivate the metal ions.

A comparison of the data on total cell counts in I. exustus exposed to the three sublethal concentrations of copper (Chapter 2;

Table 5A) with the data on the activity pattern of the two phosphatases gives very interesting results. In all the three sublethal concentrations, copper was found to induce leucocytosis, and in all the three concentrations the activity of the two enzymes was either normal or high, and at no time period the activity was found inhibited. Since there was significant leucocytosis, it is believed that there was no destabilization of lysosomes and cell death due to copper toxicity at the sublethal levels used. Although the increased level of enzyme activity has been attributed to hypersynthesis of the enzymes, at this stage it is difficult to pin point the exact reason for the high activity levels of the enzyme for the following reasons: (i) it is not certain whether the high enzyme activity was due to leucocytosis (i.e., normal enzyme activity by haemocytes already present, and also by the newly recruited cells resulting in high enzyme activity), (ii) differential leucocytosis of functionally different haemocyte cell sub-populations for enhanced activity of specific enzymes (see Mohandas, 1985; it may also be noted that Cheng and Downs (1988) had demonstrated that of the five subpopulations of C. virginica haemocytes, sub-populations 4 and 3 showed high levels of ACP and lysozyme activities), (iii) lysosomal enzymes can come from nonhaemocyte sources; nonhaemocyte sources of certain lysosomal enzymes have been reported in B. glabrata by Cheng and Rodrick (1980), and in Theba pisana by Cheng et al. (1980a), and (iv) hypersynthesis of lysosomal enzymes has been recorded under metal stress conditions.

On the contrary in L. acuminata f. rufescens such a correlation was not found. Although there was leucocytosis at all time periods (Chapter 2, Table 5B) subsequent to exposure of snails to the sublethal concentrations of copper (except at 48 hrs in 0.010 ppm), the activity levels of enzymes at a few time periods were significantly lower than those in the controls. Since this occurred at later time periods, it is believed that the metal ions have blocked the action of enzymes, inspite of the fact that there was enzyme production from the normally present haemocytes and from those immobilized into the haemolymph.

Based on the above results, it can be concluded that (i) the activity patterns of acid and alkaline phosphatases vary from species to species, and to a certain extent age/shell size is a factor which influences the enzyme activity, (ii) copper ions can cause hypersynthesis of lysosomal enzymes, (iii) hypersynthesis of alkaline and acid phosphatases need not take place simultaneously, i.e., at the same time periods, and (iv) copper ions to a certain extent can inhibit the enzyme activity.

C H A P T E R - V

EFFECTS OF COPPER ON THE ACTIVITY PATTERN OF HAEMOLYMPH GLUTAMATE OXALOACETATE AND GLUTAMATE PYRUVATE TRANSAMINASES IN *INDOPLANORBIS EXUSTUS* AND *LYMNAEA ACUMINATA* F. *RUFESCENS*

5.1 INTRODUCTION

Transaminases are a group of enzymes that catalyse the process of biological transamination. Transamination reactions involve the transfer of an amino acid to keto acid with the formation of an aminoacid from the latter, and the generation of a new keto acid. Transamination not only serves as a pathway of conversion of alpha-ketoacids to L-amino acids but also as an alternative means of replenishing pyruvate pool. Glutamate Oxaloacetate Transaminase (GOT) or Aspartate Amino Transferase (AAT, EC. 2.6.1.1), and Glutamate Pyruvate Transaminase (GPT) or Alanine Amino Transferase (AlAT, EC. 2.6.1.2) are the most important and widely investigated transaminases. Alanine and aspartate serve as two major glucogenic aminoacids which through the activities of the enzymes GOT and GPT give rise to glucose precursors (Lehninger, 1979). The aspartate and alanine aminotransferases are known to play strategic role in metabolising L-aminoacids for gluconeogenesis, and also function as links between carbohydrate and protein metabolism under altered physiological, pathological, and induced environmental stress conditions (Nichol and Rosen, 1963; Knox and Greengard, 1965; Harper et al., 1979). Changes in the activities of aminotransferases, whether induced by endogenous or exogenous factors, are often

associated with changes in metabolic functions, and may thus represent widespread alterations in the organism's physiological state.

The activities of aspartate and alaninetransferases in serum have been used to detect heart and liver damages since the first is rich in the heart and the second in the liver. When there is heart damage, or cell damage, these enzymes are released from the cell into blood. Thus, these two enzymes have been studied in connection with the diagnosis of diseases, since characteristic serum enzyme patterns have been found to be associated with known disease.

Environmental pollution appears to be one of the factors that affects aminotransferase activities in animal tissue (Lane and Scura, 1970). It has been suggested that stress in general increases the activities of aminotransferases (Knox and Greenguard, 1965; Payne and Penrose, 1975; Ahamed et al., 1978), and it is likely that toxic stress could be responsible for their elevation.

The high rate of GOT activity could result only from an enhanced rate of aspartate formation (Malhotra et al., 1986). The decreased GOT activity may be due to the damage caused to mitochondrial membranes, loss of matrix and swelling mitochondrion (Chow and Pond, 1972). The decrease in GOT may also be attributed to the decreased oxaloacetate availability. The increase in GPT activity might partly be to compensate the loss

of GOT activity or to the increased pyruvate availability (Chetty et al., 1980). All transaminases appear to require specifically either pyridoxal-5-phosphate or pyridoxamine-5-phosphate. These coenzymes are tightly bound to the apoenzymes (Guirard and Snell, 1964; Fasella, 1967). An activation of the coenzyme of copper may inturn elevate the activity of the transaminases in the tissue.

Couch (1974) reported that hepatotoxicity and hepatopathological damage are the most common responses to various xenobiotic agents in fish. Activities of hepatic enzymes were found to increase in response to xenobiotics in fish. Higher concentration of both GOT and GPT in the blood of the common Red sea harry fish Aphanius dispar, in acute and long-term mercury exposure was studied by Hilmy et al. (1981). They reported that there was an elevation in GOT activity in long term exposure of mercury and attributed this to cellular degradation by mercury. Bell (1968) reported that transaminases are indicators of degradation in salmonoids. Hilmy et al. (1981) reported that GPT showed a statistically significant increase during acute mercury poisoning due to increased permeability of the liver cell membrane leading to increased serum activity.

The liver parenchyma is a rich source of GOT second only a heart muscle and has the highest known content of GPT. The absolute amount of GPT is less when compared to GOT. Although

damage to liver tissue leads to increased serum levels of both transaminases, yet the increase in GPT levels is more specific for liver damage than GOT (Sherlock, 1968).

Dange (1986) reported the effects of toluene, naphthalene and phenol on the activities of both the transaminases in the freshwater fish Oreochromis mossambicus. Tiedge et al. (1986) investigated the effect of substituted phenols on the transaminase activity in the fish, Leuciscus idusmelanotus and reported increase in the activity of serum transaminases.

The increase of serum transaminases has been shown to be roughly proportional to the degree of cell necrosis in mice infected with hepatic virus (Friend et al., 1955). They also observed loss of analogous transaminase activity in the liver of the same animals. Zelman et al. (1959), in a similar study of human subjects, found an excellent correspondence between the extent of necrosis of liver cells and the rise in serum transaminases. An inverse relationship of the degree of necrosis of liver cells was found to some extent with both transaminases.

The occurrence of transaminases in molluscs is well known (Read, 1962; Awapara and Campbell, 1964; Goddard and Martin, 1966) but their origin remains unknown. Transaminases activities were reported in a variety of molluscan tissues such as in B. glabrata haemolymph (Rodrick and Cheng, 1974), body fluid of Viviparus bengalensis (Prasad et al., 1983), haemolymph of Lymnaea luteola

(Manohar et al., 1972) in the tissues of Pila globosa (Swami and Reddy, 1978), and the GOT levels tend to be quite a bit higher than the GPT levels in the hepatopancreas of some gastropods (Swami and Reddy, 1978; Sollock et al., 1979). Hammen (1968) reported that the levels of activity in the tissues vary with the size of the animal, indicating that animals of uniform size should be used in making comparisons of the levels of tissue activity under various experimental regimens. There are various factors affecting the levels of GOT and GPT in tissues such as difference in species and or other factors related to reason, food, size of the animals and assay procedure. In Pila globosa, the tissue levels of GPT and GOT were greater in active animals than in starving and aestivating animals (Swami and Reddy, 1978). Mohan and Dass (1969), Mohan and Babu (1975) reported that there was a considerable loss of acetyl cholinesterase, aspartate and alanine aminotransferase activities during aestivation. This loss indicated a disruption of normal pattern of acetylcholine and glutamate metabolisms in the aestivating snail.

