STUDIES ON HAEMOLYMPH CONSTITUENTS OF *PILA VIRENS* (LAMARCK) AND THE EFFECTS OF PESTICIDES ON THE ACTIVITY PATTERN OF SELECTED ENZYMES

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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. REJU, M.K., 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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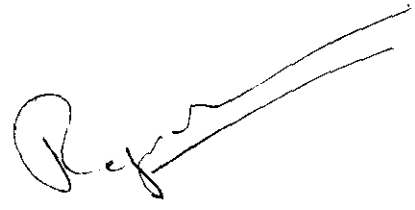
DECLARATION

I, Reju, M.K., do hereby declare that this thesis entitled "STUDIES ON HAEMOLYMPH CONSTITUENTS OF PILA VIRENS (LAMARCK) AND THE EFFECTS OF PESTICIDES ON THE ACTIVITY PATTERN OF SELECTED ENZYMES" 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

Research on gastropods has been initiated because of their important roles as agricultural pests, as intermediate hosts of a variety of parasitic worms including schistosomes, and also for academic reasons. Many of the larger aquatic snails like Pila provide cheaper source of protein-rich food for human consumption. Besides, some of the aquatic phytophagous snails may even be of value in controlling aquatic vegetation that often 'chocks' canals. Indepth knowledge on physiological, ecological, and eco-physiological aspects of these snails is vital in (i) protecting edible snails from various hazards in the environment, including man-made chemicals, (ii) appropriately using them for biological control of unwanted weeds, and (iii) to effectively control or even eradicate snails that are a health hazard to man, and the domestic animals.

Pila virens (Lamarck), the common freshwater prosobranch found in paddy fields, ponds, and streams of south India is selected as the test animal for detailed investigation. Besides their abundance in freshwater habitats, Pila is very important in maintaining normal community relations of freshwater ecosystems. They are of much use to human kind in that they (i) provide cheap protein-rich food for consumption, (ii) function as very effective biological control agents in controlling weeds like Salvinia (Thomas, 1976) which is still a serious threat to the normal

agricultural activities in many parts of Kerala, especially to paddy cultivation, (iii) being the dominant group of freshwater populations, play very important role in maintaining the community structure, (iv) are used for varied medicinal purposes (eg. for sore eyes) by local people in different parts of south India, and (v) are of paramount importance in studying the various physiological adaptations exhibited during adverse environmental conditions such as prolonged unavailability of food and longer periods of desiccation and during altered man-made environmental conditions. Under certain unknown circumstances, it is reported that Pila can play host to echinostome and amphistome cercariae (Rao et al., 1971), but under normal circumstances it is a poor intermediate host.

The freshwater prosobranchs are diverse group which present the ecologists with a rich array of material for research as these groups show interesting adaptations to the freshwater environment. Many species constitute the dominant members of their communities and are thus very important ecologically. Because all freshwater prosobranchs are derived from aquatic stock, many of their physiological adaptations differ markedly form those seen in pulmonates, which are derived from terrestrial stock (Aldridge, 1983). In line with the various diverse adaptations exhibited by amphibious prosobranchs, Pila also has evolved various

physiological adaptive measures to cope up with the environmental changes. In the present study, the physiological responses in P. virens in terms of haematological parameters are analysed under different conditions. Total haemocyte number, packed cell volume, total carbohydrate, glycogen, total protein, total lipid, urea, ammonia, sodium, potassium, calcium, and chloride are the haemolymph constituents studied in the present work. Besides these organic and inorganic constituents and formed elements in haemolymph, activity patterns of selected enzymes such as acid phosphatase, alkaline phosphatase, glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase are also analysed. All these parameters are analysed in three size/age groups of active snails, and the changes in haemolymph during imposed starvation, and aestivation are also studied. Pila, being an inhabitant of paddy fields, is continuously exposed to different types of pesticides used in agricultural operations. The effect of selected pesticides on the haemolymph enzymes activity levels, and total haemocyte number are also investigated.

Circulatory system of gastropods, being essentially open, immediately reflects changes in the external environment as well as physiological responses of the organism to these changes, in their haemolymph. Intraindividual variations in metabolic levels are possible but generally, the haemolymph metabolite concentration is taken as a direct indication of the physiological condition

or status of the organism. Very little work has been done on the various aspects of metabolic relations in normal and starved prosobranchs. Nitrogenous products such as urea and ammonia have significant roles in varying physiological and ecological conditions of the organism. As stated by Thompson et al. (1978), "Haemolymph functions as a 'sink' into which the tissues deposit ammonia and probably other metabolic end products". Besides the waste products, urea and ammonia have very important roles to play in haemolymph, especially during altered environmental and physiological conditions. The problem of ionic regulation and water balance confronted by freshwater molluscs can be better understood by studying the haemolymph ionic compositions and variations during environmental changes. Prosobranchs, being basically the descendants of aquatic ancestors (Aldridge, 1983), the problem of ionic regulation is more severe than in pulmonates, their immediate relatives. Many of these ions play crucial roles in neuromuscular functions and those related with shell synthesis and repair. The overall turnover of ions is also reflected immediately in the haemolymph of snails. One of the aspects of the present study is to determine the levels of haemolymph constituents in P. virens during active feeding, starvation, and induced aestivation. The stability and homoeostatic capacity of the circulatory system are also tested. Variations in the constituents during different growth periods or in different age/size were also analysed. Similar work in bivalves by Widdows

and Bayne, (1971); Bayne (1973), Thompson (1977); and Thompson et al. (1978) has demonstrated that seasonal changes, reproductive cycle, short term changes in the environment such as temperature, food availability and exposure to air, etc. affect the haemolymph constituents considerably.

Tissues of all species of animals contain enzymes. Alterations in enzyme levels due to any stress are immediately reflected in the functional responses of the organism. Phosphatases and transaminases are two important enzyme systems which have been subjected to considerable investigations. Alkaline phosphatase and acid phosphatase are two phophomonoesterases that catalyse transphosphorylation and hydrolysis of numerous orthophosphate esters. Transaminases are a group of enzymes that catalyse the process of biological transaminations and thus form a major link between protein and carbohydrate metabolism (Lehninger, 1979). Of the many transaminases, the most important and widely investigated ones are glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase. Molluscs generally 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 haemocytes or leak from intracellular confines of the damaged tissues, and hence serum enzyme levels are of diagnostic value (Jyothirmayi and Rao, 1987).

Gastropod haemocytes have been implicated to perform a variety of functions such as tissue repair, phagocytosis, encapsulation, digestion and distribution of food materials to organs, shell repair and regeneration, transportation of calcium, excretion of wastes etc. However, the main functions of gastropod haemocytes are correlated with their role in internal defence mechanisms (~~see~~ vide Sminia, 1981). Various changes in the number as well as cytochemistry of molluscan haemocytes are reported from time to time in response to many environmental stimuli, both biotic and abiotic (~~see~~ vide Suresh and Mohandas, 1990a,b). However, the changes in haemocytes characters such as number, cellular inclusions, enzyme content ~~etc.~~, during different environment induced changes like starvation and aestivation in gastropods remain hitherto uninvestigated. One section of the thesis illustrates the changes in the haemocyte characters consequent to the physiological changes induced by environment.

As mentioned earlier, seasonal changes, reproductive cycle, short term changes in the environment such as temperature, food availability and exposure to air ~~etc~~. were found to affect concentration of haemolymph constituents in bivalves. The second chapter of the thesis deals with the concentration of selected haemolymph constituents (both organic and inorganic), total number of circulating haemocytes, and activity pattern of selected haemolymph enzymes in three size/age groups of active P. virens.

The variations in these haematological parameters in the three age group snails, and the possible reasons for these changes are investigated and reported in this chapter.

Organisms are at the mercy of their environment for food. This is especially true of aquatic organisms which depend on plant material for their nourishment. Seasonal variations have been reported to affect the productivity of freshwater ecosystems, and the filter-feeding organisms, especially bivalves, are usually exposed to the danger of partial or complete deprivation of food material, i.e. starvation. The problem of deprivation of food is equally applicable to prosobranchs, but with reduced danger because of their ability to move from place to place in search of food. Even if there is scarcity of food, the freshwater prosobranchs, for example, Pila were reported to lead active normal life for weeks together in presence of water, albeit their capacity to aestivate (Haniffa, 1987). However, for prolonged active life without dietary intake, largescale physiological adaptations, especially metabolic adjustments, are required. Since haemolymph is the internal medium with which all the tissues of the organism communicate, even slightest changes taking place in tissues are reflected immediately in the haemolymph, as changes in haematological parameters. Chapter three of the thesis deals with the investigations on the haematological changes and the possible reasons for these changes in 10, 20 and 30 day starved P. virens.

The phenomenon of aestivation in P. virens is well known. Meenakshi (1955, 1956), Shylajakumari (1975) and Shylaja and Alexander (1974, 1975a, 1975b) have reported various physiological aspects of aestivation in P. virens. However, many aspects of aestivation, especially those pertaining to haematological changes during this period of dormancy, are still unknown. Since haemolymph acts as a 'sink' to which the tissues deposit their metabolic end products (Thompson, 1977) the composition of haemolymph gives a clear picture of the physiological mechanisms adopted by the organisms during the period of prolonged dormancy. Changes appearing in the concentration of selected haemolymph constituents, total haemocyte number, and activity pattern of selected enzymes are presented in the fourth chapter. Comparison of these changes in the haemolymph characters with those of active snails gives an insight into various metabolic and other physiologic adjustments devised by P. virens during one, two and three months of aestivation.

P. virens, the test animal of the present study, was the dominant group of organisms of freshwater population in the paddy fields of Kerala until recent past. At present the number of these snails was found to dwindle considerably in natural populations. Many reasons are suggested for the decrease of which the indiscriminate use of pesticides stands foremost, because pesticides are routinely used in paddy fields. Pesticides can adversely affect

population of organisms by being directly toxic to aquatic species or by contaminating edible organisms such as fishes and molluscs. Synthetic pesticides of varying formulations are likely to continue to be used against a variety of pests and the recent trend indicates that widespread use of organochloride pesticides will give way to organophosphate pesticides. A variety of newer chemical types with different and unique modes of action will probably become available. Current pest control methods tend to involve use of several different chemicals mixed in varying proportions (Nimmo, 1985).

Most toxic substances exert their effects on basic level in the organism by reacting with enzymes or by affecting membrane, and other functional compartments of the cell, especially lysosomes (Patel and Patel, 1985). Biochemical and physiological techniques are commonly used in laboratories to measure such effects in tissues but such attempts if combined with haematological studies can contribute most effectively to reveal the toxic mechanisms of a single group of substances (Bengtsson, 1979). The impact of pollution on an organism is initiated as disturbances at the subcellular and cellular levels (Moore, 1985). Since lysosomes are the subcellular units involved in the concentration, disintegration, and elimination of toxicants, a knowledge on the concentration of important lysosomal marker enzymes is inevitable in monitoring the extent of pollution caused by biotic and abiotic factors. Cell membranes and the confluent endoplasmic reticulum

are the first to confront pollutants. They are susceptible to the effects of pollutants as they bind to the lipoprotein layer of the membrane and induce variations in the permeability which upsets the whole cellular system. So, study of the activity of membrane bound enzymes become a useful index of the extent of pollution imposed. Investigation on the impact of pesticides on the activity of two monophospho-esterases, acid phosphatase, a lysosomal marker enzyme (see Cheng & Rodrick, 1975), and alkaline phosphatase, an enzyme bound to the cell membrane and endoplasmic reticulum (Ciro et al., 1975), is thought to be meaningful. Being effective stressors, pesticides like other pollutants are expected to disturb normal metabolic activities of the exposed organisms. Hence, activity levels of selected transaminases, which are effective indices of metabolic changes in molluscs (Cheng et al., 1980), are also studied.

Organophosphate pesticides, due to their lesser persistence level in environment when compared with organochlorine pesticides, are now gradually replacing more persistent organochlorine pesticides in use. Effects of sublethal concentrations of Dimecron^{R} (a widely used organophosphate pesticide in the paddy fields of Kerala) on the activity pattern of acid phosphatase, alkaline phosphatase, glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase are reported in the fifth chapter. Besides analysing the defence response and metabolic changes of the snails

to various sublethal concentrations of the pesticides, their behavioural responses which have direct effect on the population structure also are analysed.

Eventhough most of the widely used organochlorine pesticides such as DDT are banned in India, (Gupta & Sukumaran, 1985) at present, many formulations like Endocel 35 EC find their way in large quantities to paddy fields of Kerala. They being effective controlling agents even in small amounts, are usually preferred by farmers to other pesticides. The toxicity studies of Endocel 35 EC form the subject matter of the sixth chapter of the thesis. In the present study, the LC_{50} value of the pesticide is determined, and the effects on the activity pattern of selected enzymes and on total haemocyte counts are analysed. The results were compared with those of normal, active (control) snails and the changes were analysed in terms of change in defence response as well as in metabolism to cope up with the stress caused by the toxicant.

Studies on the sublethal effects of pesticides have gained considerable momentum in the last decade, partly because of their practical importance and partly due to academic interests. Moreover, sublethal effects of pollutants are now being recognized by regulatory agencies in formulating pollution control measures. The present investigation on the haematological aspects of P. virens especially with respect to changes in the environment (both natural and man-made) is expected to open up further detailed

research in the field of molluscan haematology and toxicology especially on pesticide induced pollution aspects in molluscan physiology.

C H A P T E R - II

HAEMOLYMPH CONSTITUENTS IN THE THREE SIZE GROUPS OF *PILA VIRENS*

2.1 INTRODUCTION

Gastropod blood cells are often called haemocytes and the other names used are leucocytes, amoebocytes, granulocytes, lymphocytes, macrophages etc. (Wagge, 1955; Cheng et al., 1970; Davies and Partridge, 1972; Cheng and Auld, 1977 ; Sminia, 1983). The morphology and functions of the cells present in the haemolymph of gastropod molluscs have been studied by many investigators (Tripp, 1970; Sminia, 1972; Sminia et al., 1973, 1974; Yoshino, 1976; Cheng and Garrabrant, 1977). Still, no agreement exists regarding the types of blood cells in gastropods. A number of investigators are of opinion that gastropods have two distinct types of blood cells, granulocytes and hyalinocytes (Harris, 1975; Yoshino, 1976; Cheng and Auld, 1977), while according to Sminia (1972) there exists only one type of blood cell in gastropods, the amoebocytes.

The number of cells per ml of haemolymph in gastropods varies from species to species and even within a species it shows large variation. The number of blood cells per ml is reported to be $3.8 - 7.2 \times 10^6$ in *Bullia laevissima* (Brown and Brown, 1965); 0.2×10^6 in *Helix pomatia* (Bayne, 1974); $0.5 - 4 \times 10^6$ in *Lymnaea stagnalis* (Sminia, 1972), and $0.1 - 1 \times 10^6$ in *Biomphalaria glabrata* (Jeong and Heyneman, 1976; Cheng and Auld, 1977; Stumpf and Gilbertson, 1978). Mohandas (1985) and Dikkeboom et al. (1985)

proposed the occurrence of different subpopulations of haemocytes with distinct enzyme content and ultrastructural peculiarities in molluscs. Dikkeboom et al. (1985) employed monoclonal antibodies to detect different subpopulations of haemocytes antigenically in juvenile and adult L. stagnalis. The total number of haemocytes in gastropods is highly variable. The various factors contributing to the intraspecific variability in the total haemocyte count are infection, snail size, age, host strain difference, temperature, wounding, oxygen tension, induced stress etc. Number of circulating haemocytes in B. glabrata (Stumpf and Gilbertson, 1978), and L. stagnalis (Dikkeboom et al., 1984) seems to be related to the age of the animal. In L. stagnalis, the density of circulating amoebocytes in the haemolymph of juvenile snails was found to be 3 to 4 times lower than that of adult snails (Dikkeboom et al., 1984). Michelson and Dubois (1975) also reported that size and strain of the snail influence the total haemocyte count. However, Feng (1965a) found no relation between the haemocyte number and size of the oyster, Crassostrea virginica. Besides the variations in total haemocyte number in juvenile and adult L. stagnalis, Dikkeboom et al. (1984) reported morphological differences also. The cells of juveniles were found to be small, round with few inclusions, high nucleus to cytoplasm ratio, low peroxidase activity, and the activity of the internal defence system was also in lower level than in the adults.

Temperature dependent variation in haemocyte number was reported by Davies and Partridge (1972), and Stumpf and Gilbertson (1978).

In B. glabrata, the number of circulating cells increased rapidly when the temperature was raised. In Patella vulgata, the haemocyte number varied from 1 x 10^6 cells per ml at 5^oC to 9 x 10^6 cells per ml at 25^oC (Davies and Partridge, 1972).

Introduction of foreign particles into the body of molluscs was found to decrease the number of total circulating haemocytes. In H. pomatia, the number of circulating cells was found to decrease after injection of foreign particles (Bayne, 1974). In B. laevissima, injection of foreign particles lead to a decrease in haemocyte number, and after the initial decrease, the number increased within 7 days upto about 2 to 5 times the initial number (Brown and Brown, 1965). Sminia (1972) reported an increase in haemocyte number within one hour in L. stagnalis after haemolymph withdrawal. The haemocyte number in B. glabrata was determined after the snails were kept for 24 hr in snail-conditioned water, and under immobilized and anaerobic conditions (Wolmarans and Yssel, 1988). In anaerobic condition, the haemocyte number increased significantly after 2 hr. Infection was reported to be yet another factor influencing the total number of haemocytes. During infection by parasites and bacterium the total number of haemocytes in gastropods was found to be increased (Michelson and Dubois, 1975; Stumpf and Gilbertson, 1980).

Gastropod molluscs possess a very effective immune system which can dispose a variety of foreign particles and organisms. Phagocytosis and encapsulation are the two major cellular processes involved in

the internal defence of gastropods (Tripp, 1961; Bayne, 1980; Renwrantz et al., 1981; Vander Knaap et al., 1981; Sminia, 1981).

Studies by Foley and Cheng (1974) on haematological parameters of Mercenaria mercenaria indicated a low percentage of Packed Cell Volume. The mean packed cell volume (PCV) in M. mercenaria was found to be 0.46% and 0.34% from specimens of two geographic regions. On comparing with higher vertebrates, the total number of cells is very low in molluscs and hence the PCVs tend to be negligible.

Generally, the energy metabolism in gastropods is carbohydrate based. (Emerson, 1967; Veldhuijzen and Van Beek, 1976). Carbohydrate is stored largely as glycogen and the specialized galactogen, and is transported and available from the blood as glucose (Livingstone and Zwaan, 1983). Glucose is the most common monosaccharide detected in the blood and tissues of many gastropods. Renwrantz et al. (1976) recorded the following concentrations of sugars in the ultrafiltrate of the haemolymph of H. pomatia: glucose, 216 µg/ml; galactose, 24 µg/ml; fructose, 8 µg/ml, and mannose, 8 µg/ml. Sugar derivatives, such as N-acetyl glucosamine and N-acetyl galactosamine, also occur but are usually incorporated into glycoproteins. The percentage of sugars in the haemolymph, free or part of macromolecules, varies among species (Livingstone and Zwaan, 1983).

In gastropod tissue, carbohydrate occurs mainly in two types of connective tissue cells : the granular cells and the vesicular connective tissue cells (Sminia, 1972). Glycogen is distributed

generally throughout the tissues with levels being high in hepatopancreas, foot and mantle. Glycogen also occurs in the muscle tissue in P. virens the levels of which were the highest in the triturative stomach muscles and the radular muscle (Suryanarayanan and Alexander, 1973). Galactogen is restricted to the albumen gland portion of the female reproductive tract of adult pulmonates and to the eggs they produce. It is synthesized solely in preparation for egg laying and is a major nutritive reserve of the embryos. It can be used as an emergency food source in starvation but only after the glycogen has been used up (Goudsmit, 1972, 1973).

Rao and Onnurappa (1979) reported the occurrence of glucose and galactose in body fluids as well as in the digestive gland of P. globosa. In L. luteola, in addition to glucose and galactose, fructose, xylose, and ribose were also detected. However, they were restricted to digestive gland and not detected in body fluid (Rao and Onnurappa, 1979). Typical concentrations of glucose (mM) in body fluids of some other gastropods are as follows: Ariolimax columbianis, 1.55 (Meenakshi and Scheer, 1968); B. glabrata, 1.07 - 1.56 (Cheng and Lee, 1971); Australorbis glabratus, 0.11 - 0.84 (Becker, 1972); Laevicaulis alte, 1.54 (Kulkarni, 1973); and B. glabrata, 0.62 (Liebsch et al., 1978). In addition to the interindividual variability in haemolymph glycogen content, intraindividual variability in a single animal also has been observed over a period of few hours. Becker (1972) noted the glucose

concentration variation of 0.06 to 0.96 mM in A. glabratus over a period of 6 hours.

Numerous factors were reported to alter the haemolymph glucose concentrations in gastropods. They include : (a) blood sampling procedures and other experimental manipulations (Subramanyam, 1973), (b) quality of food assimilated (Meenakshi and Scheer, 1968; Friedl, 1971; Scheerboom, 1978; Stanislawski and Becker, 1979), (c) quantity of food (Scheerboom, 1978). In L. stagnalis, the consumption of food itself was found to be under the control of haemolymph glucose concentrations and was found inhibited by concentrations above 120 µg/ml (Scheerboom and Doderer, 1978), (d) seasonal changes : the changes are usually in response to changes in environmental temperature and food availability and are often linked to seasonal reproductive cycles. Carbohydrate levels are generally highest in summer and autumn and lowest in winter (Marques and Pereira, 1970; Chatterjee and Ghose, 1973). The accumulation of carbohydrates generally parallels increasing food availability and food intake and may be reflected in higher blood sugar levels, as in L. stagnalis (Scheerboom and van Elk, 1978), (e) aestivation, which is characterized by a drop in oxygen consumption and haemolymph reserve carbohydrate (Heeg, 1977, Swami and Reddy, 1978; Horne, 1979), (f) circadian fluctuations, for instance 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) and (g) parasitism : decreased tissue

carbohydrate levels and/or decreased blood glucose concentrations as a result of infection in molluscs have been observed in a number of cases (Cheng and Lee, 1971; Robson and Williams, 1971; Christie et al., 1974; Vaidya, 1979; Mohamed and Ishak, 1981).

A shift in metabolism from aerobic to anaerobic was suggested during the time of parasitic and other stress conditions (Brockelman and Sithithavorn, 1980). Hyperglycemia was observed in body fluids of infected L. luteola (Manohar and Rao, 1975). Accelerated production of carbohydrate from non carbohydrate sources during stress was suggested as the reason for hyperglycemia. Ishak et al. (1975) reported depletion of glycogen in infected snails and the reason suggested was the low energy efficiency of anaerobic metabolism leading to fast depletion of carbohydrate store. As in higher organisms, hormonal control of carbohydrate metabolism is well demonstrated in molluscs. In L. stagnalis, although fixed maximum blood glucose concentrations are not apparent, a minimum level is maintained in starved animals (Veldhuijzen, 1975). Insulin-like activity and homolog of pancreatic β cells in the digestive tract have been identified in several gastropods (Goddard et al., 1964; Boquist et al., 1971; Davidson et al., 1971). It has been suggested that carbohydrate utilization process with themselves be regulated by blood sugar levels through the release of dorsal body hormone (involved in the control of female reproduction) and light green cell hormone (involved in the control of body growth) from endocrine

centres in the cerebral ganglia (Scheerboom and Hemminga, 1978). Carbohydrate metabolism is also affected by the removal of growth hormone producing light green cells which resulted in an increase in polysaccharide deposition and occassional increase in blood sugar levels (Dogterom, 1980).