Cheng et al. (1980a) reported seasonal fluctuations in GPT activity associated with the head-foot tissues of Theba pisana. There is a steady rise in glutamate pyruvate transaminase activity in the spring, terminating in winter prior to death. This is interpreted to reflect a compensatory metabolic shift prior to death. Kulkarni and Kulkarni (1987) reported enhanced AAT and AlAT activity in the gills and mantle muscles of mercury exposed clams.

The general body fluid aminotransferase activity is very high in L. luteola (Manohar et al., 1972). Compared to body fluid GPT, the GOT activity of normal L. luteola was found to be low. This might be either a reflection of the intracellular concentration or a consequence of differences in intracellular localisation, permeation through cell membrane, and the rate of inactivation and elimination of these two transferases. In view of the results of Awapara and Campbell (1964) and Hammen (1968), it is more probable that the body fluid aminotransferase activity pattern is more a reflection of the situation inside the cell.

Manohar et al. (1972) studied the activity pattern of aminotransferases in trematode cercariae infected L. luteola. They reported that the total aminotransferase activity in the body fluid rose significantly in xiphidio and furcocercarial infections and dropped in pigmented cercarial infections. Manohar and Rao (1977) observed a significant drop in both the AAT and AlAT activity levels in the tissue and highly elevated levels in the body fluid of infected snails. Of the two aminotransferases assayed, the activity of alanine aminotransferase was higher than that of the aspartate aminotransferase in the non-parasitized snails while a reverse trend was seen in infected ones. This led to the variation in the de Ritis quotient (GOT:GPT ratio). Infected snails, the haemolymph de Ritis quotient is significantly high when compared with that of uninfected ones. This is due to the significant elevation of aminotransferases during infection (Manohar and Rao, 1977). The

relative effect of different larval trematodal infection on the body fluid enzymatic profiles of the freshwater snail, V. bengalensis was reported by Prasad et al. (1983). Alanine and aspartate aminotransferase activity was lowered on Cercariae indica LXXXII infections and aspartate aminotransferase activity was increased on echinostomal cercarial infection. The GOT:GPT ratio was found to be increased in echinostome cercariae infected snails while in Cercariae indica LXXXII infected snails a more or less constant GOT:GPT ratio with respect to the uninfected snails was observed.

Transaminases might be of particular importance under conditions that impose a heavy loss on the animals store of metabolites (Goddard and Martin, 1966). The amino transferases have their own role in shell formation (Hammen and Wilbur, 1959) and in amino acid excretion (Hammen, 1968). The role of transaminases in gluconeogenesis was reported in a number of gastropods (Moran and Gonzalez, 1967; Bacila, 1970, Marshall et al., 1974; Ishak et al., 1975; Sharaf et al., 1975; Mc Manus and James, 1978; Sollock et al., 1979).

In this chapter the effect of sublethal concentrations of copper on the activity pattern of GOT and GPT is reported. From the introduction it is obvious that studies related to GOT and GPT in metal stressed animals are rare, and as far as invertebrates are concerned they are still less.

5.2 MATERIALS AND METHODS

Methods of collection of snails, acclimatisation, selection of size groups, mode of collection of haemolymph and the computation of data were the same as described in detail in section 2.2.

The toxicant used, toxicant concentration, and procedure for lethal and sublethal toxicity studies are described in section 4.2.

5.2.1 Enzyme Analysis

The activity pattern of haemolymph Glutamate-Oxaloacetate Transaminase and Glutamate-Pyruvate Transaminase in the three size groups selected, as well in one size group exposed to three different sublethal concentrations of copper were studied.

5.2.1.1 Assay of Glutamate-Oxaloacetate Transaminase (Aspartate Aminotransferase)

Glutamate oxaloacetate transaminase activity in the haemolymph was determined following the methodology of Reitman and Frankel (1957). To study the enzyme activity in I. exustus, buffer substrate of pH 7.6 was used. In the case of L. acuminata f. rufescens, the pH of the buffer/substrate mixture used was 7.4 (containing 0.1 M phosphate buffer, 0.1 M aspartate and 2 mM 2-oxoglutarate).

To 1 ml of the frozen buffer/substrate mixture, 0.1 ml of haemolymph was added and incubated for one hour at 37 $\pm$ 0.05^{o}C. At

the end of incubation, the reaction was stopped by adding 1.0 ml of chromogen solution (2,4-dinitrophenyl hydrazine) and mixed well and kept for 20 minutes at room temperature. After 20 minutes, 10 ml of 0.4 N NaOH was added and the colour developed was read spectrophotometrically at 546 nm. The enzyme activity is expressed in μ moles pyruvate liberated/minute/ml.

5.2.1.2 Assay of Glutamate Pyruvate Transaminase (Alanine Aminotransferase)

Glutamate pyruvate transaminase activity in the haemolymph was determined by the method of Reitman and Frankel (1957). Indoplanorbis exustus showed the maximum enzyme activity at pH 7.2 while Lymnaea acuminata f. rufescens at pH of 7.4. The incubation temperature was 37 $\pm$ 0.05^{o}C.

To 1.0 ml of the buffer substrate solution (containing 0.1 M phosphate buffer, pH 7.4, 0.2 M DL-alanine and 2 mM 2-oxoglutarate), 0.1 ml of haemolymph was added and the reaction mixture was incubated for one hour at 37^{o}C. After incubation, the reaction was stopped by adding 1 ml of 0.45 mM chromogen solution and allowed to stand for 20 minutes at room temperature. After 20 minutes, 10 ml of 0.4 NaOH was added and the colour developed was measured spectrophotometrically at 546 nm. Enzyme activity is expressed as μ moles pyruvate liberated/minute/ml.

5.3 RESULTS

5.3.1 Enzyme Analysis

5.3.1.1 Glutamate-Oxaloacetate Transaminase activity in the three size groups of snails

5.3.1.1.1 Indoplanorbis exustus (Table 21A)

The mean value of activity in 15 ± 1 mm size group was found to be significantly lower than the activities in 11 ± 1 mm and 7 ± 1 mm groups ($P < 0.01, 0.05$). There was no significant variation in values between size groups of 7 ± 1 mm and 11 ± 1 mm.

5.3.1.1.2 Lymnaea acuminata f. rufescens (Table 21B)

There was significant variation in the mean values among three size groups. The mean value of activity in 21 ± 1 mm size group was found higher than the values in 18 ± 1 mm and 13 ± 1 mm size groups ($P < 0.001$). The mean value in 13 ± 1 mm size group was found to be significantly lower than the activity value in 18 ± 1 mm size group ($P < 0.01$).

5.3.1.2 Glutamate Pyruvate Transaminase activity in the three size groups of snails

5.3.1.2.1 Indoplanorbis exustus (Table 22A)

The results showed significant variations in the activity pattern of Glutamate pyruvate transaminase in different size groups. The mean value of activity in 15 ± 1 mm size group was significantly

Table 21A. Haemolymph Glutamate-Oxaloacetate Transaminase (U/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	12	12	12
Mean value	0.1545	0.1774	0.1162
± SD	0.0255	0.0326	0.0523
Range	0.1334-0.2218	0.1236-0.2140	0.0244-0.2072

Table 21B. Haemolymph Glutamate-Oxaloacetate Transaminase (U/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	6
Mean value	0.0291	0.0446	0.0759
± SD	0.0099	0.0141	0.0139
Range	0.0122-0.0471	0.0253-0.0722	0.0520-0.0942

Table 22A. Haemolymph Glutamate-Pyruvate Transaminase (U/ml) in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	12	12	12
Mean value	0.0478	0.0319	0.0282
± SD	0.0118	0.0084	0.0091
Range	0.0282-0.0705	0.0181-0.0491	0.0091-0.0377

Table 22B. Haemolymph Glutamate-Pyruvate Transaminase (U/ml) in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	6
Mean value	0.0124	0.0157	0.0185
± SD	0.0043	0.0062	0.0053
Range	0.0044-0.0188	0.0065-0.0267	0.0103-0.0240

lower than the activity value in 7 ± 1 mm size group ($P < 0.001$) while there was no significant variation in values between the size groups 15 ± 1 mm and 11 ± 1 mm. When the mean value of 11 ± 1 mm size group was compared with the values in 7 ± 1 mm, the latter showed significantly higher value ($P < 0.01$).

5.3.1.2.2 Lymnaea acuminata f. rufescens (Table 22B)

Statistical analysis of the data revealed that there is significant variation in values between snails of the size groups 21 ± 1 mm and 13 ± 1 mm. The activity in 21 ± 1 mm size group was significantly higher than the activity in 13 ± 1 mm group ($P < 0.05$).

5.3.1.3 Sublethal effects of copper on the activity pattern of Glutamate-Oxaloacetate Transaminase

5.3.1.3.1 Indoplanorbis exustus (Table 23A ; Figure 11)

Snails dosed with 0.010 ppm of copper showed statistically significant elevation at 2, 6, and 12 hr post-exposure with respect to the control. At 24 and 48 hr post-exposure, no significant difference in values was observed with respect to the corresponding controls. When the mean values of 0.015 ppm copper dosed snails were compared with the respective controls, no significant variation was found at 2, 12 and 48 hr post-exposure. At 6 and 24 hr, the GOT activity was found to be significantly lower than the control values. The 0.020 ppm copper dosed snails showed significantly lower GOT activity at 6 hr than the control, and at 12 and 24 hr of exposure,

Table 23A. Haemolymph Glutamate-Oxaloacetate Transaminase Activity (U/ml) in Indoplanorbis exustus dosed with three concentrations of copper (size group 11±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	0.1818	0.1943	0.1587	0.1559	0.1658
	± SD	0.0152	0.0194	0.0328	0.0282	0.0324
	Range	0.1629-0.2003	0.0161-0.2101	0.1345-0.2130	0.1239-0.1992	0.1334-0.2120
0.010 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.2210**	0.2446*	0.3163***	0.1952	0.1719
	± SD	0.0229	0.0309	0.0373	0.0469	0.0209
	Range	0.1885-0.2665	0.2022-0.2839	0.2557-0.3511	0.1301-0.2867	0.1503-0.2101
0.015 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.1948	0.1355**	0.1438	0.1134**	0.1469
	± SD	0.0562	0.0356	0.0280	0.0106	0.0164
	Range	0.1477-0.3178	0.0940-0.2159	0.0905-0.1709	0.0922-0.1227	0.1347-0.1855
0.020 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.1562	0.1397***	0.1839	0.1981	0.2381*
	± SD	0.0284	0.0084	0.0287	0.0381	0.0424
	Range	0.1203-0.2061	0.1225-0.1486	0.1542-0.2335	0.1355-0.2557	0.2001-0.3320

Significance Level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

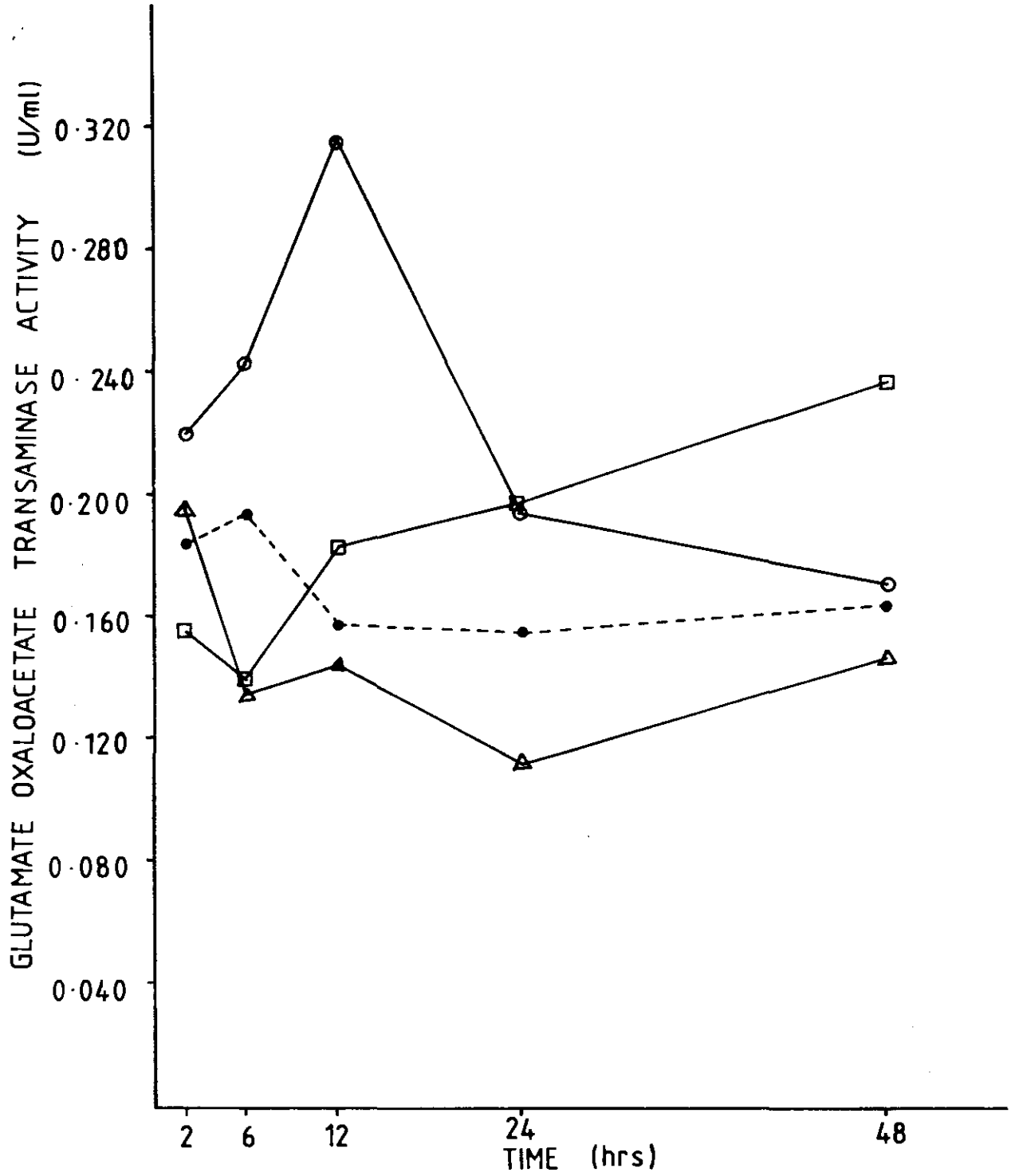

Figure 11. Haemolymph Glutamate Oxaloacetate Transaminase Activity (u/ml) in I. exustus *dosed with three sublethal concentrations of Copper. Control (—•—), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).*

a general trend in the elevation of activity was observed and at 48 hr this elevation was significantly higher.

When GOT activity in snails exposed to the three sublethal concentrations was compared at specific intervals, at 2 hr post-exposure, the enzyme activity was found to decrease as concentrations increased. The GOT activity in 0.020 ppm copper dosed snails was found to be significantly lower than the activities in snails exposed to 0.015 ($\underline{P} < 0.05$) and 0.010 ppm ($\underline{P} < 0.001$).

In 6 hr copper exposed snails, the enzyme activity was the highest at 0.010 ppm, and it was found to be significantly higher than the activities in 0.015 ppm ($\underline{P} < 0.001$) and 0.020 ppm ($\underline{P} < 0.001$).