In vertebrates, the serum proteins play an important role in maintaining the osmolarity, transport of ions, immune mechanisms and homoeostasis. In lower forms including gastropods, the haemolymph proteins have been suggested to mediate the same functions. In fresh water gastropods, the total free aminoacid concentrations are low (4 - 10 mM) in the tissues and even lower (0.3 - 1.5 mM) in the haemolymph (Simpson et al., 1959; Gilbertson and Schmid, 1975; Reddy and Swami, 1975; Stanislawski et al., 1979) on comparing with their marine counter parts (intra cellular concentrations of 50 - 400 mM, and haemolymph concentrations of 0.2 - 5 mM) (Bishop et al., 1983). Although all the aminoacids are present in gastropod haemolymph in small amounts, alanine, glycine, glutamate, aspartate, serine, and threonine are in greatest abundance. A Little or no taurine or quarternary amines have been detected. Aspartate, glutamate, glycine, alanine and serine arise from the metabolism of a variety of gluconeogenic compounds including glycogen. They can be released form the aminoacids released during peptide and protein turnover and may accumulate if catabolism is slowed (Awapara and Campbell, 1964; Simpson and Awapara, 1965; Bishop et al., 1983). The concentra-

tion of free aminoacids in the tissues and extracellular fluid compartments of mollusc varies with the diet, season, temperature, reproduction and developmental stage, and environmental stress related to desiccation, anaerobiosis, osmotic pressure, pollution and parasitism (Bishop et al., 1983). Bedford (1973), and Hanson & Dietz (1976) among others, for instance, reported increase in osmotic pressure and blood amino acids when animals were subjected to brackish water or desiccation.

Besides free aminoacids and structural proteins, respiratory proteins form a major part of total haemolymph protein. The primary function of respiratory protein is to increase the absorption coefficient of oxygen in circulatory fluids, thus augmenting the oxygen content of the blood (Ghiretti and Ghiretti-Magaldi, 1972). A total of 34 serum protein fractions has been identified in the haemolymph of B. glabrata (Gress and Cheng, 1973). Parasitism and bacterial challenge are considered very important factors controlling total haemolymph protein in molluscs. The level of serum protein in B. glabrata was found to increase when challenged with live bacterium (Cheng et al., 1978). The increase in protein content was suggested to be due to hypersynthesis of some yet unidentified host humoral protein fraction. Haemolymph protein and free amino acid concentrations in B. glabrata were found to change due to infection (Dusanic and Lewert, 1963; Gilbertson et al., 1967; Lee and Cheng, 1972). By day 70 post-exposure to the parasite

Schistosoma mansoni, the total protein content had declined to one third of that in uninfected snails (Gress and Cheng, 1973; Manohar and Rao, 1977). Starvation also is a factor contributing to haemolymph protein variability. Becker and Hirtbach (1975) reported decrease in haemolymph protein after seven days of starvation in B. glabrata.

Although knowledge on 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 their diet (Voogt, 1983). In prosobranch molluscs, gonads and digestive gland are considered as principal organs for lipid storage (Giese, 1966; Stickle, 1975). In the fresh water prosobranch Melania scabra, a reciprocal relationship was observed between the synthesis of lipid and utilization of proteins and vice versa (Muley, 1975). Composition and mechanism of transport of lipid in blood of mammals and insects are known (Sanbar, 1969; Gilbert and Chino, 1974). However, information available on the mechanism of transport and composition of lipid in haemolymph of molluscs is scanty. The most important work in this field is that of Allen (1977). According to Allen, the blood lipids of Cryptochiton stelleri are in dynamic equilibrium with the numerous lipid compartments. Structural lipids (sterols and phospholipids) comprised about 75% of the plasma lipids. Neutral lipids (triglycerides, partial glycerides and free fatty acids) constitute the form of lipid associated with energy metabolism and

are represented in the plasma of C. stelleri by alkyl diacyl glycerol, triglyceride and free fatty acids. Structural lipids predominated to an even greater extent in the blood cells making up to 95% of the total, the remaining 5% consisting of trace amounts of triglycerides and free fatty acids (Allen, 1977).

The staining and mobility properties of lipoprotein of C. stelleri were found to be similar to mammalian low density lipoprotein (Allen, 1977). However, Bevelaque et al. (1975) failed to demonstrate such a lipid transporting lipoprotein molecule in the plasma of Aplysia californica. Of further interest in this aspect are the reports on enzyme catalysing cholesterol ester hydrolysis and cholesterol esterification in the haemolymph of the snail B. glabrata (de Souza and de Oliveira, 1976), and lipase in the blood of the clam, Mya arenaria (Cheng, 1976).

Gilbert and Chino (1974) estimated the rate of entry and exit of lipid into plasma lipid compartments by in vitro experiments using either prelabelled isolated tissues and unlabelled plasma or the reverse. The ingested lipid is transported in the form of free fatty acids and triglycerides in blood. The high specific activities observed for plasma free fatty acids tempt to conclude that acyl lipid is mainly transported in the form of free fatty acids as it passes from the digestive and absorptive organs through the plasma to such tissues as gonad, mantle and foot. Blood cells are also suggested to be involved in the transport of lipids in Strongylocentrotus

purpuratus (Allen, 1974). During breeding and other specialized physiological conditions, lipids were found to be used in many prosobranchs (Chatterjee and Ghose, 1973). One of the important factors influencing haemolymph lipid level is parasitism. Live bacterium, Bacillus magaterium, stimulates elevated lipase activity in the serum of B. glabrata, indicating increased lipid hydrolysis during bacterial stress (Cheng and Yoshino, 1976a). Sex-wise analysis of haemolymph lipid constitution in molluscs (Allen, 1977) failed to yield any sex dependent variation in plasma lipid composition.

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 amount of nitrogenous degradation products produced is 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 amount of ammonia, urea and uric acid produced, which is immediately reflected in their corresponding concentration in haemolymph. Analysis of nitrogenous compounds in molluscan haemolymph is considered to be initiated by Myers (1920). Friedl (1961); Potts (1965); de Jorge et al. (1965); Vasu and Giese (1966); Little (1968); and Becker and Schmale (1975) published reports on the concentration of nitrogenous compounds in molluscan haemolymph.

Uric acid being a typical storage excretory product, is often determined in molluscs in homogenates of the whole body or in certain

organs such as hepatopancreas, kidney or foot (Becker and Schmale, 1975). Besides, Becker and Schmale (1975) reported total absence of uric acid in the haemolymph of both fed and starved gastropod snails, B. glabrata. Rao and Narayanan (1976) reported the concentration of urea and ammonia in the body fluids of four common fresh water gastropods in India, namely Indoplanorbis exustus, L. luteola, P. globosa and Viviparus bengalensis. I. exustus and L. luteola were found to differ from P. globosa and V. bengalensis in the ratio of ammonia to urea. While it was 3.6 in I. exustus, and 3.73 in L. luteola, it was 7.36 in P. globosa and 6.6 in V. bengalensis. Lymnaea and Indoplanorbis are suggested to be ureotelic while the other two as more ammoniotelic (Rao and Narayanan, 1976). Urea retension was suggested as one reason for Lymnaea and Indoplanorbis to be the preferred host for larval trematodes.

There are a few findings about the concentration of urea in the haemolymph of molluscs. de Jorge et al. (1965) reported a high value of 30.5 mg/100 ml of urea for Strophocheilus oblongus. On the other hand, Tramell and Campbell (1970) failed to detect any urea in S. oblongus. Vasu and Giese (1966) found approximately 9 mg/100 ml of urea in C. stelleri during the winter. Friedl (1961) determined 0.46 mg/100 ml of urea in L. stagnalis while Little (1968) reported 0.15 mg/100 ml of urea in amphibious prosobranch, Pomacea lineata. In B. glabrata, urea content was found to be 0.16 mg/100 ml (Becker and Schmale, 1975). B. glabrata infected with S. mansoni showed a significant increase in urea concentration in the haemolymph.

A similar effect was observed with snails starved for 5 days (Schmale and Becker, 1975). Presence of NH_4Cl (Ammonium chloride) in the external medium was found to induce highly significant increase in haemolymph NH_3-N and urea concentration of B. glabrata (Meyer and Becker, 1980).

During starvation and Schistosoma infection, an increased production of NH_3 was reported in B. glabrata. The reason suggested was increased protein degradation. The increased production of ammonia also was reflected in the level of haemolymph ammonia (Becker and Schmale, 1978). When starved Mytilus californianus begins to feed, there is a rise in the level of ammonia nitrogen in the plasma, reflecting increased protein catabolism (Thompson et al., 1978).

Hyperosmotic regulatory mechanism is employed by fresh water gastropods to keep the high osmolarity of body fluid to that of surrounding fresh water medium with low osmolarity. An osmotic gradient is continuously maintained between their hyperosmotic body fluids and tissues and the dilute, freshwater environment (from which they gain water by osmosis and to which they lose solutes by diffusion). In order to regulate body fluid and tissue concentrations at hyperosmotic concentrations, freshwater gastropods, therefore, constantly excrete excess water and actively regain lost ions against their respective osmotic gradients (Robertson, 1964; Prosser, 1973a,b; Machin, 1975). Osmolarity of freshwater prosobranch snails is much higher (74 to 113 mOsm/kg H_2O) than freshwater (1 to 10 mOsm/kg H_2O) but much lower than that of freshwater pulmonates (124 - 150 mOsm/

kg H_2O), and terrestrial pulmonates (142 - 360 mOsm/kg H_2O) (Machin, 1975). Further, prosobranchs have retained their gill and therefore, have greater relative surface areas over which water and solute exchange can occur than do gill less basommatophorans. The apparent reduction of body fluid osmolarity in freshwater prosobranchs may be an adaptation to life in freshwater that reduce ion and water exchange to controllable levels (Prosser, 1973a,b).

To keep hyperosmotic state of the haemolymph, freshwater gastropods continuously excrete water. However, the excretory fluid of freshwater pulmonates and prosobranchs are not that dilute, for they have 75% of haemolymph concentration (Robertson, 1964). Therefore, the excretory fluid represents a site of considerable solute loss which must be restored by active uptake of solutes from the environment. Various ionic regulatory mechanisms are developed by freshwater pulmonates and prosobranchs to meet the challenge of continuous flux of solutes from haemolymph through the excretory fluid. In this context, the study of composition of major haemolymph ions like Na^+, K^+, Cl^-, Ca^{2+} etc. in normal snails and those in altered physiological conditions become significant.

In marine molluscs, ionic regulations consists mainly of raising K^+ and Ca^{2+} concentrations; blood Na^+ and Cl^- remaining practically in equilibrium with the surrounding medium while in freshwater species, the concentration of all the ions including Na^+ and Cl^- appears to be regulated (Schoffeniels and Gilles, 1972). In some freshwater molluscs, the permeability to water has been estimated by measurements

of the rate of urine production. When considering the data obtained for other phyla, it can be seen that the permeability of freshwater species is lower than that of seawater animals.

Freshwater molluscs as well as terrestrial ones produce urine hypotonic to the blood (Little, 1965b, 1968, 1972). This reabsorption of ions inevitably decreases the amount of salt loss from the body. However, it compensates only part of salt lost through the urine and body wall. The loss of salt must thus be balanced in these species by an active uptake from the surrounding medium (Schoffeniels and Gilles, 1972). Active uptake of Na^+, Cl^- and Ca^{2+} by freshwater molluscs has been demonstrated. The uptake of chloride was first demonstrated in Anodonta and Lymnaea by Krogh (1939). Krogh reported the independent uptake mechanisms of sodium and chloride. Dietz (1979) also supported the idea of existence of independent uptake mechanisms for Na^{2+} and Cl^-. The chloride uptake mechanism exchanges Cl^- for bicarbonate, and sodium uptake mechanism exchanges Na^+ for hydrogen ions in freshwater gastropods (Dietz and Branton, 1979; de Wtih et al., 1980). Dietz (1978), and McCorckle and Dietz (1980) observed Na^+ and ammonia exchange in freshwater gastropods.

The haemolymph of freshwater molluscs is much more concentrated in Na^+ and Cl^- than the environmental medium. Sodium and chloride are generally the most abundant ions, while the concentrations of potassium and magnesium are very low (Schoffeniels and Gilles, 1972). The internal bicarbonate concentration can be increased and/or

decreased if necessary by appropriate changes in the relative transport rates of NaCl and CO_2. This is because, equimolar amounts of bicarbonates and hydrogen ions are eliminated to that of NaCl uptake. de With et al. (1980) reported correlation between haemolymph chloride and pH. A low internal pH appropriately stimulates exchange of sodium and hydrogen ions and inhibits exchange of chloride and bicarbonate in L. stagnalis (de With et al., 1980).

A contrasting kind of relationship is seen in the correlation between sodium and potassium levels in non marine molluscs taken collectively. In terrestrial species, there is a tendency for both ions to become diluted and concentrated together with changing body hydration, but the sodium potassium balance is altered by feeding and does not seem to be specifically regulated in the individual (Burton, 1965). Prasad et al. (1985) reported increased level of Na^+ and K^+ in the body fluids of infected L. luteola without any change in their level in the tissues studied. The reason suggested for the marked elevation are (i) tissue damage, (ii) diet, or (iii) change in total volume and water content of body fluids. However, such a marked increase in sodium and potassium in the body fluid as against the tissues will have a profound effect on the overall neuromuscular physiology of the snail (Prasad et al., 1985). Graves and Dietz (1980) reported a diurnal fluctuation in sodium influx and in the concentration of sodium and chloride in the haemolymph of Carunculina texasensis. Greenaway (1970), and Swindale and Benjamin (1976) postulated hormonal control in ion regulation in Molluscs.

Haemolymph ionic concentrations of many freshwater prosobranchs are known, and some of them are given below. Ions are expressed as millimoles per litre of blood plasma: P. globosa, Na^+ = 54.7; K^+ = 4.9 (Saxena, 1957); V. viviparus, Na^+ = 34.0; K^+ = 1.2; Cl^- = 31.0 (Little, 1965a); Pomacea depressa, Na^+ = 55.7; K^+ = 3.0; Cl^- = 52.0 (Little, 1968); Theodoxus fluviatilis, Na^+ = 45.0; K^+ = 2.2; Cl^- = 32.8 (Little, 1972). Despite its dilution, the haemolymph of prosobranchs retain some resemblance to seawater in the proportion of ions, especially in the low ratio of potassium to sodium and chloride (Burton, 1983).

In fresh waters, calcium is the major dissolved cation, the concentration ranging from 1-3 mg Ca/Kg H_2O to concentrations greater than 400 mg Ca/kg H_2O (Hunter and Lull, 1977). Freshwater gastropods maintain a continuous uptake of calcium from their environment which is sequestered as calcium carbonate, the major mineral component of the shell, against electrochemical gradient (Russel-Hunter, 1978). Vast majority of freshwater basommatophoran snails are highly calciphilic and are more successful in water of higher calcium concentrations (Young, 1975). In laboratory studies, environmental calcium concentration has been shown to have significant effect on the life history traits of freshwater gastropods. Growth rates, survivorship, and fecundity rates all decline with reduction in calcium concentration below optimal environmental values. In contrast, calcium concentrations above optimal levels for fecundity were found to increase survival rate in B. pfeifferi (Harrison et al., 1970; Thomas et al., 1974).

The calcium concentration was found to be close to equilibrium between the internal and external media of freshwater gastropods (Greenaway, 1971). When the external concentration is less than 0.5 mM, uptake is against the electrochemical gradient. Calcium is taken up by Lymnaea through the body surface (vander Borght and van Puymbroeck, 1966), as well as from ingested material (Young, 1975). According to Greenaway (1971) shell of freshwater pulmonate snails acts as calcium buffer or store from which lost haemolymph or tissue calcium can be released during periods of calcium depletion caused by short-term decrease in external Ca^{2+} concentrations such as caused by flooding. Studies of Sminia et al. (1977) demonstrated the presence of calcium cells which concentrate Ca^{2+} salts in L. stagnalis. Most important function attributed to calcium cells is the buffering of pH of body fluids besides synthesis and repair of shell and operculam (Burton, 1970; Istin and Girard, 1970). During anoxia and hypoxia, the calcium content in haemolymph was found to increase several fold. In Ligumia subrostrata, haemolymph calcium rises in anoxia as much as eight fold. In Rapana thomasiana taken out of water, the calcium content and buffer value of haemolymph rise as in bivalves (Alyakrinskaya, 1972). When P. virens are made anaerobic by being placed in boiled water, the haemolymph pH falls, for eg. to 4.5, and lactate accumulates, but neither calcium nor magnesium change in concentrations (Meenakshi, 1956). Significant increase in body fluid calcium levels with concomitant decrease in the tissues, especially mantle and digestive gland during trematode infection in L. luteola

was reported by Prasad et al. (1985). The increase in the calcium levels in the body fluids was attributed to the maintenance of pH of blood since anaerobic conditions are reported to prevail in vivo in infected snails. In Planorbis corneus, the level of calcium was found to be correlated with the level of potassium (Sorokina and Zelenskaya, 1967). Whether or not the calcium-potassium balance is regulated in other molluscs is not known. Similarly, 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 some how raises haemolymph calcium (Khan and Saleuddin, 1979).

Haemolymph concentrations of calcium ion in some prosobranch molluscs are known. Ca^{2+} ions in the following examples are expressed as millimoles per litre of blood plasma: P. globosa, 7.8 (Saxena, 1957); V. viviparus, 5.7 (Little, 1965a); P. depressa, 6.6 (Little, 1968) and T. fluviatilis, 2.3 (Little, 1972).

Several enzymes are identified in molluscan haemolymph. They include lipase in cells of the haemocytes of the oyster Ostrea edulis, C. virginica (Yonge, 1926; Takatsuki, 1934), M. mercenaria (George, 1952; Zacks and Welsh, 1953; Zacks, 1955), protease in haemocytes of O. edulis (Takatsuki, 1938); amylase, glycogenase, enzymes active on maltose, lactose, glucosidase (Takatsuki, 1934); β-glucuronidase, acid phosphatase and RNAase in M. merceneria (Janoff and Hawrylko, 1964); alkaline phosphatase in cells of gastropod H. aspersa (Wagge, 1951); lysosyme in C. virginica and M. arenaria (McDade and Tripp,

1967; Rodrick and Cheng, 1974a). Detailed work on activity pattern of acid phosphatase and alkaline phosphatase enzymes in molluscan haemolymph are reported by investigators like Eble, (1966); Cheng and Rifkin, (1970); Feng et al. (1971); and Frankboner, (1971). Read, (1962); Awapara and Campbell, (1964); Goddard and Martin, (1966) and Rodrick and Cheng (1974b) reported various works dealing with Glutamate Oxaloacetate Transaminase and Glutamate Pyruvate Transaminase enzymes in haemolymph of different molluscan species.

Enzymes by themselves are not present in the haemolymph unless they belong either to the haemocytes or leak from intracellular confines of the damaged tissue (Jyothirmayi and Rao, 1987). Saleem and Alikhan (1974) described the characters of acid and alkaline phosphatases in the haemolymph of terrestrial isopod Porcellio laevis latreille . Jyothirmayi and Rao (1988) suggested the possible origin of phosphatases in the digestive gland of L. luteola while Sminia (1972), Yoshino and Cheng (1976, 1977) and Foley and Cheng (1977) support the view that phosphatases are of haemocyte origin. Suresh and Mohandas (1990a) reported elevated levels of ACP (Acid Phosphatase) in the haemolymph of copper stressed bivalves, indicating their role in stress control. Experimentally induced elevation in acid phosphatase activity in haemolymph of B. glabrata was reported by Cheng and Butler (1979). Elevated levels of phosphatases during infection was reported by Michelson and Dubois (1973), Cheng and Yoshino (1976a,b) Cheng et al. (1977); and Cooper-Willis (1979). Jyothirmayi and Rao (1988) suggested the role of haemolymph enzyme analysis in diagnosis of

different toxicologic symptoms. Manohar and Rao (1977) expressed the relationship of GOT and GPT in term of de Ritis Quotient and found that in L. luteola, P. globosa, Planorbis sp. and V. bengalensis the activities of GOT was higher than that of GPT in the non-parasitized snails while a reverse trend was seen in the infected ones. This ëld to the variation in the de Ritis quotient.

Various functions attributed to phosphatases include (i) biosynthesis of fibrous protein (Johnson and McMinn, 1958) and mucopolysaccharides (Kroon, 1952), (ii) regulation of intracellular phosphate concentrations (Gutaman, 1959), (iii) hydrolysis of body cells, permeability process,and associated with nucleic acid synthesis (Cox et al., 1967). Acid phosphatase is associated with lysosomes and is designated as one of the marker enzymes (Edwards and James, 1987). It is a hydrolytic enzyme which takes part in the dissolution of dead cells and as such is a good indicator of stress condition in biological systems (Gupta et al., 1975; Verma et al., 1980). Under normal conditions these acid hydrolases are restricted within the lipoprotein lysosomal membrane, i.e., being in latent phase. However, if the haemocytes are challenged by a variety of abiotic or biotic factors, the lysosomes become unmasked or destabilized, i.e, the enzymes are released from lysosomes into the surrounding cytoplasm (Foley and Cheng, 1977). As a result of finding lysosome in granulocytes of different species of molluscs (Cheng, 1975; Yoshino and Cheng, 1976), and discovering that these organelles are associated with intracellular degranulation (Cheng and Cali, 1974; Cheng et al.,

1974), Cheng and Rodrick, (1975) reported that the enzyme β-glucuronidase, acid phosphatase, alkaline phosphatase, lipase, aminopeptidase, and lysozyme are associated with the cellular and serum components of C. virginica, and M. mercenaria. Earlier, Rodrick and Cheng (1974) found these enzymes in B. glabrata haemolymph.

GOT and GPT are major enzymes giving glucose precursor for gluconeogenesis (Lehninger, 1979). They form major link between protein and carbohydrate metabolism and is of high diagnostic value in physiological stress conditions (Lane and Scura, 1970). In all molluscan species investigated so far, both GOT and GPT activities have been detected in all tissues examined. The GOT level tend to be quite a bit higher than the GPT level in the hepatopancreas of some gastropods (Sollock et al., 1979; Swami and Reddy, 1978). Hammen (1968) found that the levels of activity in the tissues vary with the size of the animal. Other factors affecting the transaminase activity are difference in species, season, food, size of the animal, and assay procedures (Swami and Reddy, 1978).

In this chapter results of the investigation on various haemolymph constituents such as total haemocyte number, total carbohydrate, glycogen, total protein, lipid, ammonia, urea, sodium, potassium, calcium and chloride in the three size groups of P. virens are presented. The activity levels of haemolymph acid phosphatase, alkaline phosphatase, glutamate-oxaloacetate transaminase, and glutamate-pyruvate transaminase were also studied, and are reported in this chapter.

2.2 MATERIALS AND METHODS

2.2.1 Test Animal

The common freshwater prosobranch Pila virens (Lamarck) was selected as the test animal. Out of eight species and six varieties of the Genus Pila occurring in India, P. virens is the most common species found in south India (Rao, 1989). It is commonly found in paddy fields, slow streams, and ponds throughout the year. Besides its crucial role in freshwater ecosystem, it is regularly eaten by the poor people in many districts of Tamilnadu and Kerala. Moderate size and easy availability make it a favourable laboratory specimen for experiments.

For the present study, live specimens were collected from paddy fields of South Kalamassery, Ernakulam District, Kerala.

2.2.2 Laboratory Conditioning of Test Animals

The animals brought to the laboratory were maintained in large 50 litre capacity fibre glass tanks containing well-aerated dechlorinated tap water. They were acclimated to laboratory conditions for 48 to 96 hours, and fed with Hydrilla sp. plants (Haniffa, 1980). All animals taken for any one set of experiment belonged to the same population. Only healthy snails belonging to selected size groups were used for experiments irrespective of sex.

2.2.3 Selection of Animal Groups

To study the relative proportions of haemolymph constituents and selected enzyme (activities) in different age groups of P. virens,

the test animals were divided into three size-groups based on the shell size. The groups were (i) snails having 30 ± 2 mm shell height, (ii) 40 ± 2 mm shell height, and (iii) 50 ± 2 mm shell height. These groups corresponded to the grouping based onopercular length also i.e., 25 ± 2 mm, (ii) 32 ± 2 mm, and (iii) 40 ± 2 mm in opercular length. Selected haemolymph parameters of snails belonging to the above three size-groups were studied, and compared statistically. From each size group, for estimation of total haemocyte count, glycogen, total carbohydrate, protein, lipid, urea, ammonia, sodium, potassium, calcium and chloride twenty specimens were selected, and for enzyme analysis twelve specimens were selected.

Test animals belonging to the intermediate size group i.e., shell height 40 ± 2 mm, or opercular length 32 ± 2 mm were used for aestivation, starvation and pesticide toxicity studies. The reason for selection of intermediate group for experiments were; (i) They form the major proportion of any natural collection and (ii) Required quantity of haemolymph can be obtained from snails of intermediate size group.

2.2.4 Collection of Haemolymph

Laboratory conditioned animals were kept on filter paper for two to four hours for the removal of water and mantle fluid. An opening was made on the body whorl of the shell at the left hand top side of the operculum. The heart was identified by its rhythmic beating activity. The mucus lining the pericardium was blotted away

using a filter paper. The entire operation was done with utmost care giving minimum disturbance to the animal. Haemolymph was obtained by puncturing the heart with a needle and collected in small test tubes packed around with ice. From each snail, 1.0 to 1.5 ml of haemolymph can be collected by this method.

2.2.5 Analysis of Haemolymph Constituents

2.2.5.1 Total Haemocyte Counts

Capillary tubes rinsed in 35% formaldehyde were used for the collection of haemolymph for total haemocyte counts. Rinsing of the capillary tube with formaldehyde prevented the adherence of haemocytes to the tube and their clumping. The first few drops were discarded and the next drop was discharged on to a haemocytometer. The cells were allowed to settle for one minute. The cells in the corner four squares were counted, the number of cells per cubic millimeter was calculated by the WBC method (Coulombe, 1970), and expressed as haemocytes per mm^3.

2.2.5.2 Packed Cell Volume

The packed cell volume (PCV) was determined by Microhematocrit Method of Coulombe (1970) as described in Experiments and techniques in Parasitology (Edited by Macinnis, A.J and Voge, M., 1970). Haemolymph was collected directly into a heparinized capillary tube until it was filled to approximately one inch from the contact end. The capillary tube was rinsed with 35% formaldehyde prior to the

procedure. The dry end of the tube was sealed with sealing wax. The capillary tube was centrifuged in high speed centrifuge at 11,500 rpm for 5 minutes. Haematocrit value was measured using international circular microcapillary reader chart and is expressed as the percentage of whole blood.

2.2.5.3 Estimation of Glycogen

Glycogen present in the haemolymph was determined by Montgomery method (Montgomery, 1957). A 0.1 ml of the haemolymph sample was added to 1.0 ml of 10% Trichloroacetic acid and mixed well. It was then centrifuged at 2,500 rpm for 10 minutes. To another test tube, the supernatant was decanted carefully. From the supernatant, 1.0 ml sample was pipetted into another test tube and to this was added 1.2 ml of 95% ethyl alcohol, and mixed well. It was kept in a refrigerator for twelve hours, and then centrifuged at 2,500 rpm for 15 minutes. The supernatant was decanted carefully. 2.0 ml of distilled water and 0.1 ml of 80% phenol were added to the precipitate. 5.0 ml of concentrated sulphuric acid was added forcefully to the sample for thorough mixing and the sample was left at room temperature for 30 minutes for cooling. After cooling, the optical density was read at 490 nm. The concentration of glycogen in the sample was determined from the standard graph prepared using glucose as the standard, and expressed as µg glucose/ml.

2.2.5.4 Estimation of Total Carbohydrate

Total carbohydrate in haemolymph was determined by the method of Dubois et al. (1956). A 0.1 ml sample of the haemolymph was added

to a test tube containing 0.1 ml of 80% phenol. To this was added 1.9 ml of distilled water. After mixing, 5.0 ml of concentrated sulphuric acid was added forcefully into the test tube. The sample was mixed well and left at room temperature for 30 minutes. After cooling, the optical density was read at 490 nm. The concentration of total carbohydrate in the sample was determined from the standard graph prepared using glucose as the standard, and expressed as µg glucose/ml.

2.2.5.5 Estimation of Total Protein

The protein content of the haemolymph was estimated by the method of Lowry et al. (1951). A 0.1 ml sample of haemolymph was pipetted into a test tube containing 1.0 ml of 10% TCA. The sample was mixed well and centrifuged for 15 minutes at 2,500 rpm. The supernatant was decanted carefully and discarded. The precipitate was dissolved in 1.0 ml of 0.1 N NaOH. 0.2 ml of the sample was pipetted to another test tube and made up to 1.0 ml with distilled water. After mixing thoroughly, 5.0 ml of alkaline copper reagent was added, again mixed well and after 10 minutes, 0.5 ml of Folin's phenol reagent was added and shaken well. After 45 minutes, the optical density of the sample was read at 500 nm. From the optical density, the corresponding concentrations of protein were found out from the standard curve prepared with bovine serum albumin, and expressed as mg protein/ml of haemolymph.

2.2.5.6 Estimation of Total Lipid

The lipid content in haemolymph sample was determined by sulphophosphovanillin method described by Barnes and Blackstock (1973). A 0.1 ml sample of haemolymph was pipetted to a clean test tube. To the sample, 1 ml methanol, 2 ml chloroform, and 2 ml 2:1 solution of chloroform-methanol mixture were added in the same order. The sample was mixed well, and to this was added 0.2 ml of 0.9% NaCl solution. The whole mixture was transferred to a separating funnel and kept in a refrigerator overnight for separation of the layers. The lower layer was then separated into a test tube and placed in a vacuum desiccator over silica gel for drying. When the chloroform extract in the test tube had completely dried up, the test tubes were taken out of the vacuum desiccator, added 0.5 ml of conc. H_2SO_4 and capping with non-absorbant cotton, heated for 10 minutes in a boiling water bath. When cool, 5.0 ml of phosphovanillin reagent was added, shaken well, and after 30 minutes, the colour developed was read at 520 nm. The lipid concentration was calculated from the standard graph prepared with cholesterol standards and expressed as µg lipid/ml (as equivalents of cholesterol).

2.2.5.7 Estimation of Urea

Urea content of haemolymph was determined by Diacetyl monoxime method (Natelson, 1972). A 0.1 ml sample of haemolymph was added to 3.3 ml distilled water. To this, 0.3 ml 10% Sodium tungstate

and 0.3 ml of 2/3 N Sulphuric acid were added and mixed well. The mixture was centrifuged for 10 minutes at 3,000 rpm. 1.0 ml of the supernatant was pipetted into another test tube and to this 1.0 ml of distilled water, 0.4 ml of diacetyl monoxime and 1.6 ml of sulphuric acid-phosphoric acid mixture were added. It was then placed in a boiling water-bath for 30 minutes and later cooled. When cool, the colour developed was read at 480 nm. Standards were prepared with known concentrations of urea. The concentration of urea in the sample was calculated from the standard graph, and expressed as mg/100 ml of haemolymph.

2.2.5.8 Estimation of Ammonia

Ammonia content of haemolymph was determined as ammonia nitrogen by phenol-hypochlorite method described by Grasshoff and Johannsen (1972). 0.2 ml of haemolymph was added to 2 ml 80% ethylalcohol and centrifuged at 5,000 rpm for 5 minutes. The supernatant was transferred carefully to another test tube and made up to 5.0 ml with deionized double distilled water. 0.2 ml phenol solution and 0.2 ml nitropursside solution were added and mixed thoroughly. 1.0 ml of oxidising solution was added and mixed well. The test tube was capped tightly and placed in dark. After 2 hours, the colour developed was read at 640 nm against blank. The concentration of ammonia nitrogen was calculated from the standard graph prepared with known concentrations of ammonium chloride, and expressed as NH_3-N mg/100 ml of haemolymph.

2.2.5.9 Estimation of Sodium, Potassium and Calcium

The sodium, potassium, and calcium content of the haemolymph sample was determined by Flame photometric method of Robinson and Ovenston (1951). 2 ml of conc. Nitric acid was added to 0.2 ml of haemolymph and the precipitate formed was digested by placing the test tube in a sand bath for 15 minutes. When completely dissolved, the sample was made up to 10 ml with deionized distilled water. The made up sample was fed to the flame photometer. Blank and standards were prepared simultaneously with 0.2 ml deionized water, and 0.2 ml of sodium, potassium or calcium solutions with known concentrations of the respective salts. The concentrations of sodium, potassium and calcium were calculated from the corresponding standard graphs and expressed as micro equivalents per ml of haemolymph.

2.2.5.10 Estimation of Chloride

Chloride content of the haemolymph sample was determined using ELICO CHLORIDE METER (Model EE-34). The instrument with its associated standards and reagents gives highly accurate chloride values without the need of a calibration curve. After standardization, 0.1 ml of haemolymph was added to the titrating medium. After automatic titration, the chloride content in the sample was diplayed directly as milli equivalents of chloride per litre.

2.2.6 Activity Pattern of Selected Haemolymph Enzymes

2.2.6.1 Assay of Acid Phosphatase Activity (EC. 3.1.3.2)

Acid phosphatase activity was assayed following the methodology of Sigma Technical Bulletin (Anon, 1963), with some modifications. To 1.0 ml of 0.1 M frozen citrate buffer of pH 4.2, 0.1 ml of haemolymph was added using 0.1 ml pipette. The buffer-enzyme mixture was immediately incubated in a thermocontrolled water bath at 37 $\pm$ 0.05^{o}C. To this reaction mixture 0.1 ml of the substrate (2 mg of p-nitrophenyl phosphate sodium salt (Merck) in 0.1 ml double distilled water) was added to initiate the reaction. After 60 minutes of incubation at 37^{o}C, 2.0 ml of 0.25 N NaOH was added to the buffer-enzyme substrate reaction mixture to stop the enzyme activity. p-nitrophenol formed during incubation by the hydrolysis of p-nitrophenyl phosphate catalysed by acid phosphatase gives an yellow colour in alkaline pH, and the colour was read spectrophotometrically at 410 nm, and the value expressed as number of micromoles of p-nitrophenol released per minute per litre (U/l).

2.2.6.2 Assay of Alkaline Phosphatase Activity (EC 3.1.3.1)

The same procedure as described in section 2.2.6.1 was adopted to estimate the activity of alkaline phosphatase with the following change. Instead of citrate buffer, 0.05 M Glycine-sodium hydroxide buffer of pH 9.2 was used. The colour developed was read at 410 nm, and the alkaline phosphatase activity value expressed as number of micromoles of p-nitrophenol liberated per minute per litre (U/l).

2.2.6.3 Assay of Glutamate-Oxaloacetate Transaminase (GOT) or Aspartate Amino Transferase (AAT) Activity (EC 2.6.1.1)

The estimation of GOT activity was carried out by the colorimetric method of Reitman and Frankel (1957) as described in 'Methods of Enzymatic Analysis' (Vol.2 Edited by Bergmeyer, H.U., 1974). For estimating GOT activity, phosphate buffer-substrate solution containing 0.1 M phosphate, 0.1 M L-aspartate and 2 mM 2-oxoglutarate was used. To 1 ml of frozen buffer-substrate solution, 0.2 ml of haemolymph was added and incubated immediately at 37 $\pm$ 0.05^{o}C in a water bath. At the end of 60 minutes incubation period, the enzyme reaction was stopped by adding 1.0 ml of 1 mM chromogen in HCl (2,4-dinitrophenyl hydrazine), mixed well and kept for 20 minutes at room temperature. After 20 minutes, the reaction mixture was made alkaline by adding 10 ml of 0.4 N NaOH. The colour developed by 2,4-dinitrophenyl hydrazone of the reaction product, pyruvate, was determined spectrophotometrically at 546 nm, and the enzyme activity expressed as number of micromoles of pyruvate released per minute per litre (U/l).

2.2.6.4 Assay of Glutamate-Pyruvate Transaminase (GPT) or Alanine Amino Transferase (AlAT) Activity (EC 2.6.1.2)

Glutamate-Pyruvate Transaminase activity was determined by the colorimetric method of Reitman and Frankel (1957) as described in 'Methods in Enzymatic Analysis' (Vol.2 Edited by Bergmeyer, H.U., 1974). To 1.0 ml of frozen buffer substrate solution (containing

0.1 M phosphate, 0.2 M DL-alanine and 2 mM 2-oxoglutarate), 0.2 ml of haemolymph was added and immediately incubated in a water bath at 37 $\pm$ 0.05°C. After 60 minutes of incubation, the enzyme activity was stopped by adding 1.0 ml 1 mM chromogen solution, mixed well and allowed to stand for 20 minutes at room temperature. After 20 minutes, 10 ml 0.4 N NaOH solution was added and mixed well. The colour developed due to 2,4-dinitrophenyl hydrozone of oxaloacetate in alkaline medium was read spectrophotometrically at 546 nm, and the GPT activity expressed as number of micromoles of pyruvate released per minute per litre (U/l).

2.2.7 Computation and Presentation of Data

The results of experiments are explained with the help of graphs and Tables. Student's *t* test was employed to determine the statistically significant differences if any, in the number of haemocytes, the concentration of haemolymph constituents, and in the activity patterns of enzymes (Croxton et al., 1975) in snails of the three size groups as well as in their controls. The variations were reported significant at three levels, viz. $P < 0.05$, 0.01 and 0.001. All the computations involved in the work were carried out using a personal computer (CASIO fx 730-P).

All spectrophotometry readings were made using HITACHI Model U-2000 UV-Vis-spectrophotometer. Readings of sodium, potassium, and calcium were made in Flame Photometer (ELICO, Type 22); and chloride in Chloride Meter (ELICO, Model EE 34).

2.3 RESULTS

2.3.1 Haemolymph Constituents

2.3.1.1 Total haemocyte number (Table 1)

Total number of haemocytes in snails of the intermediate size group (40 ± 2 mm) was found to be very high when compared with the counts in the small (30 ± 2 mm), and the large (50 ± 2 mm) size group snails. Statistical analysis of the data revealed the following relationships. The mean value of total haemocyte number in the intermediate size group was significantly higher than the mean values in the two other size groups ($P < 0.001$).

2.3.1.2 Packed cell volume

The results on PCV of *P. virens* showed a very much lower value which cannot be detected using the micro haematocrit scale. The value obtained in three size groups was below 1%.

2.3.1.3 Glycogen (Table 2)

Statistical analysis showed no significant variation in haemolymph glycogen content among snails of the three size groups analysed.

2.3.1.4 Total carbohydrate (Table 3)

Statistical analysis revealed the following: Total carbohydrate content in haemolymph of the large size group snails was found to be significantly lower than that of the intermediate size group ($P < 0.001$), and the small size group ($P < 0.05$). The total carbohydrate

Table 1. Total Haemocytes/mm^3 in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	3966	6486	3697
± SD	1210.83	2255.12	1109.57
Range	2010 - 5850	3480 - 9840	1630 - 4960

Table 2. Haemolymph Glycogen (µg glucose/ml) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	93.621	82.999	98.670
± SD	29.779	35.393	35.675
Range	51.70 - 150.8	34.46 - 156.5	34.46 - 152.1

Table 3. Haemolymph Total Carbohydrate (µg glucose/ml) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	1539.67	1667.25	1248.77
± SD	408.22	285.92	346.12
Range	984 - 2215.6	1191.8 - 2236	810 - 1987

level in the intermediate size group showed no significant variation when compared with that of the small size group snails.

2.3.1.5 Total protein (Table 4)

Statistical analysis revealed no significant variation in the total haemolymph protein value in the large and the intermediate size group snails. The total haemolymph protein content in the small size group animals was found to be significantly lower than that of both the intermediate ($\underline{P} < 0.05$), and the large size group snails ($\underline{P} < 0.05$).

2.3.1.6 Total lipid (Table 5)

Haemolymph lipid level in the small size group snails was found to be significantly higher than that of both the intermediate ($\underline{P} < 0.05$) and the large size group snails ($\underline{P} < 0.001$). No significant variation in haemolymph lipid content was found between snails of the large and the intermediate size groups.

2.3.1.7 Urea (Table 6)

Statistical analysis revealed no significant variation in haemolymph urea value in snails belonging to the three size groups.

2.3.1.8 Ammonia (Table 7).

Ammonia level in haemolymph of the intermediate size group snails was found to be significantly lower than that of the small size group snails ($\underline{P} < 0.05$). No significant variation was found in haemolymph ammonia levels between large-intermediate, and small-large size groups.

Table 4. Haemolymph Protein (mg/ml) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50± 2 mm
N	20	20	20
Mean value	15.60	19.97	19.11
± SD	4.94	2.92	4.94
Range	8.42 - 24.18	13.88 - 25.22	10.83 - 29.10

Table 5. Haemolymph Lipid (µg cholesterol/ml) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	114.75	80.88	83.26
± SD	47.97	42.13	29.18
Range	44.20 - 202.21	30.94 - 172.38	35.36 - 154.70

Table 6. Haemolymph Urea (mg/100ml) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	2.855	2.605	3.959
± SD	1.453	1.507	2.711
Range	0.404 - 5.859	1.475 - 3.656	1.414 - 9.697

Table 7. Haemolymph Ammonia (mg/100ml) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	0.284	0.164	0.388
± SD	0.137	0.140	0.820
Range	0.054 - 0.568	0.042 - 0.414	0.315 - 0.610

2.3.1.9 Sodium (Table 8)

Sodium level was found to be significantly higher in the large size group snails than in the intermediate size group ($P < 0.001$). No significant variation in value was found between the large and the small size group snails whereas sodium content in haemolymph of the intermediate size group snails was found to be significantly lower than that of the small size group animals ($P < 0.05$).

2.3.1.10 Potassium (Table 9)

The haemolymph potassium level in P. virens of the small size group was found to be significantly higher than that of both the large and the intermediate size groups ($P < 0.001$). No significant variation was found between haemolymph potassium values in the large and the intermediate size group snails.

2.3.1.11 Calcium (Table 10)

No significant variation in haemolymph calcium content was found between snails of the small and the intermediate size groups. However, calcium level was found to be significantly higher in the large size group snails when compared with the values of the intermediate ($P < 0.05$) and the small size group animals ($P < 0.001$).

2.3.1.12 Chloride (Table 11)

Statistical analysis revealed no significant variation in haemolymph chloride content of snails belonging to the three size groups of P. virens.

Table 8. Haemolymph Sodium (μ equivalents/ml) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	43.13	40.48	45.13
± SD	4.26	2.60	2.78
Range	36.52 - 50.43	35.65 - 44.35	39.13 - 50.43

Table 9. Haemolymph Potassium (μ equivalents/ml) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	2.64	1.84	2.01
± SD	0.48	0.25	0.36
Range	2.05 - 3.58	1.54 - 2.05	1.54 - 2.56

Table 10. Haemolymph Calcium (μ equivalents/ml) in the three size groups of Pila virens

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	15.35	17.30	20.08
± SD	1.35	4.27	1.91
Range	13.0 - 18.0	15.0 - 20.0	16.0 - 22.5

Table 11. Haemolymph Chloride (μ equivalents/ml) in the three size groups of Pila virens

Size group	30±2 mm	40±2 mm	50±2 mm
N	20	20	20
Mean value	52.40	49.90	53.85
± SD	3.79	4.69	3.38
Range	45.0 - 60.0	46.0 - 62.0	48.0 - 60.0

2.3.2 Activity Pattern of Selected Enzymes

2.3.2.1 Acid phosphatase activity (Table 12)

Significant increase in ACP activity was observed in haemolymph of the large size group snails when compared with that of the intermediate and the small size group snails ($P < 0.05$). No significant variation was noted in activity pattern in snails of the intermediate and small size groups.

2.3.2.2 Alkaline phosphatase activity (Table 13)

Statistical analysis revealed no significant variation in enzyme activity among snails belonging to the three size groups.

2.3.2.3 Glutamate-oxaloacetate transaminase activity (Table 14)

No significant variation was observed in the activity pattern of GOT when large-intermediate, and intermediate-small size group comparisons were made. A higher activity pattern, however, was observed in the small size group animals on comparison with that of the large size group snails ($\underline{P} < 0.05$).

2.3.2.4 Glutamate-pyruvate transaminase activity (Table 15)

GPT activity was found to be significantly lower in the large size group animals when compared with that of the intermediate ($\underline{P} < 0.01$) and the small size group snails ($\underline{P} < 0.001$). No significant variation was observed in the activity pattern of GPT in the intermediate and the small size group snails.

Table 12. Haemolymph Acid Phosphatase Activity (U/l) in the three size groups of Pila virens

Size group	30±2 mm	40±2 mm	50±2 mm
N	12	12	12
Mean value	9.280	9.313	11.839
± SD	2.577	1.514	3.088
Range	7.379 - 16.574	7.265 - 13.110	7.669 - 19.788

Table 13. Haemolymph Alkaline Phosphatase Activity (U/l) in the three size groups of Pila virens

Size group	30±2 mm	40±2 mm	50±2 mm
N	12	12	12
Mean value	4.407	4.217	4.633
± SD	2.075	2.098	3.417
Range	1.787 - 7.291	1.153 - 9.251	0.822 - 11.914

Table 14. Haemolymph Glutamate-Oxaloacetate Transaminase Activity (U/l) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	12	12	12
Mean value	22.737	20.900	18.042
± SD	3.737	3.454	6.219
Range	15.664 - 30.46	16.975 - 27.42	8.268 - 30.46

Table 15. Haemolymph Glutamate-Pyruvate Transaminase Activity (U/l) in the three size groups of *Pila virens*

Size group	30±2 mm	40±2 mm	50±2 mm
N	12	12	12
Mean value	5.706	4.726	2.779
± SD	1.167	1.675	1.656
Range	3.020 - 7.534	2.916 - 7.534	0.423 - 5.857

2.3.2.5 GOT; GPT Ratio

The GOT: GPT ratio was found to be maximum in the large size group (9.33 ± SD 7.26) while in the intermediate group it was 4.79 (± SD1.32) and in the small size group it was 4.15 (± SD 1.09). A comparison of the ratio between the small and large size groups and between the intermediate and large size groups showed that the value in the large size group was significantly higher ($P < 0.05$).