When the levels of GOT activity at 3 different sublethal concentrations at 12 hr post-exposure were compared, 0.015 ppm dosed snails showed significantly lower activity than in those dosed with 0.010 ppm ($\underline{P} < 0.001$) and 0.020 ppm ($\underline{P} < 0.01$). The maximum activity was observed in those exposed to 0.010 ppm, and it was significantly higher than the activities in 0.015 ppm ($\underline{P} < 0.001$) and 0.020 ppm ($\underline{P} < 0.001$) dosed snails.

At 24 hr post-exposure, the GOT activity in 0.015 ppm was found to be significantly lower than the activities in 0.010 ppm ($\underline{P} < 0.001$) and 0.020 ppm ($\underline{P} < 0.001$).

At 48 hr also 0.015 ppm dosed snails showed significantly lower GOT activity when compared with those snails dosed with

0.010 ppm ($P<0.01$) and 0.020 ppm ($P<0.001$). The maximum activity was observed in 0.020 ppm and it was significantly higher than the activities in 0.010 ppm ($P<0.001$) and 0.015 ppm ($P<0.001$).

5.3.1.3.2 Lymnaea acuminata f. rufescens (Table 23B ; Figure 12)

The activity in snails dosed with 0.010 ppm of copper when compared with the activity in corresponding controls, significant decline was observed at 6 hr, and at 12, 24 and 48 hr post-exposure a general trend for the elevation of enzyme activity was observed. This elevation in enzyme activity was significant only at 48 hr when compared with the control. The 0.015 ppm dosed snails showed a significantly higher value at 2 and 48 hr post-exposure with respect to their controls. However at 6, 12 and 24 hr, no significant variation in enzyme activity was observed, although the trend was to go on the higher side. The GOT activity level was found to be non-significant in 0.020 ppm copper dosed snails at all time periods except at 6 hr. At 6 hr, the GOT activity was significantly lower than the control value.

When the activity levels in snails exposed to the three sublethal concentrations of copper were compared, a decrease in the activity was observed in the lowest concentration at 2 hr post-exposure and this decreased activity was significantly lower than the activities in those snails dosed with 0.015 ppm ($P<0.001$) and 0.020 ppm of copper ($P<0.001$).

Table 23B. Haemolymph Glutamate-Oxaloacetate Transaminase Activity (U/ml) in Lymnaea acuminata f. rufescens dosed with three concentrations of copper (size group 18±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	0.0399	0.0421	0.0411	0.0393	0.0386
	± SD	0.0134	0.0120	0.0123	0.0108	0.0112
	Range	0.0251-0.0562	0.0287-0.0562	0.0269-0.0593	0.0287-0.0539	0.0253-0.0539
0.010 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0289	0.0256*	0.0467	0.0520	0.0616*
	± SD	0.0105	0.0061	0.0094	0.0186	0.0184
	Range	0.0136-0.0461	0.0176-0.0339	0.0243-0.0537	0.0304-0.0878	0.0330-0.0896
0.015 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0532*	0.0563	0.0395	0.0491	0.0596**
	± SD	0.0061	0.0127	0.0107	0.0156	0.0074
	Range	0.0443-0.0609	0.0331-0.0697	0.0243-0.0547	0.0346-0.0771	0.0522-0.0681
0.020 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0533	0.0185**	0.0336	0.0477	0.0344
	± SD	0.0169	0.0073	0.0072	0.0127	0.0058
	Range	0.0287-0.0774	0.0079-0.0252	0.0243-0.0444	0.0356-0.0757	0.0269-0.0426

Significance Level : * $P < 0.05$ ** $P < 0.01$

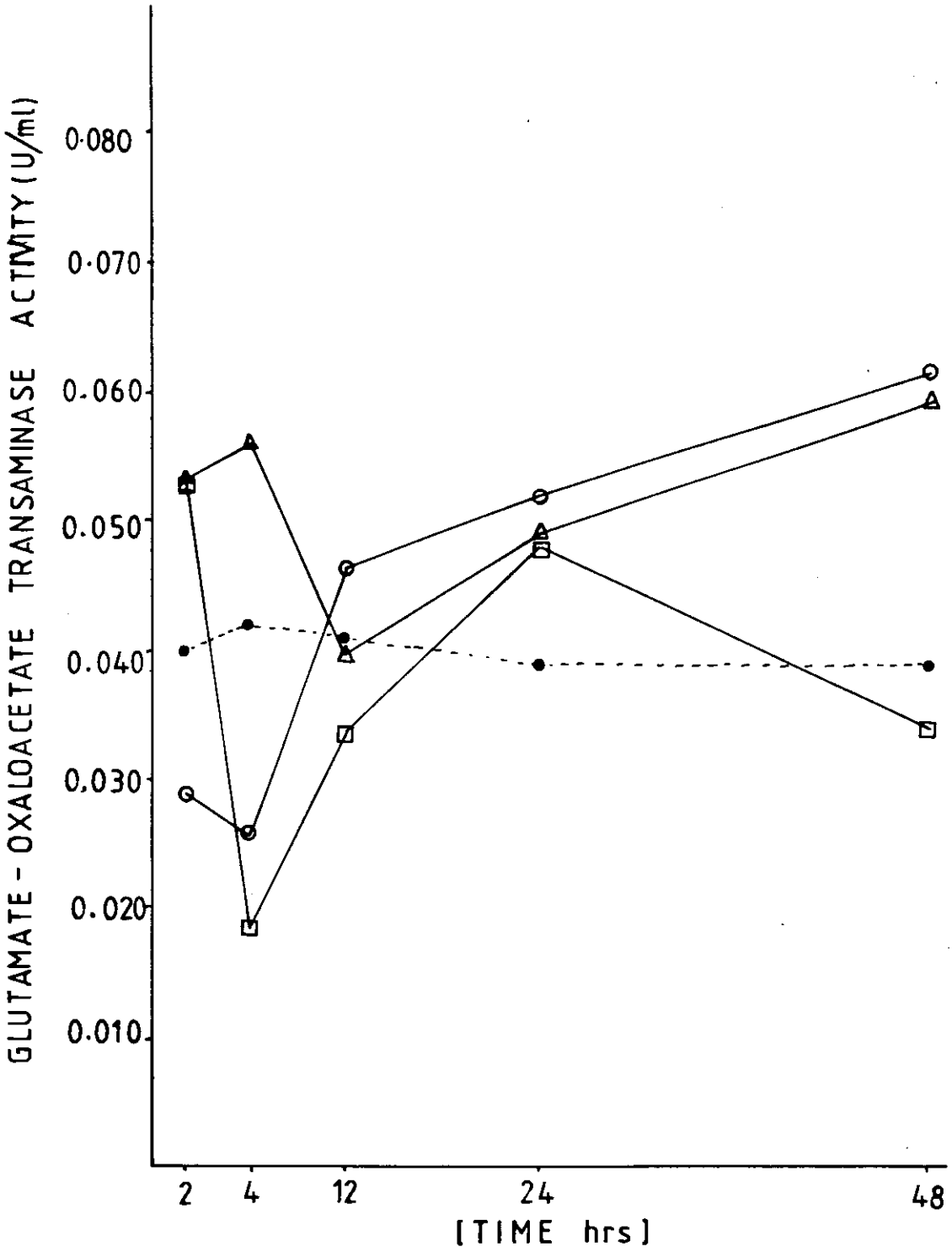

Figure 12. Haemolymph Glutamate Oxaloacetate Transaminase Activity (u/ml) in L. *acuminata f. rufescens dosed with three sublethal concentrations of Copper. Control (-•-), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).*

At 6 hr, the maximum activity was observed in 0.015 ppm dosed snails, and the activity was significantly higher than the activities in 0.010 ppm ($\underline{P} < 0.001$) and 0.020 ppm dosed snails ($\underline{P} < 0.05$). When the activity levels in 0.010 ppm and 0.020 ppm dosed snails were compared, significantly higher value was observed in 0.010 ppm dosed snails ($\underline{P} < 0.001$).

At 12 hr, the activity was found to be high at 0.010 ppm, and it was significantly higher than the activity in 0.020 ppm ($\underline{P} < 0.01$).

Snails exposed to sublethal concentrations of copper for 24 hr, however, did not show any significant variation in GOT activity, when compared among themselves.