2.4 DISCUSSION

The total haemocyte number was found to vary considerably in different size groups of P. virens, but the variations were found not to be size dependent. The highest count was noted in the intermediate size group snails, which was significantly higher than the counts in the small and the large size group snails. The increase in cell number in the intermediate size group snails could be attributed to the increase in size of the snail when compared with the small size group ones, as proposed by Stumpf and Gilbertson (1978). However, the size/age relationship to the haemocyte number is not applicable to the large size group snails. The predominance of female snails in the large size group (Hyman, 1967; Browne, 1978) could be one of the reasons for the apparent reduction in haemocyte number in the large snails. Comparable situations are reported in polychaetes by Caullery and Mensis (1898), and Dales (1964). Accordingly, in Dodecaceria concharum and Neoamphitrite figulus, the total number of blood cells was found to decrease considerably in females at maturity. The change from lipid and carbohydrate storage to protein

storage during sexual maturation is also suggested (Eckelbarger, 1976). An analogous situation is found in the case of P. virens also. The lipid and total carbohydrate concentrations in haemolymph were found to be lower while protein content was higher in the large size group snails when compared with the small size group. No information about sex-wise analysis of haemocyte number in molluscs are available so far. From the close similarity of metabolite concentrations seen in polychaetes and in P. virens, the low haemocyte number in the large size group snails can be attributed to the sex-wise difference in P. virens, and in prosobranchs, in general.

The composition of different metabolites in haemolymph gives a clear indication of the physiological state of the organism. This is especially applicable to gastropods which have an open circulatory system and lesser level of hormonal integration than higher animals.

Analysis of haemolymph glycogen values in the three size groups of P. virens showed no size-wise variation, or in other words glycogen in the haemolymph in different size/age groups is maintained at a specific level. In P. virens the concentration of haemolymph glycogen ranges from 82.9 to 98.7 µg/ml. Veldhuijzen (1975) reported a similar instance of maintenance of constant glucose level in L. stagnalis (about 20 µg/ml). The involvement of insulin-like hormone in P. virens can be considered as the reason for the maintenance of a constant level of haemolymph glycogen irrespective of size/age variation. Insulin-like activity and homolog of pancreatic

β cells in the digestive tract have been identified in several gastropods (Goddard et al., 1964; Boquist et al., 1971; Davidson et al., 1971). Dogterom (1980) demonstrated imbalance in carbohydrate metabolism in snails by removing the growth hormone producing light green cells, again supporting the role of hormonal involvement in gastropod carbohydrate metabolism. However, this finding is in clear disagreement with the observations of Gabbot et al. (1979). According to Gabbot et al. (1979), there exists no homoeostatic mechanism for the control of blood sugar level in bivalves and gastropods.

Haemocytes play a very important role in determining the level of total carbohydrate in haemolymph of molluscs. In M. californianus, haemocytes were found to carry approximately 22% of the carbohydrate present in the haemolymph (Thompson et al., 1974). In M. edulis, approximately 2% of the total glycogen was found to be represented in haemocytes alone. Thus, the number as well as the content of haemocytes, determine to a certain extent, the concentration of total carbohydrate in haemolymph (Thompson, 1977). A comparable observation is seen in P. virens also. The level of total carbohydrate and the total number of haemocytes show a close relationship. Total haemolymph carbohydrate level and total haemocyte counts were low in the small and large size group snails, but high in the medium size group snails.

Haemolymph carbohydrate is divided into two classes, (i) low molecular weight carbohydrate, and (ii) glycogen (Thompson, 1977). In M. edulis, the low molecular weight carbohydrates constituted 70

to 90% of the plasma carbohydrate. In P. virens, the low molecular weight carbohydrate concentrations in all the three size groups were found to be above 90%. In P. virens there appear size dependent variations in the proportion of glycogen to total haemolymph carbohydrate content. In the small size group snails, glycogen forms 6% of total haemolymph carbohydrate, in the medium it is 5%, whereas in the large size group, it is found to be higher (7.9%). There is no correlation between glycogen percentage and total haemocyte count in any size group. This high percentage in the large size group can be attributed more to the significantly low total haemolymph carbohydrate level in the large size group than to the non-significant high level of glycogen. Many reasons are suggested for the low haemolymph total carbohydrate concentration in the large size group of which those related to the age factor are more acceptable. Lower rates of ingestion and assimilation are reported in snails belonging to larger size/age groups (Russell-Hunter, 1970; Haniffa and Pandian, 1974; Aldridge, 1982).

Total haemolymph protein value was found to be more in the larger two size group snails than in the small size group. Various roles are being suggested for haemolymph proteins and free amino acids. Free amino acids play a very important role in regulating the osmolarity of the organism. Body water content and the size of the extracellular compartment in P. virens were reported to be stable, and this stability was maintained by the involvement of free aminoacids (Shylaja and Alexander, 1975b). Many reasons are suggested

for apparent variation in haemolymph protein content, including the composition of diet, season, temperature, infection, environmental stress, reproduction and developmental stage of the organism (Bishop et al., 1983). Fretter and Graham, (1962) reported reproduction cycle linked variations in protein and carbohydrate levels in the haemolymph of C. virginica. A similar increase in plasma protein value from 90 mg/100 ml to 250 mg/100 ml during different seasons, possibly related to reproductive cycle, was demonstrated by Thompson (1977). A comparison of data on haemolymph protein concentration in the small and the larger size groups of Pila also points to the possibility of reproductive cycle related variations in protein values because the values in the intermediate and the large size group snails (adults) were significantly higher than that in the small size group snails (juvenile). A corresponding decline in lipid content in the larger size groups also points to the same conclusion. The respiratory protein, haemocyanin, constitutes a major component of plasma proteins. Variations in the concentration of haemocyanin also can affect the total protein level of haemolymph, and it is presumed that the high level of protein in the larger two size groups might be also due to the high concentration of haemocyanin in the haemolymph. Since haemocyanin is not bound to haemocytes, a positive correlation is not possible between haemocyanin concentration and haemocyte number.

In P. virens the haemolymph total lipid concentration showed significant variation among snails of the different size groups.

Lipid level was found to be significantly higher in the small size group than in the other two size groups. Considering the intermediate and the larger size group snails as adults and hence reproductively very active, the low values can be attributed to the utilization of lipid during gametogenesis. Lipids are the metabolic storage products of the gametes (Webber, 1970) and hence, gametogenesis leads to a decline in the lipid storage content which is reflected as low haemolymph lipid level in the adults. The blood lipids of gastropods are in dynamic equilibrium with the numerous lipid compartments. Structural lipids (sterols and phospholipids) are the major component (70%) of the total plasma lipid in gastropods. In haemocytes, the percentage of structural lipids is still high, up to 95%. The remaining 5% constitutes triglycerides and free fatty acids (Allen, 1977). During breeding and other specialized physiological conditions lipids are utilized by many prosobranchs (Chatterjee and Ghose, 1973). Foot, mantle, and the digestive gland contribute lipid reserves for normal metabolic use and for gametogenesis. They are mobilized from these storage centres to the gonads for gametogenesis. The high haemolymph lipid content in small size groups snails may be an indication of large scale movement of lipid to gonads prior to gametogenesis. It is also possible that the small size group snails rely on protein rather than on lipids for energy. In adults this lipid is utilized for gametogenesis and once the process is over mobilisation is considerably reduced. This is reflected as significantly low level value in the adult haemolymph. Large scale

movement of lipids has been reported by many investigators (Lawrence and Giese, 1969; Webber, 1970; Chatterjee and Ghose, 1973). Lipids most actively incorporated into the gametes are the structural lipids. Large variations in structural lipid composition of haemolymph was observed in Placopecten magellanicus (Shieh, 1968), and in Semperula maculata (Nanaware and Varute, 1976). However, Allen (1977) investigated the sexwise lipid content of molluscs and reported that there exists no sexwise difference in lipid content.

A reciprocal relationship between the synthesis of lipids and utilization of proteins and vice versa, was reported in many molluscan species (Muley, 1975). A comparable relationship is observed in the case of P. virens also. In adults (intermediate and large size groups), haemolymph shows high concentration of protein but low concentration of lipids, and in juveniles the concentration of lipid is high but that of protein is low.

The amount of nitrogenous degradation products released is indicative of the protein and nucleic acid metabolic activities of the animal. Organisms of aquatic habitats are primarily ammonotelic. Ammonia is highly soluble in water and the external epithelial surface of aquatic species is readily permeable to it. Since the concentration of ammonia in natural environment is extremely low, aquatic organisms maintain low body concentrations of ammonia by simple diffusion from body fluids to the external environment through the epithelium (Campbell and Bishop, 1970; Campbell, 1973). In B. glabrata, high concentrations of ammonia and urea were reported in haemolymph;

corresponding to the excretory pattern. No uric acid was detected in the haemolymph of B. glabrata (Becker and Schmale, 1975). Accumulation of substantial amounts of ammonia and urea in tissues was reported in P. globosa (Swami and Reddy, 1978), and P. virens (Shylajakumari, 1975).

The present study demonstrates a size dependent variation in haemolymph ammonia concentration in P. virens. The intermediate size group snails were found to carry lesser ammonia load in haemolymph than the large and the small size groups. Size group wise difference in ammonia content was reported in Mytilus edulis also. (Bayne and Scullard, 1977). Most aquatic and amphibious freshwater prosobranchs were reported to be primarily ammonotelic, producing comparatively lower concentration of urea than ammonia (Potts, 1967; Hagag and Fouad, 1968; Campbell and Bishop, 1970; Shylaja and Alexander, 1975a; Aldridge, 1982). In the present study, however, urea concentration in haemolymph is higher than ammonia content in all the size groups. In haemolymph urea concentration also there is slight variation among size groups, though insignificant. The intermediate size group snails were found to have lower urea content than the small and the large ones. The concentration of urea and ammonia in haemolymph gives a direct indication of preferential selection of metabolite by the snail at a given period of time. Low levels of ammonia and urea in haemolymph along with low concentration of glycogen in the intermediate size group snails are clear indications of the organism's dependency

on glycogen for energy requirement. An interesting situation was reported in M. edulis by Bayne and Scullard (1977), where smaller individuals were found to be reliant on carbohydrate for energy metabolism while larger ones rely on protein catabolism leading to the production of lesser ammonia in smaller ones when compared with large amounts of ammonia and urea produced by larger M. edulis (Bayne and Scullard, 1977). But in P. virens the intermediate size group snails seem to rely on glycogen and lipid, the small ones on protein, and the large ones on protein, lipid and carbohydrates.

Haemolymph ionic concentrations in the three size groups of P. virens were also analysed. The values obtained for the different size groups were found to be different from the values reported for P. globosa , an allied species of P. virens. Saxena (1957) reported the values of sodium, potassium and calcium as 54.7, 4.9 and 7.8, respectively. In the present study, haemolymph sodium, potassium and calcium concentrations (as μ equivalents/ml) in snails of the common size group (intermediate) were found to be 40.48, 1.84, and 17.30 respectively. Among different size groups also there appeared considerable variations in the concentrations of Na^+, K^+, Ca^{2+} and Cl^- ions. Sodium and Chloride are the dominant ions in haemolymph of normally fed, active P. virens. Schoffeniels and Gilles (1972) reported that the dominant ions in haemolymph of freshwater molluscs are Na^+ and Cl^- ions. In the present study, sodium, potassium and chloride ions showed a tendency to become diluted in snails of the intermediate size gorup. Na^+, Ca^{2+} and Cl^- ions were found to be

more concentrated in the large size group snails. A comparable behaviour of sodium and potassium ions was reported by Burton (1965) and Prasad et al. (1985) in non marine molluscs. Eventhough independent mechanisms were suggested for Na^+ and Cl^- ions (Krogh, 1939; Dietz, 1979), the variations in the concentrations of these ions in different size group snails as observed in the present study seem to be related to one another.

Studies on ion regulation in prosobranchs are concentrated mostly on calcium ion which is important in shell deposition, and seems to play a crucial role in general fluid and electrolyte tolerance as well as in other physiological processes in vertebrates and invertebrates alike (Little, 1981). In Pila, eggs produced were found to be surrounded by calcified egg capsule (Prashad, 1932; Meenakshi et al., 1974; Tompa, 1980). Calcium is transported to the uterus via blood beginning immediately with the onset of egg laying. In H. aspersa, both bound and unbound fractions of blood calcium increased approximately 60%, and the elevated level was found to be maintained throughout the period of egg laying (Tompa and Wilbur, 1977). Calcium cells in blood actively take part in the rapid transportation of calcium. Pila virens is sexually dimorphic, adult females being larger in size than adult males (Hyman, 1967). During the period of egg laying largescale mobilization of calcium ions can be expected in the large size group Pila virens (most of the large size group snails belonged to female sex), reflecting higher level

of calcium ions in the haemolymph when compared with the levels in the intermediate and the small size group snails. The values obtained in the present study clearly indicate this. Meenakshi (1955) also reported considerably higher levels of calcium in female snails when compared with the levels in male snails.

Analysis of activity pattern of haemolymph phosphatases in different size group snails shows a general trend of increase with the increase in shell size. The haemolymph acid phosphatase activity was found to be significantly higher in the large size group snails when compared with the activities in the intermediate and the small size group snails. Phosphatases are reported to be involved in (i) biosynthesis of protein (Johnson and McMinn, 1958), and mucopolysaccharides (Kroon, 1952), (ii) regulation of intracellular phosphate concentrations (Gutman, 1959), and (iii) hydrolysis of body cells, permeability process, and in nucleic acid biosynthesis (Cox et al., 1967). The energy metabolism of prosobranchs in normally fed and in aerobic conditions is proved to be carbohydrate based (Veldhuijzen and Van Beek, 1976). In P. virens, the haemolymph total carbohydrate content in the large size group snails was found to be significantly lower than in the other two size group snails. Hence, phosphatases, in the large size group snails may be involved in the synthesis of mucopolysaccharides to supplement normal physiological activities. Both ACP and ALP show elevated levels of activity in haemolymph of the large size group snails. Moreover, the absence

of corresponding increase in haemocyte number along with increased ACP activity in the large snails points towards extra haemocyte source of phosphatase which is required for the elevation of the activity of the phosphatase for non-defence purpose. Non-haemocyte sources of ACP have been reported in gastropods (see Cheng and Rodrick, 1980, Cheng, 1983).

Size of the snail is considered as one of the factors influencing the levels of activities of transferases in gastropods (Hammen, 1968; Swami and Reddy, 1978). Analysis of activities of GOT and GPT shows a decreasing trend with increase in body size. The decrease is found to be more prominant and significant in the case of GPT. This significant decrease in GPT in the large size group is the main reason for the high GOT:GPT ratio seen in this size group. The decreasing trend of transferases can be attributed to the general decrease of metabolic rate and growth rate noted in gastropods along with increase in size or progress in age (Haniffa, 1980; Aldridge, 1982). In the small size group snails, increased metabolic and assimilation rates require transaminases in high concentrations while with decrease in metabolic turnover, in the large snails, the levels of transferases also show a declining trend.

C H A P T E R - III

EFFECTS OF STARVATION ON THE HAEMOLYMPH CONSTITUENTS OF PILA VIRENS

3.1 INTRODUCTION

Starvation is by definition a condition in which the animals ration is not sufficient to balance its basal demand for energy (Haniffa, 1987). Several reports are available on the level of activity of haemolymph constituents in normally fed and starved molluscs (Gilbertson et al., 1967; Friedl, 1971; Becker, 1972; Lee and Cheng, 1972; Becker and Hirtbach, 1975; Michelson and Dubois, 1975; Veldhuijzen, 1975; Stanislawski and Becker, 1979).

Reduction in metabolic rate in molluscs as a result of starvation was reported by Thompson et al. (1978), Joosse and Geraerts (1983); Umadevi et al. (1986), and Haniffa (1987). The metabolic level of one month starved Pila globosa was found to be lowered by 1/7th when compared with normally fed snails. Starved snails also showed higher metabolic cost due to poor conversion efficiency (Haniffa, 1987). In Lymnaea stagnalis, the rate of heart beat was found to be affected by starvation. The heart rate reduced from 38 to 22 beats per minute (De With et al., 1980). The decrease in the heart rate was suggested as the direct effect of reduced renal filteration, water turnover, and energy metabolism. Joosse and Geraerts (1983) reported that the snails can manipulate many aspects of its physiology by simply changing the heart rate.

A common response during periods of reduced food availability is reduction in the rate of oxygen consumption (Bayne, 1973). Some

snails show depressed oxygen uptake during starvation, eg., Bithynia leachi (Berg and Ockelmann, 1959); Marisa cornuarietis (Akerlund, 1969); Oncomelania nosophora (Yanagisawa and Komiya, 1961), and Potamopyrgus jenkinsi (Lumbye and Lumbye, 1965). On the other hand, Berg and Ockelmann (1959) reported that starvation had no marked effect on oxygen uptake rates in Bithynia tentaculata or Valvata piscinalis. Studier and Pace (1978) reported that oxygen uptake rate declines in starved Viviparus georgianus females but not in males. Bayne and Scullard (1978) recorded an exponential decline in the respiration rate of Thais lapillus during starvation with a halving of the rate over 18 days.

Carbohydrate consumption was found to increase in starved gastropods (Emerson, 1967; Christie et al., 1974; Stainslawski and Becker, 1979), although in some cases levels have been maintained, for example, in Achatina fulica (Brockelman and Sithithavorn, 1980). Haemolymph glucose concentrations were found to be maintained for 15 days in starved L. stagnalis, probably at the expense of stored glycogen (Veldhuijzen, 1975). The accumulation of carbohydrates generally parallels increasing food availability and food intake, and may be reflected in higher blood sugar levels as in L. stagnalis (Scheerboom and Van Elk, 1978); Strophocheilus oblongus (Marques and Pereira, 1970), and Thais lamellosa (Lumbert and Dehnel, 1974). Starvation was found to increase responsiveness to food but in excess was inhibitory (Jager, 1971). Joosse and Geraerts (1983) reported

gradual decrease in glycogen in the mantle of L. stagnalis during starvation. Glycogen starts to decrease only after 6 days of starvation. In gastropods, the increase in glycogen in mantle may be dependent on glucose levels in the haemolymph. Mobilization of glycogen depends on other factors and may be controlled by hormones (Joosse and Geraerts, 1983). In L. stagnails, although fixed maximum blood glucose concentration were not apparent, a minimum level is maintained in starved animals (Veldhuijzen, 1975). In regulation of carbohydrate level in haemolymph, hormonal involvement in molluscs was suggested by Veldhuijzen (1975). Insulin-like activity, and homologues of pancreatic β cells in digestive tract have been identified in several gastropods (Goddard et al., 1964; Ammon et al., 1967; Davidson et al., 1971). Among different carbohydrate components, galactogen was suggested to be used as an emergency food source during starvation, but only after the glycogen store has been used up (Goudsmit, 1972, 1973).

On starvation, Littorina littorea utilizes both carbohydrates and lipids (Holland et al., 1975). Under these conditions, T. lamellosa did not use lipids but polysaccharides and protein (Stickle, 1971); in the males even lipogenesis occurred. Stickle and Duerr (1970) suggested lipid oriented metabolism in carnivorous prosobranchs. Starvation studies by Christie et al. (1974) indicated that the glycogen reserves do not drop significantly below normal until day 21 of the experiment. At day 28, a further drop in glycogen

level was noted. In M. edulis, the energy demands of starvation in winter was reported to be met largely by the catabolism of proteins, at least on larger individuals (Gabbot and Bayne, 1973). They reported the following percentage of energy dependency during starvation; 75% due to protein, 10% to carbohydrates, and 15% lipids. However, haemolymph protein and carbohydrate levels in oysters starved for 7 days were found to be unaltered (Fisher and Newell, 1986). In Biomphalaria glabrata, on the contrary, Becker and Hirtbach (1975) reported reduction of haemolymph protein content after 7 days of starvation. Stanislawski and Becker (1979) reported hypoproteinaemia in B. glabrata fed on lipid diet in starved, and infected snails, after 30 days. A hypoglycemic response was also observed in snails fed on protein and lipid diet, for starved and infected snails. A comparison of haemoglobin/total protein ratio in starved and fed snails suggested that a proportionally large amount of non-haemoglobin protein was depleted in the haemolymph of starved snails. It was suggested to be due to the cessation or reduction of transport of proteins from the digestive gland, since there was little if any, digestion and absorption of food substances during starvation or due to elective utilization of certain proteins (Lee and Cheng, 1972). In Pila globosa, a marked decrease of 78.45% of blood copper was observed after one month of starvation. The low molecular weight proteins of the blood were also found to decrease as a result of starvation. On comparison of haemolymph protein variations due to

starvation and aestivation, decrease during starvation was found to be more prominent (Reddy and Naidu, 1978).

In Viviparus viviparus, fat is stored in the cells of connective tissues of midgut gland and in the epithelium of the stomach and intestine, which is used up during six weeks of starvation, but reappear in the midgut gland four hours after the initiation of feeding (Hyman, 1967). Duncan et al. (1987) reported a decline of about 12% in the mean total lipid content in snails after 2-3 weeks of starvation. In bivalves, starvation experiments showed lipid to be the most important reserve in Crassostrea gigas (Riley, 1976). In Paphia laterisulca however, lipid content remained constant after starvation for 12 days, glycogen content decreased, and water content was found to increase (Nagabhushanam and Dhamne, 1977). In freshwater limpet Ancylus fluviatilis, Streit (1978) reported the utilization of lipid first during starvation, followed by carbohydrates.

Thompson et al. (1978), while studying the effect of starvation on M. edulis, reported that starvation for short duration has no effect on the total haemocyte number or their biochemical composition. Whether the same observation is applicable to gastropods is not known.

The haemolymph functions as a sink into which the tissues deposit ammonia and probably other metabolic end products. In B. glabrata, when feeding in water, ammonia production is about four

times the urea production, whereas when starved, the ammonia production falls only slightly (20%), or remain constant while urea excretion increases 4 to 5 fold resulting in an overall two to three fold increase in nitrogen excretion after 5 days. Uric acid levels in the kidney and tissues were very low or below detectable limits (Stanislawski et al., 1979). High level of protein catabolism resulting from starvation induced urea and uric acid synthesis in B. glabrata. After only 5 days of starvation, haemolymph ammonia concentration in B. glabrata increased by 45% but haemolymph urea showed an extraordinary 16 fold increase. (Becker and Schmale, 1975). Increase in haemolymph urea concentration in starved individuals was shown to correspond to increase in the specific activity of selected urea cycle enzymes (Schmale and Becker, 1977). However, uric acid could neither be detected in the haemolymph of normally fed nor of starved snails (Becker and Schmale, 1975). Bayne and Scullard (1977) reported an increase in ammonia excretion and a decrease in O:N ratio in T. lapillus during starvation. A different report was provided by Thompson et al. (1978), in which the level of ammonia in haemolymph of the mussel Mytilus californianus was shown to increase when starved mussels began to feed, suggesting increased protein metabolism during feeding than during starvation. Becker and Schmale (1978) discussed the differences in percentage of ammonia and urea in the excretory products in fed and starved snails. Nitrogen excretion increase in starved and infected snails by 62 and 79%, respectively. While in fed

condition 80% of the nitrogen is excreted as ammonia, in starving or infected snails, nitrogen by over 50% is excreted as urea (Becker and Schmale, 1978). Stickle (1971) reported that T. lamellosa increased ammonia excretion during starvation as a result of protein utilization from the foot. With littorines (Littorina planaxis) after 66-70 days of starvation, the free aminoacid levels in the tissues fell about 60%, the uric acid content rose from 0.62 to 4.29 mg/g dry weight, and lipid content fell about 40%, whereas the total carbohydrate and protein contents remained fairly constant (Duerr, 1967; Emerson and Duerr, 1967).

Starvation was reported as one of the reasons affecting the reproductive capacity of molluscs. In B. glabrata, a decline in egg laying was noted 7 days after start of starvation. After 14 days of starvation, egg production dropped to nil. Same effect was reported in L. stagnalis also (Veldhuijzen, 1975).

In gastropods, starvation was reported to produce marked effect on ionic concentration of haemolymph. The changes are mostly completed in one day in the case of sodium and potassium (De With, 1978). A marked reduction in sodium, potassium, and calcium content and increase in chloride content were noted in starved L. stagnalis when compared with fed snails (fed snails : Na^+, 55.3; K^+, 1.7; Ca^{++}, 4.4 and Cl^-, 36.2 mM; starved snails; Na^+, 47.7; K^+, 1.2; Ca^{++}, 3.5 and Cl^- 40.7 mM) (De With and Sminia, 1980). Both potassium and calcium concentrations tend to rise after feeding in marine molluscs

(Burton, 1968). De With and Sminia (1980) showed that (Ca^{++}) (CO_3^{--}) is lowered in starvation, but the relationship of this to the solubility of calcium carbonate is controversial. The role of the shell here is unclear, but it helps to maintain haemolymph when Ca^{++} is lost to the medium (Greenaway, 1971). De With (1980) proposed a direct correlation between haemolymph chloride and pH- the higher the pH, the higher the Cl^- level. Starvation is reported to elevate the pH of haemolymph of molluscs (De With, 1980).

In this chapter, the effects of starvation on the various haemolymph organic and inorganic constituents, haemocyte number, and the activity pattern of selected enzymes of P. virens are reported.

3.2 MATERIALS AND METHODS

Methods of collection of test animal, laboratory conditioning of the animal, collection of haemolymph, estimation of total haemocyte count, haemolymph glycogen, total carbohydrate, total lipid, total protein, urea, ammonia, sodium, potassium, calcium, and chloride were the same as explained in detail in section 2.2.5. Activity patterns of GOT, GPT, ACP, and ALP also were determined following the methodology described in section 2.2.6 . For computation and presentation of the results, the methods explained in section 2.2.7. were followed.

3.2.1 Selection of animal groups

Healthy P. virens belonging to the common size group (intermediate size group i.e., 40 $\pm$ 2 m shell height) were selected for

starvation studies. Snails selected belonged to the same population. They were laboratory conditioned for 72 hrs and segregated into test and control groups. A batch of 240 snails of comparable body weight was segregated into two groups of 120 each. The first group was the test group which was further divided into three sets of 40 each, and placed in three fibre glass tanks (50 L capacity) containing dechlorinated, aerated tap water. The second group, also divided into three sets and reared in three 50 L fibre glass tanks served as the controls. The former group was not given food while the latter was fed with Hydrilla (Haniffa, 1978). Water was replenished completely every 24 hours, and aerated regularly. The tanks were cleaned daily to prevent algal growth. Snails from the first tank of the first group were taken for haemolymph analysis after 10 days of starvation. Snails in the first tank of the second group served as the controls. Similarly, snails in the second set of the first group and those in the second set of the second group were used for 20 days starvation studies. For 30 days starvation studies, snails of the final set of both the groups were used. Mortality rate during the entire experiment was nil. For estimation of total haemocyte counts, for each time period 15 specimens were used as experimentals and 10 as controls. From the 15 specimens chosen for each time period as experimentals, randomly selected 10 were used for estimation of other haemolymph constituents and activity levels of enzymes. From the remaining 25 specimens maintained as

experimentals (for each time period) and 30 as controls; duplicates were run.

3.3. RESULTS

3.3.1 Haemolymph constituents

3.3.1.1 Total haemocyte count (Table 16)

A general trend of decrease in total haemocyte number was observed in starved snails. But the decrease was not statistically significant.

A significant decrease in total counts was observed in 20 and 30 day starved snails when compared with the count in 10 day starved snails ($P < 0.05$).

3.3.1.2 Glycogen (Table 17)

Statistical analysis of the results revealed no significant variation in haemolymph glycogen in starved snails when compared with their respective controls.

The variation in the levels of haemolymph glycogen among 10, 20 and 30 day starved snails also were found to be statistically insignificant.

3.3.1.3 Total carbohydrate (Table 18)

After 10, 20 and 30 day of starvation, significant decrease in total haemolymph carbohydrate levels was observed in starved snails when compared with the control values ($P < 0.001$, 0.01 and 0.01 respectively).

Table 16. Total Haemocytes/mm^3 in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	15	10	15	10	15
Mean value	6262.00	6245.00	5820.00	4244.00	5783.00	4290.00
± SD	2960.80	2109.43	1752.71	1929.07	1700.96	1889.28
Range	4510.00-10440.00	2350.00-10360.00	3900.00-9490.00	1830.00-10030.00	3900.00-9460.00	1560.00-7180.00

for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	167.09	136.36	157.70	121.11	125.14	99.91
± SD	72.95	69.33	80.49	75.92	56.43	30.49
Range	56.80-326.80	86.58-266.60	45.22-250.80	11.08-248.60	42.94-236.80	49.18-134.36

Table 18. Haemolymph Total Carbohydrate (µg glucose/ml) in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	1658.34	536.10***	1363.82	471.75**	1027.78	254.76**
± SD	493.50	201.92	596.86	199.61	597.31	77.34
Range	1062.00-2956.00	281.20-881.80	403.80-2356.00	241.00-834.40	294.40-2356.00	156.52-415.60

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

Statistical analysis of values among 10, 20 and 30 day starved snails also yielded interesting results. No significant variation in total carbohydrate level was observed in haemolymph of 10 and 20 day starved animals whereas in 10 and 30 ($\underline{P} < 0.01$), and 20 and 30 ($\underline{P} < 0.001$) day starved snails when the values were compared sharp decline in total carbohydrate level was observed in 30 day starved animals.

3.3.1.4 Total protein (Table 19)

When compared with the control values, the levels of activity of total protein in 10, 20 and 30 day starved snails were found to be significantly low ($\underline{P} < 0.001$). However, no significant variation was observed in protein level when comparisons were made between the levels of activity in the three sets of starved snails.

3.3.1.5 Total lipid (Table 20)

10, 20 and 30 days of starvation resulted in statistically significant reduction in lipid content in the haemolymph of starved snails. The fall in lipid level was more conspicuous in 20 and 30 day starved snails ($\underline{P} < 0.001$) than in 10 day starved snails ($\underline{P} < 0.05$), when compared with the control values.

The fall in haemolymph lipid content was found to be significant between 10 and 20 day starved snails ($\underline{P} < 0.05$), and 10 and 30 day starved snails ($\underline{P} < 0.05$) where the lipid level in 10 day starved snail was significantly lower. No significant variation in values was found between 20 and 30 day starved snails.

Table 19. Haemolymph Protein (mg/ml) in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	15.01	4.46***	17.34	5.22***	19.92	7.47***
± SD	7.55	1.75	7.80	1.38	6.78	4.17
Range	6.17-31.64	2.43-7.87	6.17-28.96	3.29-6.88	7.72-28.96	1.57-10.24

Table 20. Haemolymph Lipid (µg cholesterol/ml) in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	60.43	34.77*	78.09	52.04***	70.84	50.04***
± SD	29.89	16.52	14.05	9.86	10.53	11.45
Range	24.28-119.50	21.04-75.80	53.14-97.38	44.51-73.65	58.00-90.64	34.53-69.60

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

3.3.1.6 Urea (Table 21)

Analysis of data from the three sets of experimental snails regarding urea levels showed a marked elevation in urea content in the haemolymph of starved snails when compared with the control values ($\underline{P} < 0.001$).

When comparisons were made among the experimentals, i.e., between 10-20, 20-30 and 10-30 day starved snails, the first two sets showed marked variation, i.e. significantly higher values in 20 day starved snails ($\underline{P} < 0.001$ and $\underline{P} < 0.01$ respectively) while in the third set no significant variation was observed.

3.3.1.7 Ammonia (Table 22)

After 10 and 20 days of starvation, significant elevation ($\underline{P} < 0.05$ and $\underline{P} < 0.001$) in the level of haemolymph ammonia was observed in the experimentals while 30 day starved snails registered a significant decline ($\underline{P} < 0.05$) in ammonia level on comparison with the control values.

When the values in 10, 20 and 30 day starved snails were compared for haemolymph ammonia, statistically significant decrease was observed in 30 day starved snails than in 10 day starved ones ($\underline{P} < 0.001$) and 20 day starved ones ($\underline{P} < 0.001$).

3.3.1.8 Sodium (Table 23)

Statistically significant decrease in sodium content was noted in 10, 20 and 30 day starved snails on comparison with the control

Table 21. Haemolymph Urea (mg/100 ml) in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	1.78	3.58***	2.51	6.36***	1.80	3.88***
± SD	0.81	0.84	0.94	1.91	0.43	0.74
Range	0.65-2.78	2.42-5.45	1.37-3.79	2.26-9.01	1.29-2.26	2.71-5.25

Table 22. Haemolymph Ammonia (mg/100 ml) in Pila virens starved for 10, 20 and 30 days.

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	0.29	0.44*	0.24	0.48***	0.27	0.13*
± SD	0.07	0.16	0.08	0.15	0.14	0.09
Range	0.22-0.48	0.21-0.55	0.13-0.41	0.24-0.74	0.12-0.52	0.21-0.27

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

values. The sharp decline in the 10 day starved ($\underline{P}<0.001$) group was gradually minimized in 20 ($\underline{P}<0.01$) and 30 day starved ($\underline{P}<0.05$) groups with the progress in starvation days.

No statistically significant variation was observed in haemolymph sodium content in 10, 20 and 30 day starved snails when comparisons were made between the groups.

3.3.1.9 Potassium (Table 24)

Statistically significant decline in haemolymph potassium levels was observed in 10, 20 and 30 day starved snails when compared with the control values ($\underline{P}<0.001$ and $\underline{P}<0.01$)

No significant variation in potassium level was observed when the values in the three sets of starved snails were compared among themselves.

3.3.1.10 Calcium (Table 25)

Statistically significant decrease ($\underline{P}<0.001$) in calcium content of haemolymph was observed in 10, 20 and 30 day starved snails when compared with the control values.

A comparative study between the values in 10 and 20 day starved, and 10 and 30 day starved snails also showed a sharp decline in the calcium content in 20 and 30 day starved ones ($\underline{P}<0.001$). No significant variation in values was observed on comparison between 20 and 30 day starved sets.

Table 23. Haemolymph Sodium (μ equivalents/ml) in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	41.82	34.87***	43.66	36.70**	40.96	37.13*
± SD	1.56	2.82	3.25	4.67	3.75	3.07
Range	39.13-43.48	31.30-40.00	37.39-47.63	29.57-41.74	37.39-46.95	32.17-41.74

Table 24. Haemolymph Potassium (μ equivalents/ml) in Pila virens starved for 10, 20 and 30 days.

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	3.38	1.44***	2.56	1.44**	2.41	1.54**
± SD	0.43	0.32	0.68	0.74	0.54	0.42
Range	2.56-4.10	1.03-2.05	1.54-3.59	0.51-2.56	1.54-3.08	1.03-2.05

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

10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	26.00	23.70***	21.80	18.40***	23.20	19.50***
± SD	1.56	1.34	1.40	1.77	1.03	2.07
Range	24.00-28.00	20.00-25.00	19.00-23.00	17.00-21.00	21.00-25.00	17.00-22.00

Table 26. Haemolymph Chloride (µ equivalents/ml) in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	51.40	48.90	48.70	41.90***	49.90	44.30**
± SD	1.58	4.43	1.95	3.38	2.18	4.60
Range	48.00-53.00	43.00-57.00	46.00-51.00	38.00-48.00	45.00-52.00	38.00-50.00

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

3.3.1.11 Chloride (Table 26)

Statistically significant reduction in haemolymph chloride level was observed in 20 ($\underline{P} < 0.001$) and 30 day ($\underline{P} < 0.01$) starved snails when compared with their respective controls.

Significant decline in chloride level was observed 20 and 30 day starved groups on comparison with the value in 10 day starved group ($\underline{P} < 0.05$). However, no significant variation in values was observed in 20 and 30 day starved snails.

3.3.2 Activity Pattern of Enzymes

3.3.2.1 Acid phosphatase activity (Table 27)

A slight decrease in the activity levels of ACP was observed in the 3 sets of starved snails when compared with their controls. The fall in enzyme activity level was statistically significant in 20 day starved snails when compared with the control value ($\underline{P} < 0.001$).

When the activity levels in snails of the 20 and 30 day starved sets were compared, significant increase in enzyme activity level was found in the latter group ($\underline{P} < 0.001$).

3.3.2.2 Alkaline phosphatase activity (Table 28)

In 10 and 20 day starved snails, the alkaline phosphatase activity levels were found to be significantly lower than the control levels ($\underline{P} < 0.01$). No significant variation was found in activity levels when comparison was made between 30 day starved snails and its control.

Table 27. Haemolymph Acid Phosphatase Activity (U/l) in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	8.52	7.51	9.02	5.53***	9.19	8.91
± SD	1.05	4.37	2.39	0.96	0.78	1.65
Range	6.53-9.65	4.94-19.58	6.81-14.19	4.08-7.26	7.89-10.84	6.47-11.41

Table 28. Haemolymph Alkaline Phosphatase Activity (U/l) in Pila virens starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	5.78	2.68**	6.54	2.23**	3.75	4.88
± SD	2.53	1.39	3.22	0.99	1.54	3.13
Range	2.05-9.11	0.84-4.87	3.23-13.83	1.18-3.75	1.64-6.34	1.61-11.53

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

When the activity levels in starved snails of the 3 sets were compared among themselves, no significant variation in enzyme activity level was observed between 10 and 20 day starved ones. But significant increase in enzyme activity level was found in 30 day starved snails when compared with the level of activity in 20 day starved snails ($\underline{P}<0.05$).

3.3.2.3 Glutamate-oxaloacetate transaminase activity (Table 29)

No significant variation was observed in 10 and 20 day starved snails in enzyme activity levels when compared with the control values. In 30 day starved snails, however, significant fall ($\underline{P}< 0.001$) in enzyme activity level was observed.

Comparison of the activity pattern of GOT in 10, 20 and 30 day starved snails among themselves yielded the following results. Significant rise in enzyme activity level was observed in 20 day starved snails than in 10 day starved ones ($\underline{P}< 0.001$). Significant reduction in activity level was observed in 30 day starved snails when compared with the activity levels in 10 ($\underline{P}< 0.01$) and 20 day starved snails ($\underline{P}< 0.001$).

3.3.2.4 Glutamate pyruvate transaminase activity (Table 30)

No significant variation in enzyme activity pattern was observed in 10 and 20 day starved snails when compared with the pattern in the controls. However, 30 day starved snails registered a significant fall in enzyme activity than the controls ($\underline{P}< 0.001$).

Table 29. Haemolymph Glutamate [illegible]
starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	14.91	14.22	14.43	19.79	20.50	6.95***
± SD	3.74	5.54	3.63	8.01	6.15	3.73
Range	11.31-20.02	8.26-20.02	10.87-23.00	10.87-35.72	13.15-30.05	3.48-12.18

Table 30. Haemolymph Glutamate-Pyruvate Transaminase Activity (U/l) in Pila virens
starved for 10, 20 and 30 days

Period	Control	10 days	Control	20 days	Control	30 days
N	10	10	10	10	10	10
Mean value	2.87	2.13	4.81	3.22	4.95	2.42***
± SD	0.89	1.36	2.38	1.57	1.29	0.64
Range	1.58-4.04	0.63-4.39	2.64-10.00	0.50-6.33	3.29-7.09	1.03-3.21

Significance Level : *** $P < 0.001$

No significant variation in activity pattern was observed when the values in 10, 20 and 30 day starved groups were compared among themselves.

3.3.2.5 GOT:GPT Ratio

The GOT:GPT ratios in 10 (8.07 ± 2.95) and 20 day (8.78 ± 7.28) starved snails were found to be higher than the control values (5.65 ± 2.01 and 3.98 ± 1.83) but not significant. In 30 day starved snails, the value (3.19 ± 2.5) was lower than the control (4.23 ± 1.09), but not significant.

When the ratios among the experimentals were compared, the ratio in 30 days starved snails was significantly lower than the ratios in 10 ($P < 0.01$) and 20 day ($P < 0.05$) starved ones.

3.4 DISCUSSION

On comparing the total haemocyte number in P. virens during aestivation and starvation, it is obvious that total count is little affected by prolonged starvation. In 10 day starved snails, the total number of circulating haemocytes was almost the same as that of control snails. When the period of starvation was extended to 20 and later to 30 days, there appeared a slight decrease in number, though statistically insignificant, when compared with the control values. During aestivation, the significant rise in total count in one month aestivated snails was attributed to their involvement in the transportation of food

reserves (section 4.4). Since the aestivating animals are protected from external environment to a certain extent the role of haemocytes in defence is practically nil. Hence, a major proportion of haemocytes can be spared for transportation of nutrients or for other metabolic purposes. In 20 and 30 day starved snails, the marginal decrease in cell number could be due to the general decline in metabolic status of snails as a result of continued unavailability of food. Haniffa (1987) reported 1/7th reduction in metabolic activities in P. globosa when they were starved for 30 days. Similar observations on the capacity of snails to lower the metabolic levels and oxygen uptake mechanisms were made by many investigators (Berg and Ockelmann, 1959; Yanagisawa and Komiya, 1961; Akerlund, 1969; Heeg, 1977). During aestivation and starvation, since there is no food intake, it may appear that freshwater gastropods may resort to the same metabolism during these periods. However, it may be noted that for freshwater gastropods in general, and for P. virens in particular, prolonged periods of aestivation is natural and part of the life cycle, and the animal will adjust to that condition in a slow and systematic way; but starvation in nature seldom occurs; but is always induced and sudden. Hence, the snails undergoing aestivation and starvation will fail to respond in identical manner. Most of the adjustments or adaptations noticed in starved snails are to conserve the available energy reserves and to meet the challenges by adapting

to varied metabolic habits designed according to the nature of the metabolite available for energy metabolism. Reports on the effects of starvation on haemocyte number in gastropods are not available. However, comparable results are available on bivalves during short term starvation (Thompson et al., 1978).

Analysis of haemolymph glycogen and total carbohydrate levels in starved snails showed a decreasing trend in their concentration, which was found to be more pronounced in the case of haemolymph total carbohydrates. The fall in glycogen level was found to be insignificant, again, supporting the role of insulin-like hormone in maintaining a comparatively constant level of haemolymph glycogen even when dietary addition is nil. Veldhuijzen (1975) reported a similar instance of maintenance of a minimum level of blood glucose in starved L. stagnalis. Goddard et al. (1964), Ammon et al. (1967), Davidson et al. (1971), among others have also suggested hormonal involvement in gastropods for maintenance of a constant haemolymph glucose level. Joosse and Geraerts (1983) also supported the view that hormones are involved in the mobilisation of glycogen. The total carbohydrate concentration in haemolymph declined sharply in 10 day starved snails. The reduction in 10 day starved snails was 68% of the control values and in 20 days starved snails, the reduction in haemolymph total carbohydrate was found to be 65%, a little better than that of 10 day starved snails. This could be due to depletion

of carbohydrate reserves from different storage organs in the absence of dietary addition. Gluconeogenic activities aided by transaminase enzymes in 20 day starved snails is suspected and this suggestion is supported by increased levels of activity, though insignificant, of haemolymph GOT in 20 day starved snails. Carbohydrate reduction in 30 day starved snails was found to be 75%, a very significant reduction from the control values. This could be attributed to the prolonged total unavailability of food, depletion of carbohydrate reserves in storage organs as well as to the significantly lower activity levels of GOT and GPT. The significant fall in total carbohydrate levels and the maintenance of consistent levels of haemolymph glycogen in starved snails indicate two possibilities: (i) from the total carbohydrate, glucose was utilized for metabolism but not glycogen, or (ii) glycogen was also utilized but to maintain the minimum level; glucose from total carbohydrate pool was used with the involvement of insulin-like hormone. Christie et al. (1974), while studying the effects of starvation on B. glabrata observed a time dependent decrease in glycogen content. Glycogen was found to decline significantly only after 21 days of starvation. At day 28, the glycogen fell sharply. In L. stagnalis also a gradual decrease in glycogen in the mantle and in other parts of the body was observed after 6 days of starvation (Joosse and Geraerts, 1983).

Shifting of metabolic dependency from carbohydrate to other metabolites such as lipid, and protein also can be suggested during starvation. This is evident from the significantly lower concentrations of protein and lipid in the haemolymph of the starved ones than the corresponding levels of lipid and protein in the controls. Metabolite preference during starvation metabolism is reported to vary from species to species. During starvation, Littorina littorea utilized both carbohydrate and lipids (Holland et al., 1975). Thais lamellosa, during starvation, did not use lipids but polysaccharides and protein (Stickle, 1971). In the present study, the metabolic preference during starvation was found to vary according to the period of starvation as explained below.

The percentage reduction observed in carbohydrate level was found to be more applicable to low molecular weight constituent of haemolymph carbohydrates. The ratio of glycogen to total carbohydrate was found to be between 10 and 12 in control snails while in 10 and 20 days starved snails it was approximately 25, and in 30 days starved ones, 39. This indicates large scale utilization of the low molecular weight carbohydrates, preferentially over haemolymph glycogen.

Haemolymph protein concentrations in 10, 20, and 30 day starved snails were found to be significantly lower than those of control snails. The low levels of haemolymph protein indicates large scale catabolism of protein, evidently supported by the

presence of high concentrations of haemolymph urea. Lee and Cheng (1972) reported a similar instance of significant reduction in non-haemoglobin protein in the haemolymph of starved snails. They suggested that cessation or reduction of transport of protein from the digestive gland and utilization of protein for energy metabolism as the apparent reason for the decrease. Similar observations were made in starved M. edulis (Gabbot and Bayne, 1973), and B. glabrata (Becker and Hirtbach, 1975; Stanislawski and Becker, 1979). An interesting trend of slow increase in the concentration of haemolymph protein emerged with increase in starvation period when values in 10, 20 and 30 day starved snails were compared among themselves.