At 48 hr post-exposure, GOT activity was found to be lower in 0.020 ppm dosed snails, and it was significantly lower than the activities in 0.015 ppm ($\underline{P} < 0.001$) and 0.010 ppm dosed snails ($\underline{P} < 0.001$).

5.3.1.4 Sublethal effects of copper on the activity pattern of Glutamate Pyruvate Transaminase

5.3.1.4.1 Indoplanorbis exustus (Table 24A ; Figure 13)

The snails dosed with 0.010 ppm of copper did not show any statistically significant variation in GPT activity at 2, 6, 24 and 48 hr post-exposure with respect to the control values. The only significant variation in GPT activity at 0.010 ppm was at 12 hr at which the activity was significantly higher than the control.

Table 24A. Haemolymph Glutamate-Pyruvate Transaminase Activity U/ml) in Indoplanorbis exustus dosed with three concentrations of copper (size group 11±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	0.0420	0.0392	0.0401	0.0402	0.0382
Control	± SD	0.0080	0.0104	0.0086	0.0081	0.0080
	Range	0.0282-0.0490	0.0221-0.0503	0.0267-0.0488	0.0280-0.0488	0.0282-0.0475
	N	8	8	8	8	8
0.010 ppm of	Mean value	0.0465	0.0455	0.0758***	0.0390	0.0415
Cu^{2+}	± SD	0.0042	0.0031	0.0072	0.0052	0.0024
dosed	Range	0.0417-0.0522	0.0417-0.0503	0.0647-0.0857	0.0274-0.0427	0.0372-0.0447
	N	8	8	8	8	8
0.015 ppm of	Mean value	0.0449	0.0424	0.0354	0.0298*	0.0417
Cu^{2+}	± SD	0.0048	0.0055	0.0048	0.0033	0.0029
dosed	Range	0.0382-0.0535	0.0372-0.0544	0.0307-0.0427	0.0274-0.0357	0.0377-0.0475
	N	8	8	8	8	8
0.020 ppm of	Mean value	0.0372	0.0443	0.0607**	0.0636*	0.0600**
Cu^{2+}	± SD	0.0051	0.0027	0.0081	0.0201	0.0097
dosed	Range	0.0264-0.0425	0.0397-0.0475	0.0467-0.0723	0.0311-0.0857	0.0531-0.0817

Significance Level : * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

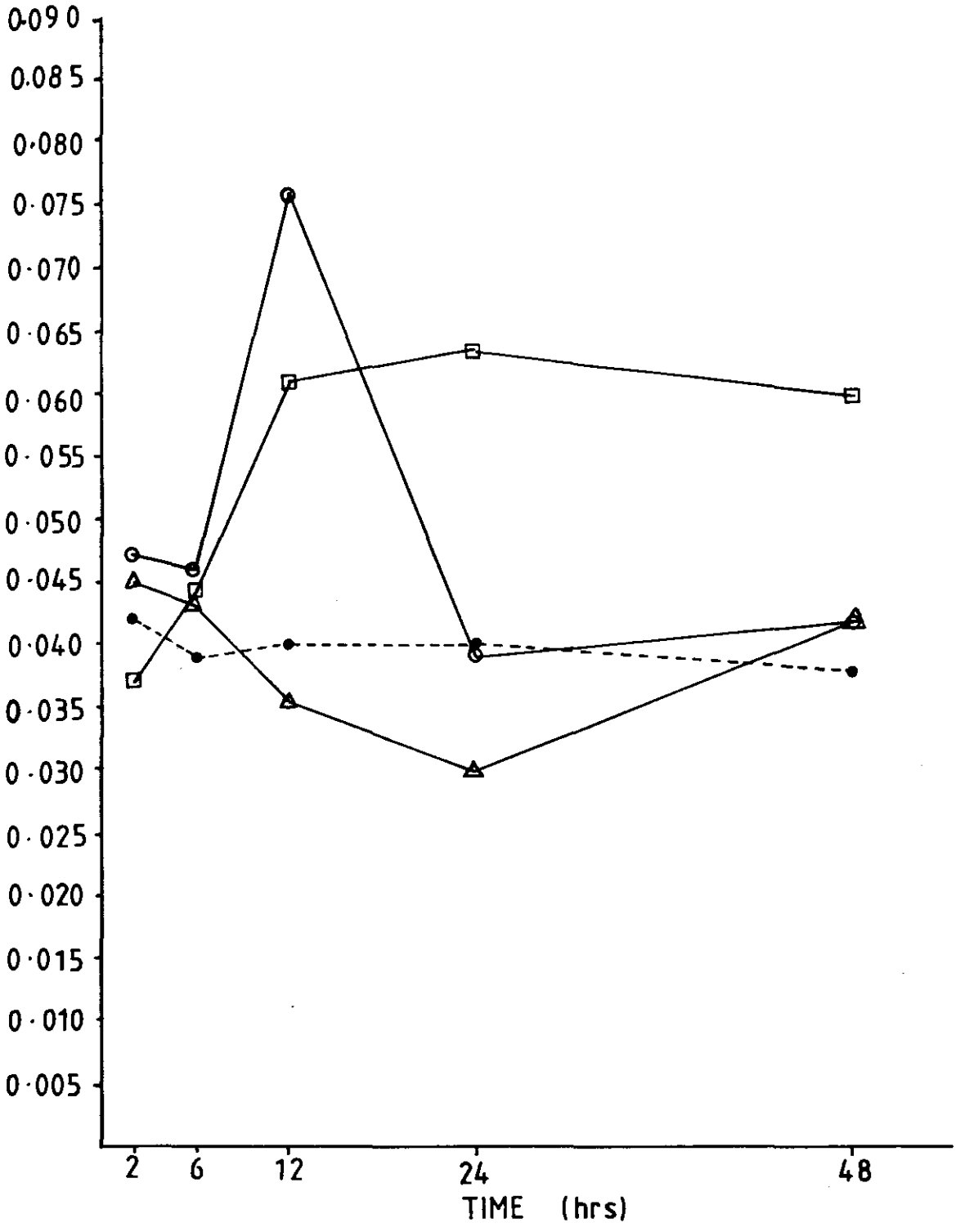

Figure 13. Haemolymph Glutamate Pyruvate Transaminase Activity (u/ml) in I. *exustus dosed with three sublethal concentrations of Copper. Control (-•-), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).*

The 0.015 ppm copper dosed snails showed significantly lower value at 24 hr than the control. No significant change was observed at 2, 6, 12 and 48 hr post-exposure. In snails dosed with 0.020 ppm of copper, significantly higher GPT activity was observed at 12, 24 and 48 hr post-exposure with respect to the controls.

When GPT activity among experimentals were compared at specific intervals, the enzyme activity was found to be higher at 2 hr post-exposure in 0.010 ppm dosed snails, and the activity was significantly higher than the activities in 0.015 ppm ($\underline{P}<0.05$) and 0.020 ppm ($\underline{P}<0.001$) dosed ones.

At 6 hr post-exposure, no significant change in enzyme activity among different sublethal concentrations was observed.

At 12 hr post-exposure, the GPT activity in 0.015 ppm dosed snails was found to be significantly lower than the activities in 0.010 ppm ($\underline{P}<0.001$) and 0.020 ppm dosed snails ($\underline{P}<0.001$). When the enzyme activity levels in 0.010 and 0.020 ppm were compared, the former showed significantly higher value than the latter ($\underline{P}<0.001$).

The GPT activity was found to be higher in 0.020 copper dosed snails at 24 hr, than the activities in 0.010 ppm ($\underline{P}<0.001$) and 0.015 ppm dosed snails ($\underline{P}<0.001$). The enzyme activity was found to be significantly higher in those snails dosed with 0.010 ppm ($\underline{P}<0.001$) when compared with the value in 0.015 ppm.

At 48 hr post-exposure, the GPT activity in 0.020 ppm dosed snails was significantly higher than the activities in 0.010 ppm ($\underline{P}<0.001$) and 0.015 ppm ($\underline{P}<0.001$) dosed snails.

5.3.1.4.2 Lymnaea acuminata f. rufescens (Table 24B ; Figure 14)

Snails dosed with 0.010 ppm of copper showed significantly lower value than the control at 6 hr post-exposure. There was no significant change in GPT activity at 2, 12, 24 and 48 hr post-exposure. The 0.015 ppm and 0.020 ppm copper dosed snails did not show any significant change in GPT activity at 2, 6, 12, 24 and 48 hr post-exposure.

When the enzyme activity was compared among experimentals at specific intervals, GPT activity at 2 and 6 hr post-exposure was found to increase significantly in 0.015 ppm exposed snails compared with 0.010 ($\underline{P}<0.001$) and 0.020 ppm dosed ones ($\underline{P}<0.001$). When compared with 0.010 ppm, the GPT activity was found to be significantly higher in those dosed with 0.020 ppm at 2 hr ($\underline{P}<0.05$) and 6 hr ($\underline{P}<0.001$) post-exposure.

At 12 hr post-exposure, the GPT activity was maximum in those snails exposed to 0.020 ppm of copper, and it was significantly higher than the activity in 0.010 ppm dosed snails ($\underline{P}<0.01$).

There was no significant variation in GPT activity at 24 and 48 hr post-exposure, when the activities in snails exposed to the three different sublethal concentrations of copper were compared.

Table 24B. Haemolymph Glutamate-Pyruvate Transaminase Activity (U/ml) in Lymnaea acuminata f. rufescens dosed with three concentrations of copper (size group 18±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	0.0141	0.0145	0.0136	0.0123	0.0122
	± SD	0.0078	0.0080	0.0072	0.0054	0.0049
	Range	0.0059-0.0267	0.0054-0.0237	0.0064-0.0224	0.0067-0.0211	0.0717-0.0200
0.010 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0076	0.0043**	0.0120	0.0168	0.0133
	± SD	0.0013	0.0004	0.0029	0.0078	0.0058
	Range	0.0062-0.0096	0.0035-0.0047	0.0096-0.0157	0.0092-0.0342	0.0041-0.0202
0.015 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0214	0.0127	0.0145	0.0120	0.0122
	± SD	0.0041	0.0036	0.0042	0.0064	0.0029
	Range	0.0159-0.0264	0.0071-0.0172	0.0096-0.0193	0.0058-0.0253	0.0065-0.0166
0.020 ppm of Cu^{2+} dosed	N	8	8	8	8	8
	Mean value	0.0101	0.0072	0.0170	0.0120	0.0118
	± SD	0.0033	0.0033	0.0039	0.0086	0.0031
	Range	0.0065-0.0172	0.0024-0.0105	0.0107-0.0213	0.0030-0.0286	0.0081-0.0174

Significance Level : ** $P < 0.01$

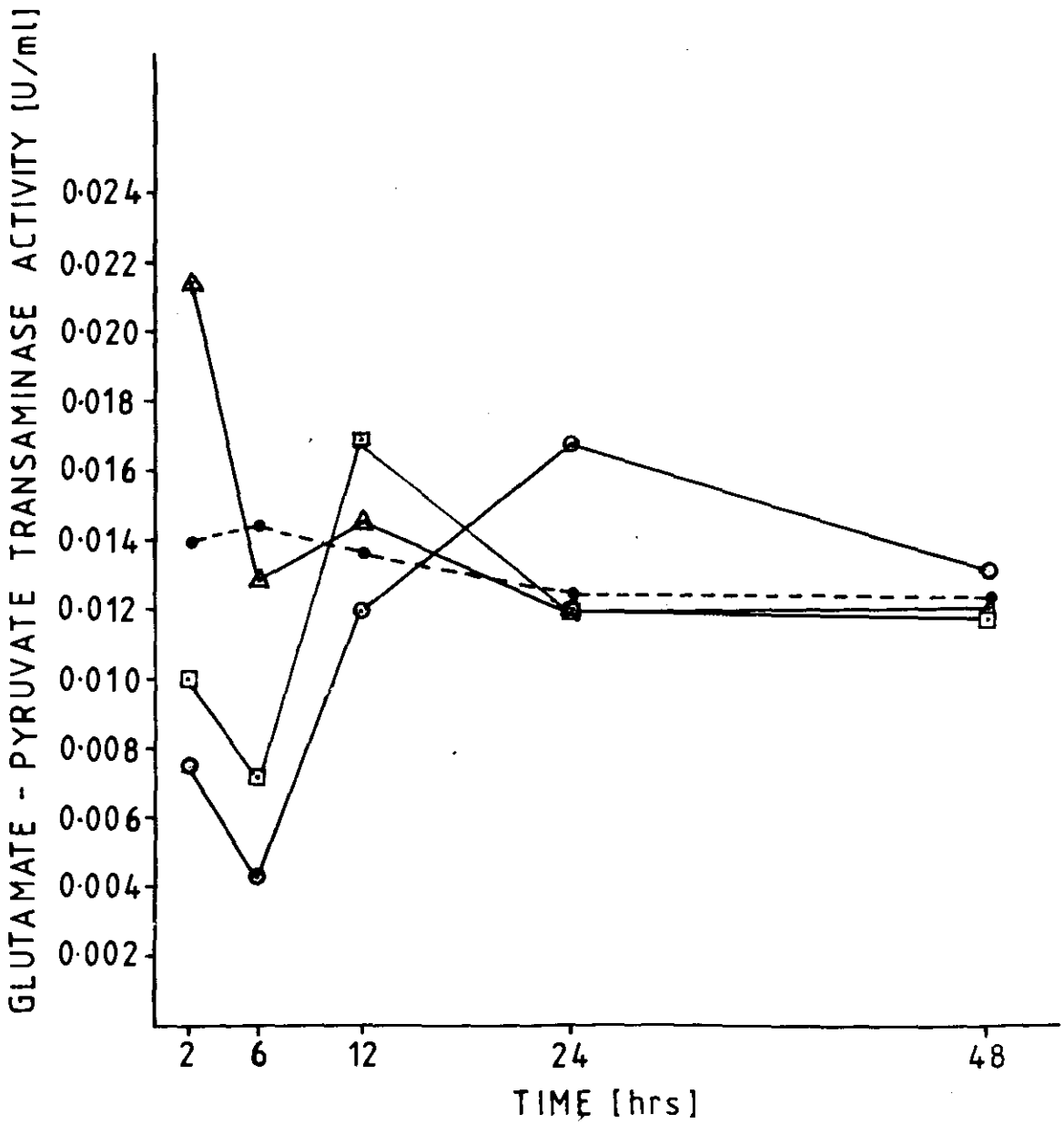

Figure 14. Haemolymph Glutamate Pyruvate Transaminase Activity (u/ml) in L. acuminata f. rufescens dosed with three sublethal concentrations of Copper. Control (-•-), 0.010 ppm (⊙), 0.015 ppm (△), and 0.020 ppm (⊡).

5.3.1.5 GOT:GPT Ratio

5.3.1.5.1 Indoplanorbis exustus

Among size groups, the ratio was significantly higher in snails of the 11 ± 1 mm size group than in those of the 7 ± 1 mm size group ($P < 0.001$ Table 25A).

Except for the values at 6 hr post-exposure, which were significantly lower in 0.015 and 0.020 ppm copper dozed snails than in the controls ($P < 0.05$), the ratio did not show any significant variation (Table 26A).

Among the experimentals when the ratio was compared, it was significantly higher at 24 and 48 hr post-exposure in 0.010 ppm dosed ones than in 0.015 ppm dosed ones ($P < 0.05$ and $P < 0.01$, respectively), at 6, 12 and 24 hr post-exposure in 0.010 ppm than in 0.020 ppm dosed ones ($P < 0.001$, $P < 0.01$, and $P < 0.01$ respectively); and at 12 hr post-exposure in 0.015 ppm than in 0.020 ppm dosed ones ($P < 0.05$).

5.3.1.5.2 Lymnaea acuminata f. rufescens

Among size groups, the ratio was significantly higher in the largest size group than in the smallest size group ($P < 0.001$) (Table 25B)

The ratio did not show any significant variation between the control and the experimentals at any time period (Table 26B).