Lipid concentrations in haemolymph was also found to be significantly lower in 10, 20 and 30 day starved snails. This clearly demonstrates the much reported role of lipids in supplementing energy metabolism in the absence of or when there is shortage of the primary metabolite, the carbohydrate. Comparable decrease in lipid content during starvation was reported in many molluscan species (Stickle and Duerr, 1970; Riley, 1976; Nagabhushanam and Dhamne, 1977; Streit, 1978; Duncan et al., 1987).

Comparative analysis of the levels of haemolymph metabolites helps to elucidate many of the physiological adaptations, in terms of metabolic adjustments, the snails resort to during prolonged periods of starvation. In 10 day starved snails, the haemolymph

total carbohydrate level has shown a considerable decrease. The glycogen concentration is kept at the normal level in spite of the very low, total carbohydrate level. The protein reserves are also utilized at this period. This is evident from the high values of ammonia and urea in the haemolymph in 10 day starved snails. The level of haemolymph lipid or the less percentage loss could be attributed to the lesser mobilization of lipids from storage centres. From the percentage loss of metabolites, it is evident that 10 day starved snails were utilizing more of stored carbohydrates and protein, and less of lipids. In 20 day starved snails also there was reduction in haemolymph total carbohydrates. A remarkable increase in haemolymph lipid content, when compared with the value in 10 day starved snails is also observed. Very high concentrations of haemolymph ammonia and urea, along with comparatively slight decrease in carbohydrate level indicate a shift to protein catabolism at this period. Lipids are also utilized at this time period. ACP and ALP activity levels were found to reach all time low levels, suggesting clear dominance of catabolism over anabolism. The role of phosphatases in biosynthesis of protein, mucopolysaccharides and nucleic acids is well established (Johnson and McMinn, 1958; Kroon, 1952; Cox et al., 1967). A slightly elevated level of GOT in haemolymph at this period suggests active involvement of the transaminase in gluconeogenesis, probably to maintain the level of haemolymph glycogen more or less constant, in spite of the non-availability of food.

Comparatively low level of haemolymph ammonia along with comparatively (when compared with the values in 10 and 20 day starved snails) higher concentrations of lipid suggest the predominance of lipid oriented metabolism in 30 day starved snails. Total carbohydrate level was also found to be very low, probably due to decline in gluconeogenic activities at this period. Significantly low activity levels of GOT and GPT also point towards this direction.

Haemolymph urea content was found to be significantly higher in 10, 20 and 30 day starved snails than in the controls. This is because of increased nitrogen metabolism to compensate the lack of dietary addition of carbohydrates for metabolism. Urea concentration was comparatively very high in 20 day starved snails. This, along with very high ammonia concentration, clearly indicates dominance of protein catabolism for energy at this time period (see Lee and Cheng, 1972). In 30 day starved snails, ammonia concentration falls below that of control values. Urea concentration was also found to be lower than that of 20 day starved snails. A shift to lipid metabolism can be suggested as the reason for these changes. Many investigators have reported the presence of high concentrations of both urea and ammonia in the haemolymph of starved molluscs (Schmale and Becker, 1975; Bayne and Scullard, 1977; Becker and Schmale, 1978).

In freshwater gastropods, major part of the ions lost from body to the surrounding medium is compensated through dietary intake of ions. In starved snails, due to complete lack of feeding, the

ionic contents lost from body fluids are not compensated by dietary addition, and hence decline in ionic contents can be expected. Comparative analysis of haemolymph ionic concentrations of starved and fed (control) snails justifies the expected dilution of ions in starved snails. All the ions analysed (Na^+, K^+, Ca^{2+} and Cl^-), showed significantly lower concentration in the haemolymph of starved snails than in their controls. In 10, 20 and 30 day starved snails a reduction of 17, 16, and 10 per cent in Na^+ ionic concentration from the control values; 9, 16, and 16 per cent reduction in the case of Ca^{2+} ions, and 5, 14, and 11 per cent reduction in the case of Cl^- ions were observed. The reduction in ionic concentration was found to be more marked in the case of K^+ ions. In 10, 20 and 30 day starved snails, the percentage reduction of K^+ ions was found to be 57, 44, and 36 respectively. Nagabushanam and Dhamne (1977) reported increase in water content and dilution of ionic content in Paphia laterisulca during starvation. Decrease in calcium content during starvation, and the role of shell in supplementing Ca^{2+} ionic loss were reported in many gastropods (Greenaway, 1971; De With and Sminia, 1980; Mc Mahon, 1983). Very high concentration of nitrogenous products present in haemolymph during starvation could be possibly to maintain the osmolarity of the body fluid, which was upset by considerable dilution. A homoeostatic mechanism for maintenance of osmolarity of haemolymph irrespective of food intake is evidently present in P. virens.

Study of the activity patterns of selected enzymes in P. virens during 10, 20 and 30 days of starvation gives information regarding their role in metabolism. Phosphatase enzyme activities are found to be very low in the haemolymph of starved snails when compared with the activity levels in active snails. The activity level of ACP was found to be significantly low in 20 day starved snails, and for ALP in both 10 and 20 day starved snails. Information regarding the effect of starvation on activity pattern of enzymes is scarce. However, the highly reduced metabolic activity reported during starvation could possibly be the reason for the low level activities of phosphatases. Yet another reason may be that there is no increase in haemocyte number during starvation and in fact the total counts at all time periods were low, if not significantly. Low levels of transaminase activities were also noted during starvation. The decrease in activity levels of both GOT and GPT was found to be significant in 30 day starved snails. The most important role of GOT and GPT is to act as a link between protein and carbohydrate metabolism (Lane and Scura, 1970). In 30 day starved snails, both protein and carbohydrate reserves were minimum resulting in the switching over to lipid metabolism. When the levels of the precursors are low, both GOT and GPT have no significant role to play, and hence the extremely low activity levels. The activity patterns of enzymes during starvation give us a clear picture of time dependent metabolite preference in starved

P. virens. During early periods of starvation, carbohydrate and proteins were preferred. Later, when starvation was prolonged to 30 days, a clear shift in metabolite selection from protein-carbohydrate to lipid was observed. The varying concentrations of haemolymph nitrogenous products at different periods of starvation also support this.

C H A P T E R - IV

EFFECTS OF AESTIVATION ON HAEMOLYMPH CONSTITUENTS OF *PILA VIRENS*

4.1 INTRODUCTION

Pila virens, aestivates in summer when ponds, streams, and paddy fields inhabited by them dry up (Meenakshi, 1956, 1958; Shylajakumari, 1975). During this period, the snail withdraws into the shell, tightly closes the shell aperture with the operculum, and remains in a state of torpor until the advent of rain. Reports of detailed investigations on the various aspects of physiological adaptations seen in snails during this period of inactivity, are available. However, information in the field of haematology, especially regarding changes in haemolymph constituents of aestivating prosobranchs, is scarce.

Aestivation in the broadest sense is a widespread phenomenon among molluscs (Boss, 1974), involving lowered metabolic demands along with physical isolation from the environment to cope in sites with adverse environmental conditions such as temperature extremes, and desiccation. It is a very common adaptation among freshwater prosobranchs (Ramanan, 1903; Dundee, 1957; Komiya, 1961; Meenakshi, 1956, 1958, 1964; Little, 1968; Shylaja and Alexander, 1975a,b). Metabolism during aestivation was reported to be aerobic in *Pila ovata* (Coles, 1968) and *Pomacea urceus* (Burky et al., 1972), and anaerobic in *P. virens* (Meenakshi, 1956, 1957) and *P. globosa* (Krishnamoorthy and Brahmanandam, 1970). Reddy and Swami (1976)

reported accumulation of lactate in aestivating P. globosa. Earlier, Meenakshi (1956, 1958, 1964) found progressive depletion of the glycogen reserves and accumulation of lactic acid in the tissues of the snail during aestivation, followed by a repayment of oxygen debt and disappearence of lactic acid during the revival of the snail in the post-aestivation period. A number of detailed studies on aestivating cyclophoraceans have been made. Biomass loss during aestivation was found to be considerable (35-60%), the most important being water loss (Burky et al., 1972; Haniffa, 1978).

Glycogen provides most of the energy used by P. globosa and P. virens during aestivation (Meenakshi, 1956; Reddy and Swami, 1976). Marisa cornuarietis also utilizes primarily carbohydrates during aestivation (Horne, 1979). Oxygen uptake rates in aestivating snails are reduced to a fraction of their active levels (Coles, 1968; Burky et al., 1972; Rao et al., 1972; Haniffa, 1978). Horne (1979) in his detailed studies on physiology of aestivation reported that during aestivation, M. cornuarietis utilized primarily carbohydrate (56%) and some protein (33%) for energy requirements. During aestivation, the rate of respiration in Bulimulus dealbatus dropped to 16% of the resting rate within 3 days and remained at that level. The R.Q. was about 0.82 with extended aestivation up to 120 days indicating utilization of protein reserves. After 70 days of aestivation, there appeared a little or no decline in lipid or water content, but there was 85% loss in carbohydrate reserves,

and 21% loss in body protein. The loss of protein nitrogen was found to be balanced by the increase in urea nitrogen (Horne, 1971, 1973a,b).

Burky (1969, 1971) reported a specialized physiological adaptation "reverse acclimation" in aestivating Ferrissia rivularis, which is characterised by conservation of energy stores by reduction of O_2 uptake and adjustments in metabolism. Alternate anaerobic pathways are reported in many freshwater pulmonates during aestivation for conservation of energy (von Brand et al., 1950; Mehlman and von Brand, 1951). In B. glabrata, during aestivation, the rate of oxygen consumption declined to less than 20% that of normal snails, and 50 to 60% of the body organic matter especially protein was found depleted after 128 days of aestivation (von Brand et al., 1957). Lactic acid which accumulates during early periods disappeared within 30 days of aestivation indicating aerobic catabolism during aestivation. Water loss in freshwater pulmonates during aestivation was found to be prevented by secretion of layers of dried foamy mucus (Machin, 1975). There was 75% reduction in purine metabolism, indicating an overall reduction in the metabolic rates in Otala lactea (Pulmonata) during aestivation (Horne, 1973a, Machin, 1975). Drop in oxygen consumption and Kreb's cycle oxidation followed by consumption of tissue and haemolymph body reserves, particularly carbohydrates were reported by many (Singh and Nayeemunnisa, 1976; Heeg, 1977; Krupanidhi et al., 1978; Swami and Reddy, 1978; Horne, 1979).

Many metabolic adaptations of P. globosa and P. virens during aestivation are known. Meenakshi (1964) reported many aspects of metabolic adjustments in 6 month aestivated P. virens. Anaerobic glycolysis coupled with lipid synthesis was suggested in P. virens during aestivation. When compared with normal snails, the metabolic level of one month starved P. globosa was found lowered by 1/7th while in aestivating snails the decline was 1/18th of that of normal snails (Haniffa, 1987). Similar observations on the capacity of the snail to lower its metabolic levels have also been made in P. virens (Meenakshi, 1964), P. ovata (Visser, 1965), and Morula granulata (Umadevi et al., 1986). The view that aestivating P. virens consumes no oxygen and is totally anaerobic has been challenged by experiments with P. globosa in which injected (U-^{14}C) glucose was converted to $^{14}CO_2$ (Reddy and Ramamurthi, 1973); however, in this case a totally anaerobic metabolism is still possible as CO_2 can be produced via the succinate pathway. While studying the physiology of aestivation in P. globosa, Reddy and Swami (1968) reported the occurrence of concentrated cytochrome proteins (helicorubin) in the gut of P. globosa, suggesting large scale morphological changes in mitochondria of aestivating snails implying metabolic adjustments during aestivation. Further, Reddy and Swami (1967) reported that the level of ATP was significantly higher and that of ADP lower in aestivating snails than in active snails.

von Brand et al. (1957) suggested proteins as the primary energy source of aestivating snails. Terrestrial molluscs such

as Helix aspersa and O. lactea showed changes in free amino acid levels in the tissues and haemolymph during desiccation (Campbell and Speeg, 1968; Wieser and Schuster, 1975). The total protein level in aestivating snails was reported to be 30-40% of the normal level reported in active snails (Swamy and Reddy, 1978). In Bulinus africanus (pulmonata), the main reserve was found to be protein, and lipids were metabolized preferentially over carbohydrate during aestivation of Cryptozoa ligulata (pulmonata).

During the early stages of aerial aestivation, freshwater pulmonates have been reported to shift from ammonia to urea production as the major nitrogenous waste. Urea appeared to be retained in the body fluids of aestivating snails (von Brand et al., 1957; Newman and Thomas, 1975). Reports on the excretory pattern of normal and aestivating P. globosa (Saxena, 1952; 1955; Reddy and Swami, 1963, 1975; Reddy et al., 1974; Swami and Reddy, 1978; Chaturvedi and Agarwall 1979) and P. virens (Shylajakumari, 1975; Shylaja and Alexander, 1975a) are available. Reddy et al. (1974) reported a decrease in ammonia and urea levels in aestivating P. globosa. Shylajakumari (1975) reported an increase in urea and uric acid contents of P. virens in all the tissues examined. However, the urea content decreased when aestivation prolonged beyond three months. The uric acid content showed increase in concentration along with progress in the period of aestivation. Conversion of metabolically produced ammonia to urea is a homoeostatic mechanism that allows retention of large quantities

of nitrogenous wastes in the haemolymph, while ammonia remains well below toxic levels (Horne, 1971). In B. glabrata, a decided shift towards ureotelism was reported during longer periods of aestivation. In snails aestivated for 30 days, the percentages of nitrogenous wastes detected were 74% urea, 13% uric acid, and 13% ammonia. During longer periods of aerial aestivation (97-174 days) in Bakerilymnae cockerelli, a decided shift to uricotelism was observed (49-58% uric acid, 17% ammonia nitrogen, and 24-34% urea nitrogen) (Newman and Thomas, 1975). During this transition from the ammonotelic - uricotelic pattern of the active feeding snails (B. dealbatus) to the ureotelic pattern of the aestivating snails, there is a sharp increase in the levels of all the urea cycle enzymes, and a 10 fold increase of ^{14}C incorporation into urea from $^{14}CO_2$ (Horne, 1971, 1973b). At the same time, the small amount of urease in feeding snails seems to be lost or inhibited in aestivating snails. Therefore, the urea accumulated during aestivation results from activation of the urea cycle biosynthetic pathway, and a suppression or inactivation of the urease activity. Horne (1973a) concluded that the enhanced urea biosynthesis during aestivation is probably an ammonia detoxifying process. The increased concentration of urea may add to the osmotic pressure within the tissues and could play a role in reducing water loss by evaporation during aestivation.

Several authors have reported variations in osmotic pressure and ionic composition in the haemolymph of gastropods during aestivation and hibernation (Burton, 1965, 1969; Grainger, 1969). In hibernating H. pomatia, osmotic pressure and ion content were found to be markedly higher than in the active snails. Little (1968) reported that in Pomacea lineata and Pomacea depressa, blood and urine osmolarities doubled during aestivation. Wieser (1980) reported the formation of alanine in snails during anoxia for maintenance of osmotic balance. Meenakshi (1958) found that lactate was a major anaerobic end product in P. virens. The pH of the blood of active P. virens was found to be on the alkaline side, 7.1 to 7.3 (Meenakshi, 1956). But when the snails were made anaerobic by being placed in boiled water, the haemolymph pH fell to 4.5, and lactate accumulated (Meenakshi, 1956). De With et al., (1980) reported that a low internal pH appropriately stimulated exchange of sodium and hydrogen ions and inhibited exchange of chloride and bicarbonates in L. stagnalis. In Rapana thomasiana, exposed to air, the calcium content and buffer value of haemolymph were found to rise as in bivalves (Alyakrinskaya, 1972). Reddy and Naidu (1978) reported significant increase in the blood copper level in P. globosa upon aestivation while a reverse trend was observed in hepatopancreatic copper. The increase in blood copper level during aestivation was found to be associated with corresponding increase in the haemocyanin content.

Literature on the effect of aestivation on certain other haematological parameters such as haemocyte number are still wanting. However, the effect of exposure to air on haemocyte number in aquatic molluscs was studied by Thompson et al. (1978). Thompson et al. (1978) reported an increase in the number of circulating haemocytes when Mytilus californianus was exposed to air. The high number of haemocytes was found to be maintained throughout the exposure period of 25 hours.

Many reports on enzyme activities in haemolymph and other tissues of active and aestivating gastropods are available. Variation in the levels of glutamine synthetase was reported in tissues and haemolymph of active and aestivating pulmonate snails (Campbell and Bishop, 1970; Chetty et al., 1979). Reddy and swami (1975) reported a doubling in the glutamine synthetase levels in the hepatopancreas of P. globosa during aestivation. In a series of feeding-starvation-aestivation experiments at different temperatures, arginase levels in the hepatopancreas of O. lactea were found to be the highest in starved or aestivating snails at 23°C, and lowest in feeding animals. In B. dealbatus, activity levels of arginases in the hepatopancreas were found to increase almost linearly with the increase in days of aestivation (Horne, 1973b). Lower activity levels of various enzymes involved in different metabolic activities were reported in aestivating snails. The activities of succinic dehydrogenase, glutamic dehydrogenase

and cytochrome C oxidase were found to be decreased by 50 to 60% in different tissues of the snail during aestivation (Reddy and Swami, 1968). In H. aspersa, lactate dehydrogenase was found to function in anoxic conditions to direct pyruvate to lipid synthesis, and to convert lactate to pyruvate (Livingstone and de Zwaan, 1983). Shylaja and Alexander (1974), after studying the activity pattern of alkaline phosphatase in P. virens during active and aestivating conditions, reported that the levels of ALP activity were considerably lower in all tissues examined in aestivating snails. Similarly, Swami and Reddy (1978) reported low levels of activity of GOT and GPT in aestivating P. globosa when compared with active counterparts.

In this chapter the effects of prolonged, and induced aestivation on total haemocyte counts, on haemolymph organic and inorganic constituents and on the activity levels of certain haemolymph enzymes are reported.

4.2 MATERIALS AND METHODS

Methods of collection of test animal, laboratory conditioning, collection of haemolymph, estimation of total haemocyte count, estimation of haemolymph glycogen, total carbohydrate, total protein, total lipid, urea, ammonia, sodium, potassium, calcium, chloride, and ACP, ALP, GOT and GPT activity levels were the same as explained in section 2.2. For computation and expression of the results, methods explained in section 2.2.7 were followed.

4.2.1 Selection of animal groups

Healthy P. virens belonging to the intermediate size group were selected for aestivation studies. They were laboratory conditioned for 72 hours and placed on filter paper for drying. The dried specimens were weighed and numbered with marking pencil. The selected snails were then buried in dry sand kept in wooden boxes and placed in undisturbed place of the laboratory where there was plenty of ventilation. At the interval of one, two, and three months, the aestivating snails were taken out of the wooden boxes directly used for studies. Anticipating mortality, for each time period 35 specimens were conditioned as experimentals, and 45 specimens were maintained for each time period as controls in 50 L capacity fibre glass tanks filled with water and fed with Hydrilla. In control tanks water was replenished completely every 24 hr; aerated regularly, and cleaned daily to prevent algal growth. In the experimental groups (35 each), 15 specimens from each group were used for estimation of total haemocyte counts, and from the same 15 randomly chosen 12 were used for estimation of haemolymph organic and inorganic constituents, and activity levels of haemolymph enzymes. From the control group, 20 specimens were used for estimation of total haemocyte counts, haemolymph glycogen, total carbohydrates, protein, lipid, sodium, potassium, calcium, and chloride, and from the same 20 randomly selected 15 specimens were used for estimation of haemolymph ammonia and urea, and 12 for

estimation of activity levels of the four enzymes. All the snails belonged to the same population. From the remaining specimens duplicates were run as experimentals and controls.

4.3 RESULTS

4.3.1 Haemolymph Constituents

4.3.1.1. Total haemocyte number (Table 31)

Total haemocyte counts in one month aestivated snails were found to be significantly higher than the counts in active snails ($P < 0.001$). No significant variation in counts was seen on comparison of the counts in active and two month aestivated snails, while the total number of haemocytes was found to be significantly lower in three month aestivated snails ($P < 0.001$).

Comparison of total counts among the three sets of aestivated snails (i.e., one, two and three months of aestivation) revealed very interesting results. Statistically significant fall in total haemocyte count was observed with progress in time of aestivation. The fall in count was significant in two month aestivated snails when compared with the count in one month aestivated ones ($P < 0.001$), and significantly lower in three month aestivated snails than in two month aestivated ones ($P < 0.001$). The highest haemocyte number was seen in one month aestivated snails, higher than in the active snails ($P < 0.001$), and lowest in three month aestivated snails, lower than in the active snails ($P < 0.001$).

Table 31. Total haemocytes/mm^3 in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	20	15	15	15
Mean value	6486.00	10183.00***	6474.00	3810.00***
± SD	2255.10	1656.20	1257.60	1057.10
Range	3480 - 9840	8120 - 13400	4270 - 8160	1266 - 5000

Significance level : *** $P < 0.001$

4.3.1.2 Glycogen (Table 32)

Haemolymph glycogen contents in one and three month aestivated snails were found to be significantly higher than the value in active snails. ($\underline{P} < 0.001$ and $\underline{P} < 0.01$, respectively). No significant variation in values was seen in two month aestivated snails from the control values.

Statistical analysis of the glycogen values of the three sets of aestivated snails revealed the following. Glycogen content of two month aestivated snails was found to be significantly lower than that of one month ($\underline{P} < 0.01$), and three month aestivated snails ($\underline{P} < 0.05$). No significant variation in value was observed in one and three month aestivated snails.

4.3.1.3. Total carbohydrate (Table 33)

Statistically significant variations were absent when haemolymph total carbohydrate content of one and three month aestivated snails were compared with that of active snails. However, significant decline in value ($\underline{P} < 0.01$) was observed in two month aestivated snails when compared with that of active snails.

When toal carbohydrate levels in the three sets of aestivated snails were compared, no significant variation in values was found in one month and three month aestivated snails, while the total carbohydrate content in the haemolymph of two month aestivated snails was found to be significantly lower than that of one month ($\underline{P} < 0.01$) and three month aestivated snails ($\underline{P} < 0.05$).

Table 32 Haemolymph Glycogen (μg glucose/ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	20	12	12	12
Mean value	82.99	153.16***	71.16	144.89**
± SD	35.39	41.98	67.69	76.83
Range	34.46 - 156.50	106.26 - 215.40	9.00 - 218.20	76.88 - 268.80

Table 33. Haemolymph Total Carbohydrate (μg glucose/ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	20	12	12	12
Mean value	1667.25	2169.36	1150.88**	1984.16
± SD	285.91	1073.69	518.75	1110.83
Range	1191.80 - 2236	1090.20 - 4145.4	534.20 - 2200	631.20 - 4584

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

4.3.1.4 Total protein (Table 34)

When protein values of aestivated snails were compared with that of active snails the following pattern emerged. The total protein content of one month aestivated snails registered no significant variation with the haemolymph protein content of active snails. Two and three month aestivated snails registered significant time dependent decrease in protein content, when compared with the value of active snails ($\underline{P} < 0.01$ and $\underline{P} < 0.001$).