Among the experimentals, the ratio in 0.015 ppm dosed ones was significantly higher at 24 hr than in 0.010 ppm dosed ones

Table 25A. GOT:GPT ratio in the three size groups of Indoplanorbis exustus

Size group	7±1 mm	11±1 mm	15±1 mm
N	12	12	12
Mean value	3.323	5.854	5.291
± SD	0.551	1.860	4.550
Range	2.612-4.731	4.110-10.840	0.840-16.752

Table 25B. GOT:GPT ratio in the three size groups of Lymnaea acuminata f. rufescens

Size group	13±1 mm	18±1 mm	21±1 mm
N	12	12	6
Mean value	2.410	3.233	4.282
± SD	0.502	2.039	0.831
Range	1.440-3.220	1.142-5.333	3.241-5.401

Table 26A. GOT:GPT ratio in Indoplanorbis exustus dosed with three concentrations of copper (size group 11±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	4.488	5.250	4.118	4.110	4.570
Control	± SD	1.133	2.167	1.219	1.707	1.726
	Range	3.640-6.472	3.192-8.881	3.492-5.800	2.902-7.111	2.920-7.512
	N	8	8	8	8	8
0.010 ppm	Mean value	4.775	5.365	4.216	5.065	4.145
Cu^{2+}	± SD	0.600	0.559	0.614	1.269	0.491
dosed	Range	4.130-5.512	4.710-6.340	3.251-5.192	3.041-6.741	3.732-4.911
	N	8	8	8	8	8
0.015 ppm	Mean value	4.326	3.176*	4.077	3.823	3.515
Cu^{2+}	± SD	1.014	0.567	0.800	0.418	0.238
dosed	Range	3.260-5.951	2.210-3.961	2.820-5.320	3.341-4.350	3.153-3.902
	N	8	8	8	8	8
0.020 ppm	Mean value	4.242	3.153*	3.073	3.287	3.990
Cu^{2+}	± SD	0.801	0.199	0.632	0.722	0.554
dosed	Range	3.160-5.612	2.862-3.444	2.382-4.011	2.632-4.432	3.261-4.882

Significance Level : * $P < 0.05$

Table 26B. GOT:GPT ratio in Lymnaea acuminata dosed with three concentrations of copper (size groups 18±1 mm)

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	3.722	4.054	3.924	3.832	3.628
Control	± SD	3.240	3.553	3.032	2.436	2.196
	Range	1.941-9.530	1.731-10.300	1.890-9.230	1.731-7.990	2.172-7.522
	N	8	8	8	8	8
0.010 ppm of	Mean value	4.028	6.086	3.981	3.200	5.435
Cu^{2+}	± SD	1.989	1.739	1.041	0.565	2.926
dosed	Range	1.740-6.470	3.821-8.691	2.482-5.430	2.502-4.331	2.981-12.251
	N	8	8	8	8	8
0.015 ppm of	Mean value	2.553	4.550	2.866	4.566	5.096
Cu^{2+}	± SD	0.533	0.936	1.017	1.445	1.186
dosed	Range	2.050-3.521	2.782-6.031	2.010-4.640	2.432-5.962	3.972-6.000
	N	8	8	8	8	8
0.020 ppm of	Mean value	5.481	2.975	2.020	6.095	2.992
Cu^{2+}	± SD	1.828	1.576	0.425	4.537	0.499
dosed	Range	4.350-9.630	1.300-5.420	1.280-2.680	2.491-14.880	2.331-3.652

($\underline{P} < 0.05$), at 6, 12 and 48 hr post-exposure in 0.010 ppm dosed ones than in 0.020 ppm ($\underline{P} < 0.01$, $\underline{P} < 0.001$ and $\underline{P} < 0.05$ respectively) and at 6 and 48 hr post-exposure significantly higher in 0.015 ppm dosed ones than in 0.020 ppm dosed ones ($\underline{P} < 0.001$), but at 2 hr post-exposure significantly lower ($\underline{P} < 0.001$).

5.4 DISCUSSION

The activity patterns of GOT and GPT in the two snail species were entirely different. Generally, the activities of both the enzymes were found elevated in the larger size group in L. acuminata f. rufescens, while in I. exustus the GOT activity level was maximum in the intermediate size group, and GPT in the small size group. This difference in pattern can be explained as species difference. Hammen (1968) reported that the levels of GOT and GPT activity in the tissues vary with the size of the animal. Bishop et al. (1983) have also reported that other factors affecting the levels of these transferases in tissues are difference in species and/or other factors related to season, food, size of the animal, and assay procedure.

In both the species, studied, the GOT activity was found to be higher than GPT activity in all the three size groups. Similar results were reported by Sollock et al. (1979),and Swami & Reddy(1978) in the hepatopancreas of some gastropods. Since there is higher GOT activity than GPT activity, and as aspartate and glutamic acids seem to be abundant in snails (Senft, 1967), it is possible that reactions involving oxaloacetate seem to gain more importance than others involving pyruvate. This change from pyruvate-oriented to oxaloacetate-oriented metabolisms might have something to do with glycogen reserves. It is possible as suggested by Manohar et al. (1972) that the aminotransferase activity pattern is more a reflection of the situation inside the cell.

I. exustus dosed with three sublethal concentrations of copper showed significant increase in GOT activity at 0.010 ppm till 24 hr post-exposure than the controls. The maximum activity was found at 12 hr post-exposure, then the activity gradually decreased to normal level at 24 and 48 hr post-exposure. Similarly, the GPT activity also showed significant increase at 12 hr post-exposure, and then gradually normalised at 24 and 48 hr post-exposure. Snails dosed with 0.015 ppm of Cu^{++} showed decreased activity levels of both transferases. At 24 hr post-exposure, the activity levels of GOT and GPT were significantly lower. At 0.020 ppm of Cu^{++} exposure initial decrease was followed by significant elevation in GOT activity at 48 hr, and significant elevation in GPT activity was also noticed at later time periods.

In L. acuminata f. rufescens, Cu^{++} did not produce significant change in activity pattern of GPT (except at 6 hr in 0.010 ppm), while significant changes occurred in the activity pattern of GOT. At 0.010 ppm and 0.020 ppm the initial decrease was compensated later, and in 0.010 ppm the activity reached significantly higher level at 48 hr. At 0.015 ppm the GOT activity, in general, was found to be high, and significantly higher at 2 and 48 hr post-exposure.

It has been suggested that stress in general increases the activities of aminotransferases, and it is very likely that copper stress could be responsible for the elevation of activity levels of transferases as observed in the present study. The increase in

both GOT and GPT may be due to stress induced metabolic shift which results in gluconeogenesis. Metabolic shift during heavy metal exposure has been well documented. Since many aminoacids such as alanine, aspartate, glutamate etc. serve as gluconeogenic precursors, and mostly the free amino acid nitrogen in the invertebrates is distributed in few aminoacids like alanine, aspartate and glutamate which form a link with the citric acid cycle (See Manohar and Rao, 1977), it is highly probable that gluconegenesis might be operating in copper-stressed snails. Alanine and aspartate are the two major glucogenic aminoacids which through the activities of the enzymes GOT and GPT give rise to glucose precursors (Lehninger, 1979). The higher level of GOT activity may be due to increase in aspartate formation as suggested by Malhotra et al. (1986). Yet another reason for the elevation in GOT level may be due to cellular degradation by copper; particularly the hepatopancreas. The increase in GPT activity also might be due to cellular degradation because transaminases are both cytoplasmic and mitochondrial enzymes; or due to pyruvate availability as indicated by Chetty et al. (1980). Catabolism of alanine in most gastropods involves transamination to pyruvate (Livingstone and de Zwaan, 1983). Transamination is of particular importance under conditions that impose a heavy drain on the animal's store of metabolites (Goddard and Martin, 1966). Transamination not only serves as a pathway of conversion of α-keto acids to L-amino acids but also as an alternative means of

replenishing the pyruvate pool. Feng et al. (1970) have suggested the possibility of oxaloacetate, resulting from transamination, being converted to pyruvate. However, the predominance of GOT activity over GPT activity, indicates a shift towards oxaloacetate oriented metabolism, and this may not be unusual since Senft (1967) has reported the abundance of aspartate and glutamate or their amines in snails.

At certain time-periods decrease in transferases activities were noticed. This decrease may be due to competition by glutamate dehydrogenase in the presence of ammonia for NADH and α-ketoglutarate (D'Apollonia and Anderson, 1980). Although Chow and Pond (1972) attributed this to the damage caused to mitochondrial membrane, loss of matrix, and swelling of mitochondrion, it does not seem that the observed decrease in activity can entirely be attributed to the explanation given by Chow and Pond (1972), since the activity levels were normalized at later time periods. It could be more due to lesser availability of precursors due to the heavy utilization of amino acids to counter the stress as indicated earlier by Chow and Pond (1972). It could also be due to inhibition of proteolytic enzymes by the toxicant because it leads to depletion of glucogenic amino acids; i.e., aspartate and alanine, among others. This inhibition reaction, however, is reversible (Hilmy et al., 1981), and this might be the reason for the normalization of the activity levels at later time periods.

C H A P T E R - VI

SUMMARY

The problem investigated is on the haematological aspects of two freshwater pulmonate snails, Indoplanorbis exustus (Deshayes), and Lymnaea acuminata f. rufescens (Gray). An important aspect of the present investigation is to emphasize the utilization of freshwater organisms as models for research directed at understanding the basic biomedical problems that remain unresolved. Another aspect is to demonstrate how haemolymph can be treated as a tissue because of late, it has been shown that several parameters of blood can be taken as reliable indicators for diagnostic purposes, and also to monitor environmental pollution. The various haematological parameters studied are total haemocyte number, packed cell volume, haemoglobin (in I. exustus), and inorganic as well organic constituents in three size groups of both the snail species. The effect of copper toxicity was measured in terms of total haemocyte count, and the activity pattern of selected phosphatases and transaminases.

The thesis is divided into six chapters. The first chapter is the general introduction, the second chapter is on haemocytes, and the third one deals with the inorganic and organic constituents in the haemolymph of three size groups of both the snail species. The fourth chapter deals with variations in the haemolymph acid and alkaline phosphatases activities in snails of the three size

groups, as well as subsequent to challenge with copper ions. Variations in the activities of haemolymph glutamate-oxaloacetate, and glutamate pyruvate transaminases in snails of the three size groups, and the activity pattern of haemolymph transaminases under copper stress are outlined in the fifth chapter. Summary of the work forms the sixth chapter, followed by the list of references.

The first chapter, the general introduction, includes the significance of the work, the present status of haematological studies on invertebrates, and molluscs in particular, and the importance of haematological studies in monitoring pollution.

Studies on total haemocyte number in the three size groups of the two species of snails, packed cell volume, and haemoglobin levels in I. exustus are the subject matter of the second chapter. The effect of selected abiotic as well as biotic factors - temperature, pH, copper toxicity, and snail-conditioned water - on the total haemocyte counts at different time periods in the intermediate size groups snails was also investigated. Size group/age dependent increase in haemocyte number was observed in both the snail species studied, while packed cell volume did not show any significant difference, as it was found to be below the detectable level of 1%. Haemoglobin concentration in I. exustus was significantly higher in the intermediate size group but in the large size group also the level was high. The results on total haemocyte counts in different size groups, and the effects

of abiotic as well as biotic factors on total counts revealed the following: (a) shell size/age is a factor which influences the number of haemocytes in the two species studied, (b) abiotic factors such as temperature, and pH affect total cell counts to a certain extent in both the snail species, (c) snail-conditioned water does not influence the total count, and (d) copper ions induce leukocytosis in both the species of snails.

In the third chapter, observations on the inorganic as well as organic constituents of haemolymph in the three size groups of snails are reported. The various inorganic constituents studied are haemolymph sodium, potassium, calcium, chloride, and ammonia, while the organic constituents are urea, total carbohydrate, glycogen, total protein, and total lipids. The results showed an almost size dependent variation in constituents except in the case of chloride and ammonia in both the species. No significant variations in the concentrations of chloride and ammonia were noticed in both the snail species. The concentrations of urea, total carbohydrate, glycogen, and protein were found to increase with increase in size/age while those of sodium, potassium, and lipid were found decreased. The results are discussed in this chapter, and it is concluded that the concentrations of organic and inorganic constituents present in the haemolymph are dependent on the size/age of the snails.

In the fourth chapter, the activity patterns of acid and alkaline phosphatases inthe three size groups, as well as the effect of copper on the activity patterns of the two phosphatases in the haemolymph of the intermediate size group of both the snail species were reported. Based on the LC_{50} value, the haemolymph acid and alkaline phosphatase activity patterns in snails exposed to three sublethal concentrations of copper, 0.010, 0.015 and 0.020 ppm, were studied at 2, 6, 12, 24 and 48 hr post-exposure. Among the size groups, no significant change in acid phosphatase activity was observed in the case of I. exustus, while in L.acuminata f. rufescens the activity pattern was generally found to be high in the largest size group studied. In the case of alkaline phosphatase, a general trend of decreased activity pattern was observed in I. exustus, while a general trend of increased enzyme activity was observed in L. acuminata f. rufescens. In copper dosed I. exustus both alkaline and acid phosphatase activity levels were found elevated in the highest concentrations studied. In the case of L. acuminata f. rufescens the ACP activity was found to be elevated than the controls at almost all time periods while ALP activity showed significant variations at lower concentrations. The results on the phosphatase activity indicated the followings, (i) the activity patterns of acid and alkaline phosphatases vary from species to species, and age/size is a factor which to a certain extent influences the enzyme activity, (ii) copper ions can cause hypersynthesis of lysosomal enzymes, (iii) copper ions to a certain

extent can inhibit the activity of phosphatases, (iv) copper ions can cause variations in total haemocyte count and (v) hypersynthesis of alkaline and acid phosphatases need not take place simultaneously.

Studies on the haemolymph transaminase activity in the three size groups of both the snail species, and under copper toxicity are the subject matter of the fifth chapter. The haemolymph glutamate-oxaloacetate transaminase activity was found to be higher than glutamate-pyruvate transaminase activity in both the snail species studied. Among the different size groups, both GOT and GPT activities were found generally decreased in the largest size group in I. exustus, while in L. acuminata increased activity towards the largest size group was observed. In copper dosed I. exustus the GOT activity in 0.010 ppm copper dosed snails was found to be high at early time periods till 24 hr post-exposure and at 48 hr the activity was normalised. In 0.015 ppm copper dosed snails the GOT activity was below the control values at 6, 12, 24 and 48 hr post-exposure. In 0.020 ppm copper dosed snails the GOT activity declined at early time periods but tends to increase towards 48 hr post-exposure. In L. acuminata f. rufescens the GOT activity in 0.010 ppm dosed ones showed a general trend of decreased activity at early time periods and after 6 hr post-exposure increased activity was observed. In 0.015 ppm dosed snails a general trend of increased activity at all time periods was observed, while in 0.020 ppm dosed ones at 6 hr post-exposure a significant decrease in the activity was observed. After the

initial significant decrease the activity was normalised. The GPT activity was found to be very high in 0.020 ppm copper dosed I. exustus at later time periods, while at 0.015 ppm the enzyme activity was normal except at 24 hr post-exposure where the activity was significantly lower. In 0.010 ppm copper dosed snails a highly significant increased activity was observed at 12 hr post-exposure. In the case of L. acuminata f. rufescens, the GPT activity was almost normal at all time periods except at 6 hr in the lowest concentration. The results showed that (i) the GOT activity is always higher than the GPT in both the snail species, (ii) age/size is a factor which influences the activity pattern of both transaminases, and (iii) activity patterns of transaminases, to a certain extent are stress indicators.

It is concluded that enzyme activity levels can be taken as reliable indicators to monitor pollution. Age is a factor that determines several of the physiological, biochemical and metabolic activities since there are variations in several haemolymph parameters. This study also indicates that haemolymph can be taken as an organ system to study the various changes taking place at organ systems levels.

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