When the haemolymph protein content in the three sets of aestivated snails was compared among themselves, the protein content in two month aestivated snails was found to be significantly lower than that of one month aestivated snails ($\underline{P} < 0.05$). The same trend was seen when protein contents in two and three month aestivated snails were compared ($\underline{P} < 0.05$). Similarly, the protein content in the haemolymph of three month aestivated snails was found to be significantly lower than that of one month aestivated snails ($\underline{P} < 0.01$).

4.3.1.5 Total lipid (Table 35)

Aestivation upto two months produced no significant variation in haemolymph lipid level. However, three month aestivated snails registered a significant fall in the level of total lipids in the haemolymph when compared with that in active snails ($\underline{P} < 0.05$).

Table 34. Haemolymph Protein (mg/ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	20	12	12	12
Mean value	19.97	20.19	13.91**	11.92***
± SD	2.92	6.35	6.08	5.08
Range	13.88-25.22	10.54-30.54	5.41-24.40	3.71-19.10

Table 35. Haemolymph Lipid (µg cholesterol/ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	20	12	12	12
Mean value	80.88	89.32	88.31	47.77*
± SD	42.13	34.13	20.53	19.78
Range	30.94-172.31	49.72-156.91	68.51-143.65	30.21-95.49

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

Total haemolymph lipid content was found to be significantly lower in three month aestivated snails when compared with that in one month ($\underline{P} < 0.01$) and two month aestivated snails ($\underline{P} < 0.001$). No significant change in lipid content was observed when the value in one month and two month aestivated groups were compared.

4.3.1.6 Urea (Table 36)

No significant change in urea content was observed in one month aestivated snails when the value was compared with that in active snails. But two month aestivated snails registered significant fall in urea content ($\underline{P} < 0.05$), and three month aestivated ones showed significant rise in urea level ($\underline{P} < 0.001$) when compared with the level in active snails.

Statistically significant increase in urea level was observed in three month aestivated snails when the value was compared with those in one ($\underline{P} < 0.001$) and two month ($\underline{P} < 0.001$) aestivated snails.

4.3.1.7 Ammonia (Table 37)

Haemolymph ammonia levels in snails during aestivation were found to be significantly higher than the level in active snails. The increase was in the following degrees; one month aestivated - ($\underline{P} < 0.05$), two month aestivated - ($\underline{P} < 0.001$), and three month aestivated - ($\underline{P} < 0.05$).

When haemolymph ammonia levels in one, two, and three month aestivated snails were compared, no statistically significant variation was observed.

Table 36. Haemolymph Urea (mg/100 ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	15	12	12	12
Mean value	2.61	2.69	2.05*	15.81***
± SD	1.50	1.38	1.41	7.43
Range	1.48-3.66	1.01-6.66	0.81-5.45	8.48-29.49

Table 37. Haemolymph Ammonia (mg/100 ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	15	12	12	12
Mean value	0.16	0.30*	0.39***	0.33*
± SD	0.14	0.14	0.05	0.20
Range	0.04-0.41	0.19-0.62	0.33-0.47	0.06-0.73

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

4.3.1.8 Sodium (Table 38)

Haemolymph sodium content in aestivating snails was found to be significantly higher than that of normal active snails. One, two and three month aestivated snails recorded the increase in the following degrees ($\underline{P}<0.001$), ($\underline{P}<0.001$), and ($\underline{P}<0.05$).

When comparisons were made among the aestivated groups, significant decrease in sodium level was seen in three month aestivated snails ($\underline{P}<0.01$) when compared with the level in two month aestivated snails. Similarly, significant decrease was observed in three month aestivated snails ($\underline{P}<0.001$), when compared with the level in one month aestivated snails.

4.3.1.9 Potassium (Table 39)

In one ($\underline{P}<0.001$), two ($\underline{P}<0.001$), and three month ($\underline{P}<0.01$) aestivated snails, significant increase in potassium level was observed on comparison with the level in active snails.

When comparisons were made among one, two, and three month aestivated snails, potassium level in two month aestivated snail was found to be higher than that in three month aestivated snails ($\underline{P}<0.001$), but lower than that in one month aestivated snails ($\underline{P}<0.001$). In three month aestivated snails, potassium level was significantly lower than that in one ($\underline{P}<0.001$), and two month aestivated snails ($\underline{P}<0.01$).

able 38. Haemolymph Sodium (μ equivalents/ml) in Pila virens aestivated for 1, 2 and 3 months

eriod	Active snails	1 month	2 months	3 months
	20	12	12	12
ean value	40.48	50.79***	52.39***	43.55*
SD	2.59	2.69	7.22	4.30
ange	35.65-44.35	47.80-54.78	40.87-68.69	38.26-49.57

Table 39. Haemolymph Potassium (μ equivalents/ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	20	12	12	12
Mean value	1.84	4.14***	2.99***	2.14**
± SD	0.25	0.56	0.40	0.19
Range	1.54-2.05	3.07-5.13	2.05-4.62	2.05-2.56

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

4.3.1.10 Calcium (Table 40)

Haemolymph calcium contents in one, two, and three month aestivated snails were found to be significantly higher ($P < 0.001$) than that in active snails.

Haemolymph calcium level in two month aestivated snails was found to be significantly higher than that in one ($P < 0.05$) and three month aestivated snails ($P < 0.01$). When calcium levels in three and one month aestivated snails were compared, no significant variation was found.

4.3.1.11 Chloride (Table 41)

Chloride content was found to be significantly higher in one month ($P < 0.001$) two month ($P < 0.001$), and three month ($P < 0.05$) aestivated snails than the values in active ones.

No significant variation was found when the values in one month and three month aestivated snails were compared, while two month aestivated snails have significantly higher level of chloride than in one month aestivated ($P < 0.01$), and three month aestivated snails ($P < 0.001$).

4.3.2 Activity Pattern of Enzymes

4.3.2.1 Acid phosphatase activity (Table 42)

Acid phosphatase activity level was found to be little affected by aestivation. In one and three month aestivated snails,

Table 40. Haemolymph Calcium (μ equivalents/ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	20	12	12	12
Mean value	17.30	26.08***	31.83***	25.16***
± SD	4.27	2.81	6.53	2.52
Range	15.00-20.00	22.00-31.00	22.00-42.00	21.00-28.00

Table 41. Haemolymph Chloride (μ equivalents/ml) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	20	12	12	12
Mean value	49.90	59.75***	69.33***	56.16**
± SD	4.69	6.89	8.63	5.32
Range	46.00-62.00	48.00-70.00	52.00-87.00	48.00-66.0

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

no significant variation in enzyme activity level was observed. But in two month aestivated snails, significant fall in enzyme activity was observed when compared with the level in active snails.

When enzyme activities in the haemolymph of three sets of aestivated snails were compared among themselves, no statistically significant variation was found.

4.3.2.2 Alkaline phosphatase activity (Table 43)

Significant elevation in haemolymph ALP activity level was noticed in snails aestivated for one month ($P < 0.001$). However, when the period of aestivation was extended to two and three months, no significant variation was found in the activity levels in aestivated and active snails.

The increase in haemolymph enzyme activity observed in one month aestivated snails was found to be significantly higher than that in two ($P < 0.01$) and three month aestivated snails ($P < 0.01$). Statistically significant variation was absent when activity levels in snails aestivated for two and three months were compared.

4.3.2.3 Glutamate-oxaloacetate transaminase activity (Table 44)

Significant variation was not found when the level of activity of the enzyme in one, two and three month aestivated snails was compared with that in active snails.

Among aestivated snails also the values did not show significant variation.

Table 42. Haemolymph Acid Phosphatase Activity (U/l) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	12	12	12	12
Mean value	9.31	9.04	7.16**	7.72
± SD	1.51	4.46	2.30	2.74
Range	7.27-13.11	4.43-20.66	5.34-12.66	5.73-15.01

Table 43. Haemolymph Alkaline Phosphatase Activity (U/l) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	12	12	12	12
Mean value	4.22	23.06***	8.07	8.39
± SD	2.09	12.75	6.85	7.17
Range	1.15-9.25	7.12-47.55	1.47-18.27	1.27-21.87

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

Table 44. Haemolymph Glutamate-Oxaloacetate Transaminase Activity (U/l) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	12	12	12	12
Mean value	20.90	23.47	21.90	18.77
± SD	3.45	9.56	3.98	8.03
Range	16.97-27.42	16.53-41.41	16.10-30.05	10.84-34.41

Table 45. Haemolymph Glutamate-Pyruvate Transaminase Activity (U/l) in Pila virens aestivated for 1, 2 and 3 months

Period	Active snails	1 month	2 months	3 months
N	12	12	12	12
Mean value	4.73	4.84	7.15**	3.36
± SD	1.67	4.08	2.30	1.79
Range	2.91-7.53	0.95-13.06	5.33-12.66	1.11-7.41

Significance Level : ** $P < 0.01$

4.3.2.4 Glutamate pyruvate transaminase activity (Table 45)

When the enzyme activity levels in active and aestivated snails were compared, no significant variation was found in the activity levels in one and three month aestivated snails from the controls. However, two month aestivated snails registered significant increase in GPT activity when compared with the level of activity in active snails ($\underline{P} < 0.01$).

When the three sets of aestivated snails were compared among themselves, the enzyme activity level was found to be significantly higher in two month aestivated snails ($\underline{P} < 0.001$) than in three month aestivated snails. Significant variations were not found when the activity levels in one and three month aestivated snails and in one and two month aestivated snails were compared.

4.3.2.5 GOT:GPT Ratio

The GOT:GPT ratio in one (7.04 ± 3.50), two (7.22 ± 2.19), and three month (5.83 ± 3.19) aestivated snails were found to be higher than that of active snails (4.79 ± 1.32). But the increase was found to be statistically significant only in two month aestivated snails ($\underline{P} < 0.01$).

When the GOT:GPT ratios of the experimentals were compared among themselves, no statistically significant variation was observed.

4.4 DISCUSSION

Many of the physiological adaptations in P. virens during aestivation have been investigated. The present study aims to find out the effects of aestivation on different haematological parameters of P. virens. The total number of circulating haemocytes was found to be greatly influenced by aestivation. In one month aestivated snails, the total count was found to be very high when compared with control values. P. virens usually aestivate for six months, corresponding to the drought period prevalent in different parts of S. India, but are capable of aestivating for years together (Shylajakumari, 1975). The high cell count in one month aestivated snails can be considered as an initial response to aestivation to boost the functions of haemocytes, such as nutrient digestion and transport, excretion etc., which are essentially required during extended periods of dormancy. Gradual but steady decrease in total haemocyte number with progress in the time of aestivation justifies this. Increase in haemocyte number as an immediate reaction to disappearance of water in the environment was reported in M. californianus (Thompson et al., 1978). Analysis of other haemolymph constituents especially concentrations of haemolymph carbohydrate, glycogen, and ALP activity level in aestivating snails, clearly suggests the role of haemocytes in these functions. Large scale increase in haemocyte number was observed in P. virens when they were exposed to sublethal concentrations of pesticides. The

significant increase in the number of haemocytes during the initial periods of pesticide (Dimecron^{R}) exposure was accompanied by significant increase in the activity levels of phosphatases also. The increase in the number of haemocytes in one month aestivated snails was accompanied by elevation in ALP activity level but not in ACP activity level, and the haemolymph concentrations of carbohydrate and glycogen also increased. This clearly demonstrates the differences in biochemical constitution of haemocytes normally present and those produced/mobilised during these two different types of environmental stress. In the former condition, the haemocytes responded by increasing the levels of enzymes while in the latter, the synthesis and transport of metabolites were given priority. This further supports the proposition that there exists different subpopulations of haemocytes with distinct cytochemical and functional roles in molluscs (Mohandas, 1985; Dikkeboom et al., 1985) which was proved later by Cheng and Downs,(1988).

Concentrations of glycogen and total carbohydrate in haemolymph of P. virens showed considerable variations during different periods of aestivation. A significant rise in haemolymph glycogen content was observed in 1 and 3 month aestivated snails but the corresponding rise in total carbohydrate levels was not significant. In 2 month aestivated snails, the level of total carbohydrate was found to be significantly lower but glycogen concentration was almost the same as that of active snails. The

possible role of insulin-like hormone in maintenance of a constant haemolymph glycogen level has been suggested in gastropods (see section 2.4). However, the observed glycogen levels in aestivating snails tend to modify the suggestion in the sense that, insulin-like hormone, if present, is probably to keep a minimum level of glycogen in haemolymph by removing glucose from haemolymph through stimulating the synthesis of glycogen as indicated by Joosse and Geraerts (1983). But elevation in the level does not appear to be under hormonal control. In other words, the hormonal control suggested in maintenance of haemolymph glycogen level in P. virens is meant for keeping a minimum level in haemolymph beyond which it is not allowed to decrease. A similar instance of maintenance of a minimal level of haemolymph glycogen was reported in L.stagnalis (Veldhuijzen, 1975). Accordingly, the level of haemolymph glycogen in L. stagnalis is not allowed to fall below the level of 20 μg/ml through endocrine control.

Increase in haemolymph carbohydrate content in one month aestivated snails can be partly attributed to the high elevation in total number of haemocytes. The capacity of molluscan haemocytes in transporting large quantities of carbohydrates is well documented (Devoigne and Sparks, 1968; Armstrong et al., 1971). In two month aestivated snails, total carbohydrate level was found to fall below the control values but glycogen level though low was not significantly lower than the control level. The significant fall

in total carbohydrate level may be indicative of the fact that a significant quantum of glucose was removed from haemolymph by stimulating the synthesis of glycogen so as to maintain a minimum level of glycogen in the haemolymph. It is also possible that at this period glycogen was not utilized but simple sugars were utilized to maintain minimum metabolism, and hence the lower total carbohydrate level. The total number of haemocytes also declined, but the decrease was not significantly lower than the control level, indicating the many other functions played by haemocytes than transportation of metabolities. In three month aestivated snails, surprisingly, both haemolymph glycogen and total carbohydrate levels increased. Since the snails were not feeding during this period, the hypersynthesis of carbohydrate may be through intermediary metabolism. The transport of carbohydrates by haemocytes from the storage organs can be ruled out on the basis that the total number of haemocytes at this time period was found to be minimum; significantly lower than that of active snails.

Many of the metabolic adjustments in Pila during aestivation are known. Meenakshi (1964) reported anaerobic glycolysis coupled with lipid synthesis in P. virens during aestivation. High concentrations of glycogen, total carbohydrate, and lipid during early periods of aestivation noted in the present study also support the reports of Meenakshi (1964). Metabolically expensive anaerobic glycolysis along with lack of dietary intake will result in the

heavy utilization of the body reserves within a short period. But this has been overcome by greatly reducing the metabolic level in aestivating Pila. Haniffa (1987) reported a 1/18th fold reduction in metabolic level in 1 month aestivated P. globosa. This kind of reduction in metabolic level explains the apparent slow expenditure of metabolites in aestivating P. virens also. Similar observations on the capacity of the snails to lower its metabolic levels have also been reported in P. ovata (Visser, 1965), Morula granulata (Umadevi et al., 1986), and in P. virens (Meenakshi, 1964). Anaerobic glycolysis, (von Brand et al., 1950; Mehlman and von Brand, 1951), drop in oxygen consumption, and Kreb's cycle oxidation during aestivation were reported in many gastropods (Singh and Nayeemunnisa, 1976; Heeg, 1977; Krupanidhi et al., 1978; Swami and Reddy, 1978).

In two month aestivated snails, a clear shift in metabolic activities is evident from the value of haemolymph proteins. Protein level showed considerable decline unlike in one month aestivated snails. At this period utilization of protein through transamination of aminoacids can be considered as a reason for the sharp decline in haemolymph protein concentration. This suggestion is supported by the high activity level of GPT noticed at this period which is essentially involved in aminotransferase activity. Significant increase in haemolymph ammonia at this time period also suggests protein degradation. In 3 month aestivated snails also haemolymph

protein level was found to be significantly lower. This significant decrease in 3 month aestivated snails can be attributed to the direct catabolism of proteins, which is evidently supported by the high value of haemolymph urea concentration at this time period.

In 3 month aestivated snails, the haemolymph is characterised by a sudden fall in lipid level which is significantly lower than that of active snails. The fall in lipid level can be explained as its catabolism may be for carbohydrate synthesis, evidently supported by the high values of haemolymph glycogen and total carbohydrates at this time period. The rise in haemolymph carbohydrate level at this period cannot be attributed to the transportation of this nutrient by haemocytes because the total number of haemocytes at this period was well below the control values. At this time period, the lipid reserve can be considered to be involved in providing energy for varied physiological activities.

The excretory pattern of aestivating P. virens was extensively worked out by Shylajakumari (1975). Accordingly, up to 3 months of aestivation the tissue urea content increases steadily while after 3 months the accumulation of urea ceases, instead uric acid starts to accumulate. However, the level of NH_3-N in tissues is kept at a low level throughout the period of 12 months of induced aestivations. In the present study, the haemolymph ammonia concentration was found to be significantly higher

than that of active snails but the level was not found to increase with increase in the time of aestivation. From these observations it can be stated that the haemolymph level of ammonia is always kept below the toxic concentration even in periods of aestivation and the level of activity seen in aestivating snails may be within the limit of tolerance. Haemolymph urea concentration reflects the rate of nitrogen catabolism. In one month aestivated snails, the urea content in haemolymph was found to be the same as that in normal active snails, indicating normal level of nitrogen metabolism. In 3 month aestivated snails the highly significant fall in haemolymph protein level accompanied by low activity levels of transferase enzymes, point to the possibility of increased protein catabolism for energy production, as evidenced by very high level of urea concentrations in the haemolymph.

Since the aestivating snails are completely isolated from water environment, the pattern of ionic regulation is expected to be different from that of active snails. The major problem facing active snails is that of the danger of dilution of body fluids by the effect of hypotonic external medium while in aestivating snails, the major problem is that of water loss. Water and body fluid loss are minimised in aestivating snails. Since there is no excretory water loss, ionic loss also is practically nil. In addition to the morphological and anatomical modifications to prevent water

loss during aestivation, physiological adjustments are also made to minimise water loss. Body fluids are made more concentrate during this period by concentrating metabolites, nitrogenous products and ions. All the ions studied (Na^+, K^+, Ca^{2+}, and Cl^-) were found to be concentrated in the haemolymph of aestivating snails, much above the normal values. Na^+ and Cl^- ions behave more closely than Na^+ and K^+ ions. They were in highest concentrations in the haemolymph of 2 month aestivated snails than in one and three month aestivated snails. Ca^{2+} ions were also found to be the highest in two month aestivated snails. In three month aestivated snails, all the ions were in lowest concentrations which was, of course, significantly higher than those in active snails. Whether the comparatively high concentrations of ions in two month aestivated snails is to compensate the decreased levels of haemolymph glycogen, total carbohydrate, protein and urea in haemolymph of two month aestivated snails is not known. Comparable results of higher concentration of haemolymph ions during aestivation were reported in many gastropods (Burton, 1965; 1969; Little, 1968; Grainger, 1969). Besides their important role in many physiological functions, one of the major roles played by haemolymph Ca^{2+} ions is that of buffering the pH of the medium. The possible lowering of haemolymph pH during aestivation can stimulate increased levels of Ca^{2+} ions for buffering. Alyakrinskaya (1972) reported similar increase in haemolymph Ca^{2+} ions content in R. thomasiana when withdrawn from water.

Results of the present investigations on the level of activities of the enzymes, acid phosphatase, alkaline phosphatase, glutamate-oxaloacetate transaminase, and glutamate-pyruvate transaminase disagree with the reports of Shylaja and Alexander (1974), and Swami and Reddy (1978). Shylaja and Alexander (1974) reported that the activities of tissue ALP was considerably low in aestivating snails on comparison with those in active snails. But in the present study the ALP activity levels in haemolymph of aestivating snails were found to be high, and significantly higher in one month aestivated snails. The possible reason for the high level activity of ALP in one month aestivated snails could be their active involvement in various synthetic activities meant for preparing the animal for longer periods of dormancy. Phosphatases are proved to be involved in various synthetic activities (Johnson and Mc Minn, 1958; Kroon, 1952; Gutman, 1959; Cox et al. 1967). Yet another reason may be the significant increase in total haemocyte count at this time period.

However, the activity level of ACP in the haemolymph of aestivating snails was found to be below the normal level of activity. The fall in activity level was significantly lower in two month aestivated snails. Another interesting aspect observed in enzyme analysis is that of selective hypersynthesis of ALP alone in one month aestivated snails. A positive correlation can be observed while comparing the activity level of ALP and total

haemocyte number. Since ACP fails to register a high level of activity along with increase in haemocyte number, again, the concept of subpopulations of haemocytes with clear cytochemical difference, surfaces.

Analysis of GOT activities in the haemolymph of aestivating P. virens shows a gradual decrease in levels of activity, but the decrease was not statistically significant. GPT behaves in a different way. The activity level of GPT was found to be significantly higher in two month aestivated snails when compared with the activity level of active snails. GPT, being a freely reversible transaminase enzyme, the high level of activity in two month aestivated snails can be attributed to large scale fluctuations in haemolymph carbohydrate and protein levels observed in two month aestivated snails when compared with the levels in one month and three month aestivated snails. The high level activity of GPT in two month aestivated snails is again in disagreement with the observation of Swami and Reddy (1978) who stated that the GOT and GPT activities in P. globosa were below the level in active snails. This clearly shows species difference in Pila.

C H A P T E R - V

EFFECT OF DIMECRON^{R} ON THE ACTIVITY PATTERN OF HAEMOLYMPH ACID PHOSPHATASE, ALKALINE PHOSPHATASE, GLUTAMATE OXALOACETATE TRANSAMINASE, AND GLUTAMATE PURUVATE TRANSAMINASE OF PILA VIRENS

5.1 INTRODUCTION

The use of chemicals to kill pests is not a new concept. As far back as sixteenth century, the chinese elaborated the use of arsenic sulphide as an insecticide. The use of arsenical compounds is being continued even in the 20th century. The use of synthetic organic pesticides gained momentum around 1940, and a large number of manufacturing and formulating units have sprung up all over the world after the development of D.D.T. This has progressed rapidly to the extent that today about 1,000 pesticide formulations are in common use around the world, of which a large number is commonly used as insecticides, herbicides, fungicides, nematicides, and as other chemicals in agriculture (Kant, 1987).

There are about 3,75,000 cases of human poisoning by pesticides in developing countries every year, with some 10,000 deaths. Lack of protein in the food of the rural workers is an additional factor that makes these chemicals even more toxic and more dangerous. According to Kant, (1987), volatile herbicides sprayed on sugarcane plantations may easily be carried away by the wind, and reach neighbouring crops such as cotton, beans, tomatoes and papaya and destroy them. Over intensive aerial applications of pesticides have been known to pollute air and affect people in nearby towns, causing headache, dizziness, and nausea. The indiscriminate and extensive

use of pesticides in agriculture has also been responsible for the development of resistance seen in agricultural pests. Now, compounds of different chemical groups are required to control them. It has also been found that the total number of pest species has increased because predators have also been exterminated by pesticides.

At present in India, about 1,20,000 metric tonnes of pesticides are used annually. At least 70 percent of these belong to the category of agrochemicals banned or severely restricted in a number of countries, and identified by WHO as excessively toxic or hazardous (Handique, 1987). We in India, have to face the problem of environmental pollution for two main reasons. The major one is because of poverty, under development, illiteracy and lack of awareness, and the second one is due to the efforts and consequences of rapid industrialization, urbanization and developmental works. The technology development has not given sufficient time to man to see the deleterious consequences on the environment or ecosystem as a whole. The green revolution has brought with it the indiscriminate use of pesticides and agricultural chemicals resulting in the build-up of residues in man.

In soil, insecticides act in a cumulative or chronic manner so that as animals move through pesticide treated soil, they slowly contact and acquire residue that may eventually be large enough to kill them. Because residue can reach animals in many indirect

ways, chronic toxicity of insecticides cannot be measured in laboratory experiments and most toxicological studies are on the acute toxicity of one application.

Pesticides reach the aquatic environment either through direct application to control aquatic pests, or through erosion resulting from heavy rains or from irrigation. Pesticides are carried with the soil particles, at times, for considerale distances and get deposited in the bed of streams, ponds and lakes and on land areas subject to flooding. Present knowledge indicates that long before this water becomes hazardous for human consumption, it becomes potentially hazardous for aquatic life and fish eating birds in the locality.

The first major publication highlighting the problems of pesticide pollution was the book 'Silent Spring' (Carson, 1962), which by greatly exaggerating the potential hazards of persistant pesticides, focussed much more attention on the problem, and in this way helped to stimulate an awareness of the need for research, and thus indirectly contributed to our knowledge on the state of pesticide residues in the various sections of the environment, although we still know little more of the significance of the residue.

The concept of 'target' and 'non-target' organisms has arisen from the understanding of the dual role of many pesticides - vital controlling agents of injurious pests on one hand and dangerous contaminants of the environment on the other. In most freshwater

habitats where control measures are directed against a particular undesirable organism, some other organisms (non-target) whose destruction is not intended also are affected. The target concept has given way to the synthesis of pesticides with selective toxicity, i.e., toxicity only to target species. The selective toxicity is related to the natural physiological mechanisms of the non-target organisms by which the toxicant is detoxified metabolically. For instance, the high selective toxicity of malathion has been attributed to high carboxy esterase activity in mammals, in contrast to its low activity in susceptible insects (Krueger and O'Brien, 1959).

Apart from immediate or delayed expression of acute toxicity, exposure to pesticides in natural communities may exert various indirect effects on the capacity of an organism to survive. Many authors have noted behavioural effects on various major taxa, generally including irritability, loss of co-ordination, and finally immobility, that precede death or occurs with sublethal pesticide exposure. Of particular interest is the observation made in bivalves. Reduced pumping rate and increased shell movements were noticed in bivalves in response to several organochlorine insecticides (Matsumura, 1975). Lethargy and swelling of the body are two prominent effects noted in the common south Indian earth worm Lampeto mauritii exposed to phosphomidon (Bharathi and Rao, 1984). While studying the toxic effects of organophosphate pesticides, Reddy et al. (1982, 1983) reported a decreased rate of limb growth, moulting rate, and ovarian

maturation in crabs. The insecticides lindane, phosphomidon, phorate and trichlorofon were found to inhibit the filtering activity of Anodonta cygnea at sublethal concentrations (Salanki and Varanka, 1979). All these morphological as well as behavioural changes induced by pesticide toxicity weaken the chance of survival of the affected organisms. Individual mortalities as well as drastic changes in populations and in community structure will be the net result.

Various physiological changes analysed in organisms exposed to pesticides include metabolic, hormonal, neuronal, immune as well as enzymatic responses. Ram and Sathyanesan (1985) reported cythion induced reduction in protein, RNA and DNA contents in different tissues of Channa punctatus. Besides, organophosphate pesticide cythion was shown to control protein metabolism through phosphorylation and methylation of proteins. Modifications in tissue metabolism were evident in the muscles of penaeid prawn exposed to sublethal concentrations of phosphomidon (Reddy and Rao, 1986). Casida and Maddrell (1971) suggested the release of a diuretic factor from the central nervous system into haemolymph of fifth stage larvae of Rhodnius probixus during insecticide toxicity. This resulted in induced diuresis. Increased secretory activity in the gut by oozing of secretory globules by organophosphate pesticide toxicity was reported by Saxena and Saxena (1970). The effect of pesticides on sodium and potassium activated adenosine triphophatase and its effect on maintenance of hydromineral balance of fish have also been explained (Matsumura, 1975). A marked decline in immunological competency in

the clam Mercenaria mercenaria exposed to organic pollutants was reported by Anderson et al. (1981). Untreated clams routinely cleared more than 90% of the bacteria injected into their haemolymph while majority of the pollutant exposed clams showed significantly impaired ability to clear bacteria.

The impact of pollutants on an organism is realized as perturbations at different levels of functional complexity (Moore, 1985). Many ways in which the structure and or function of organelles and cells can be disturbed by toxic contaminants have been conveniently classified by Slater (1978) into four main categories. (i) Depletion of stimulation of metabolites or coenzymes, (ii) Inhibition or stimulation of enzymes and other specific proteins, (iii) Activation of a xenobiotic to a more toxic molecular species, and (iv) Membrane disturbances. In the present study, the pattern of activities of selected enzymes are taken as the explorative tool for the analysis of impact of the toxicant on the functional complexity of the organism. The importance of enzymes and their role in metabolism is paramount (Mahler and Cordes, 1988). The ability to accurately characterise enzymes with respect to their distribution and kinetics makes them attractive indices of stress (Dillon and Lynch, 1981). Two phosphatase enzymes (Acid phosphatase and Alkaline phosphatase), and two aminotransferase enzymes (Aspartate aminotransferase and Alanine aminotransferase) are selected for the analysis of pattern of activity in response to different concentrations of an organochloride (Endocel 35 EC) and an organophosphate (Dimecron^{R}) pesticides.

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 pH, respectively (Chin-Yin and Hiroyuki, 1987). These enzymes are concerned with biosynthesis of fibrous protein (Johnson and Mc Minn , 1958), mucopolysaccharides (Kroon, 1952), or they may serve as a regulator of intracellular phosphate concentration (Gutman, 1959). They are also hydrolytic enzymes which play an active part in the dissolution of the body's cells. ACP and ALP are also believed to be involved in permeability processes and associated with nucleic and synthesis (Cox et al., 1967). Stimulation or inhibition of these enzymes will thus result in disturbances in metabolism.

Acid phosphatase is associated with lysosome and is designated as one of the marker enzymes (Edwards and James, 1987). It is a hydrolytic enzyme which takes part in the dissolution of dead cells and as such is a good indicator of stress conditions in biological systems (Gupta et al., 1975; Verma et al., 1980).

Molluscs generally have low enzyme levels and information on their specific role 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 enzymes levels are of diagnostic value (Jyothirmayi and Rao, 1987).

It has been established that molluscan haemocytes, especially granulocytes, represent a major source of lysosomal enzyme synthesis (see Cheng, 1983). Under normal conditions these acid hydrolases are restricted to within the lipoprotein lysosomal membranes, i.e. being in latent phase. However, if the granulocytes are challenged by a variety of abiotic and biotic factors, the lysosomes become unmasked or destabilized, i.e., the enzymes are released from lysosome into the surrounding cytoplasm (see Mohandas et al., 1985). Along with the finding of lysosomes in granulocytes of several species of molluscs it was also discovered that these organelles release the enzymes through degranulation upon challenge (see Mohandas et al., 1985). Cheng and Rodrick (1975) reported that β-glucuronidase, acid phosphatase, alkaline phosphatase, lipase, aminopeptidase and lysozyme are associated with both the cellular and serum components of C. virginica and M. mercenaria, and Rodrick and Cheng (1974b) found the same enzymes in B. glabrata haemolymph.

Besides the degranulation process proposed for acid phosphatase release into serum, an endocrine involvement for the enzyme is also suggested. In stressed conditions, the stress sensitive endocrine glands such as the pituitory, and adrenal glands increase the rate of production of plasma corticosterone and consequently glucocorticoid responsive enzymes in liver such as alkaline phosphatase. The magnitude of increase in the enzyme activity depends not only upon the plasma corticosterone level, but also upon the rapidity and

duration of this response. However, investigations on alkaline phosphatase activity in molluscs are comparatively few (Larraneta and Ronz, 1954; Muller, 1965; Albert, 1966; Sumner, 1969; Virute and Patil, 1971; Rodrick and Cheng, 1974b; Cheng and Rodrick, 1975). Shylaja and Alexander (1974), reported histochemical and biochemical assay of alkaline phosphatase in the alimentary canal, and nephridium of the normal and aestivating specimens of Pila virens. Tissues which are involved in active secretory activity were shown to have high levels of alkaline phosphatase activity in P. virens.

The effects of numerous organic pollutants, including pesticides, on alkaline and acid phosphatase activities were worked out by various investigators in different target as well as non-target organisms. Acid phosphatase, a typical lysosomal enzyme, present in lysosome in a latent state, can be activated not only by the disruption of particle membrane invitro, but also by a number of stress conditions in vivo (DeDuve et al., 1955). Increase in acid phosphatase activity after pesticide exposure seems to be characteristic of tissue damage and became a useful diagnostic and experimental tool (Tietz, 1970).

Dalela et al. (1980) studied the effect of sublethal concentrations of phenol and pentachlorophenol on hepatic acid and alkaline phosphatases and observed significant inhibition. Synergistic effects of phenol and dinitrophenol on acid and alkaline phosphatase were also observed by Verma et al. (1980).

Yap et al. (1975) and Desaiah (1978), after PCP exposure, pointed out that uncoupling of oxidative phosphorylation is the main reason for inhibition of phosphatases. Uncoupling of oxidative phosphorylation was also suggested by Dalela et al. (1980), and Verma et al. (1980) for the inhibition of acid and alkaline phosphatases. Simon (1953) stated that concentrations higher than those needed to prevent oxidative phosphorylation injured the mitochondrial system so greatly as to block the action of enzymes concerned with oxidative metabolism. The inhibitory role of phenol was also suggested by Ravichandran and Anantharaj (1984). ACP, ALP, ATPase, glucose-6 nucleotidase, SDH and PDH were reported to be inhibited in brain, liver, kidney and gills of Notopterus notopterus exposed to sublethal concentrations of phenol (Gupta et al., 1983; Gupta, 1985, 1986).

An increase in acid phosphatase was noted in tissues of crab, Ophiotephlusa senex senex, following exposure to methyl parathion (Reddy et al., 1986). Shastry and Sharma (1980) reported reduced alkaline phosphatase activity in the brain of fish exposed to diazion (an organophosphate pesticide) for 96 hrs. Acid phosphatase was found to decrease to the lowest level after 30 days of exposure whereas alkaline phosphatase level was found to increase 30 days post-exposure. Hormonal involvement in the elevation of alkaline phosphatase activity was suggested by Murphy (1969). Accordingly, organophosphate pesticides induce the release of plasma corticosterone

from adrenal by exposure. It inturn increases the release of alkaline phosphatase in liver. Reddy et al. (1984) reported an increase in acid phosphatase activity in hepatopancreas of crab exposed to four subacute concentrations of sumithion for 24 hours up to 10 days. Increased acid and alkaline phosphatase activities in serum of fish Mysta vittatus exposed to pesticides and their different combinations were reported by Verma et al. (1984).

This chapter deals with the effect of Dimecron^{R}, an organophosphate pesticide, on the activity pattern of selected enzymes in P. virens. Dimecron^{R} is an extensively used pesticide all over India, especially in paddy fields of Kerala. The use of organophosphate pesticides has increased in recent years due to the ban or restriction on chlorinated hydrocarbons. These pesticides degrade rapidly in biological systems and their metabolic products persist in irrigation and drainage canals, and affect aquatic fauna. The major metabolic breakdown process of phosphomidon, which is the active ingredient of Dimecron^{R}, is P-O-vinyl hydrolysis, resulting in the formation of dimethyl phosphate and alpha-chloro di-ethyl acetamide, which can be dehalogenated and further degraded. Demethylation also occurs as a minor degradation pathway. Another metabolic pathway is oxidative N-deethylation which yields toxic-N-diethyl phosphomidon and phosphomidon amide. The N-hydroxy ethyl intermediates are also converted into carbohydrate conjugates (O'Brien, 1967). Due to the rapid biodegradability, organophosphate

pesticides are more favoured, and hence they are now produced in greater quantities than the chlorinated hydrocarbon insecticides.

The following review deals with the action of organophosphate pesticides on the enzyme system of selected organisms. In certain cases of organophosphate poisoning, liberation of cellular enzymes, or complex changes in the molecular architecture of membrane bound enzymes may be induced. Malathion was found to stimulate the release of arylsulfatase from liver lysosome in vivo and in vitro. It interacts with a structural component of the lysosome membrane and alters the permeability (Ntifora and Stein, 1967). Williams and Keys (1970) demonstrated the increase of serum β-glucuronidase due to daily administration of parathion. Organophosphate compounds were shown to have profound effect on nerve cells in organisms affected and that too is inhibition of cholinesterase activity (Mehrotra and Lal, 1970; Endean, 1972; Kucera, 1987). The Glutamate dehydrogenase activity was found to increase in the foot and mantle of Lamellidens marginalis, exposed to methyl parathion toxicity. The increased glutamate oxidation provides 2-ketoglutarate to meet the energy demands under toxic impact by entering the TCA cycle (Mohan et al., 1987).

Besides the focus enzymes acid and alkaline phosphatases, review of research works on the impact of pesticides on other enzymes are also available. Reduced ATP production due to uncoupling of

oxidative phosphorylation by sublethal concentrations of sodium penta chlorophenate was reported by Webb and Brett (1973). Desaiah (1978) also came to the above conclusion by his works in rat tissues. William and Keys (1970) reported an increase in level of serum β-glucuronidase in serum of rats exposed to parathion. The blocking of acetyl choline receptors by high concentrations of organophosphate pesticides was reported by Eldefran et al. (1971). However, such high concentrations seldom occur in natural environments. Acetylcholinesterase inhibition is considered as the immediate effect of organophosphate pesticides. Acetyl choline and cholinesterase are reported in nerve endings and heart muscle of gastropods (Endean, 1972). The inhibition of this enzyme disturbs the normal nervous function, finally resulting in the death of animals (Eto, 1974). The inhibitory activity of organophosphate esters against cholinesterase was first found in 1941 by Adrian and coworkers (Eto, 1974). Some organophosphate pesticides inhibit 'SH enzymes' probably owing to alkylation of SH group. Alkylation of DNA and thereby inhibition of protein synthesis is also suggested. Fungicidal activity of organophosphate esters is by inhibition of chitin biosynthesis (Eto and Ohkawa, 1970). Organophosphate pesticides were known to methylate and phosphorylate cellular proteins. Cythion induced reduction in protein, RNA and DNA content was noted in different tissues of C. punctatus by Ram and Sathyanesan (1985).

Alanine and aspartate serve as two major glucogenic aminoacids which through the activities of the enzymes alanine and aspartate transferases give rise to glucose precursors (Lehninger, 1979). Aspartate and alanine aminotransferases (Glutamate oxaloacetate transaminase and Glutamate pyruvate transaminase, respectively), thus play a strategic role in gluconeogenesis and also function as links between carbohydrate and protein metabolism under altered physiological, pathological and environmental stress conditions (Nichol and Rosen, 1963; Knox and Greengard, 1965; Harper et al., 1979).

The changes in the activities of aminotransferases, whether induced by endogenous or exogenous factors, are often associated with changes in the organism's physiological state. Environmental pollution appears to be one of the factors that affects aminotransferase activities in animal tissues (Lane and Scura, 1970). Hepatotoxicity and hepato pathological damage are the most common responses to various xenobiotic agents in fish (Couch, 1974). Levels of both GOT and ALP enzymes utilized during increased metabolic activity and tissue synthesis increase in response to hepatotoxicity folllowing xenobiotic contamination. Elevated tissue levels of hepatic enzymes are indicative of increased metabolism and tissue repair following hepatotoxicity (Bell, 1968).

The glutamate oxaloacetate transaminase content is more in heart muscles when compared with liver parenchyma cells in human. The damage in liver tissue leads to increased levels of both

transaminase but the increase in GPT levels is more specific for liver damage than GOT (Sherlock, 1968). However, the absolute amount of GPT was found to be less when compared with GOT. Elevated serum GOT values indicate liver cell destruction and not liver cell disfunction. Wroblewsky and Duve (1955) extended their work on SGOT and found that acute and chronic hepatic diseases are associated with quantitative and serial elevations of SGOT activity which are sufficiently characteristic to permit diagnostic differentiation. The ratio of GOT:GPT in the serum is often taken as an index for liver diseases (Von Brand, 1946).

The decrease in GOT activity may be due to the damage caused to mitochondrial membrane, loss of matrix and swelling mitochondron (Chow and Pond, 1972) or due 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). However, it has been generally accepted that stress in some cases like mercury poisoning may be due to leakage of the enzymes into blood because of the damage of hepatopancreas.

As in mammalian clinicial studies, the transaminases are considered to have diagnostic role in histopathological studies of other organisms too. Bell (1968) considered it as a tool in the study of tissue damage in salmonoids. GPT was shown to increase during mercury poisoning due to liver damage. The increase in serum

transaminases has been shown to be roughly proportional to the degree of cell necrosis in mice infected with hepatitis virus (Friend et al., 1955). Zelman and Wang (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.

In all molluscan species investigated, both aminotransferases were detected in all tissues examined. The GOT levels tend to be quite a bit higher than the GPT levels in the hepatopancreas of some gastropods (Sollock et al., 1979, Swami and Reddy, 1978). Hammen (1968) found that the levels of activity (units/g) 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. Other factors affecting the transaminase activity are, difference in species, season, food, size of the animal and assay procedures.

For instance, the tissue levels of AlAT and AAT were greater in active snails than in starving and aestivating P. globosa (Swami and Reddy, 1978). In L. luteola, xiphidio-and furco cercarial infections were shown to increase the level of GOT and decrease the level of GPT in body fluids (Manohar et al., 1972). The general body fluid aminotransferase activity was found to be very high in L. luteola. The De Ritis quotient (GOT:GPT) was found to be less than 1.0, (0.8) as body fluid GOT activity was lower than body fluid

GPT activity. This condition was found in many bivalve molluscs (Hammen, 1968). The ^{14}C incorporation studies (Hammen and Wilbur, 1959; Awapara and Campbell, 1964; Bryant et al., 1964) on snail tissue homogenates also suggest similar high levels of activity of aminotransferases.

Aminotransferases are of particular importance under conditions that impose a heavy drain on the animals store of metabolites (Goddard, 1960). The important role these enzymes play has been documented during shell formation (Hammen and Wilbur, 1959), and in relation to aminoacid excretion. During infection, depletion of host glycogen (Cheng, 1963) and amino acids (Targett, 1962; Cheng, 1963; Richards, 1969; Senft, 1969; Feng et al., 1970) has been reported, exerting a metabolic burden on the host. Therefore, it was suggested that the infected snail raises its intracellular aminotransferase levels to replenish the metabolites lost, which is reflected in the high aminotransferase levels in body fluids. Similar elevation in GOT and GPT was observed when the organisms are exposed to toxicant stress also (Payne and Penrose, 1975).

The information regarding the effect of pesticides and allied hydrocarbons on the activity pattern of serum GOT and GPT are scanty. GOT, GPT and lactate dehydrogenase were shown to be significantly elevated in blood serum and other tissues of the fish N. notopterus chronically exposed to phenolic compounds and their combinations (Verma et al., 1981; Gupta et al., 1983; Gupta, 1985). Murty et al.

(1985) demonstrated the influence of Lindane on the activity pattern of GOT and GPT of Oriochromis mossambicus and the relationship between aminotransferase enzymes and metabolic alterations due to stress. Gross physiological change in terms of aminotransferase activity was elaborately described by Lane and Scura (1970) on the basis of their studies regarding the effects of dieldrin on aminotransferase in Poecilia latipinna. An elevation in the activity of both enzymes accompanied by a reduction in their soluble protein content was demonstrated in Tilapia by the toxicity of Naphthalene, Toluene and Phenol (Storer, 1967; Dange, 1986).

Among molluscs, literature on aminotransferases is limited to a few contributions. Mohan and Babu (1976) studied the effect of aestivation on the activities of aminotransferases in nervous system of P. globosa. They found a general decrease in activity of both GOT and GPT during aestivation of the snail. The increased level of GOT and GPT in Theba pisana prior to the mass mortality was studied by Cheng et al. (1980). Effect of different larval trematode infections on enzyme activities of body fluids of the banded pond snail Viviparus bengalensis was studied by Prasad et al. (1983). They demonstrated that both GOT and GPT levels were low in Cercaria indica LXXXII infections while in echinostoma cercarial infections the GOT level was found to be high. Rodrick and Cheng (1974b) analysed the activity pattern of GOT and GPT in the whole haemolymph of B. glabrata and found that the percentage composition of GOT was

higher than that of GPT. Recently, Mohan et al. (1987) demonstrated the enhancement of activities of aminotransferases in L. marginalis during parathion toxicity.

As very little is known about the activity and release pattern of enzymes in snails exposed to insecticides, it was thought worthwhile to investigate this aspect in P. virens exposed to sublethal concentrations of Dimecron^{R} which is the most commonly used insecticide in paddy fields of Kerala. The results of the study are reported in this chapter.

5.2 MATERIAL AND METHODS

Methods of collection of test animal, laboratory conditioning, selection of size group, mode of collection of haemolymph, and the statistical analysis of the data were the same as described in detail in section 2.2.

5.2.1 Test Medium

Dechlorinated tapwater, aerated to full saturation, was taken as the test medium. The pH of the test medium was 7.0 to 7.5. The addition of toxicant did not bring about any appreciable variation in the pH. All the experiments were carried out at laboratory temperature, 30 $\pm$ 1.5^{o}C.

5.2.2 The Toxicant - Dimecron^{R}

Dimecron is the commercial product of Hindustan Ciba-Geigy Ltd. It is a water soluble organophosphorus pesticide containing

850 gm phosphomidon (2-chloro-2-diethyl carbonyl-1-methyl vinyl-dimethyl phosphate) as active ingredient in one kilogram of product. It is a systemic insecticide-cum-acaricide effective against sucking, chewing and mining insects on paddy, maize, barley, wheat ~~etc.~~ Dimecron^{R} is extensively marketed and used in Kerala for diverse agricultural practices.

5.2.2.1 Toxicant concentrations

Initially, stock solution of the toxicant was prepared by mixing weighed out quantities of commercial formulations of the toxicant in glass distilled water. At the time of dosing, media with desired ppm of the toxicant were prepared by pipetting known volume of stock solution into the test medium.

5.2.3. Toxicity Studies

5.2.3.1 Lethal toxicity studies

Before conducting acute toxicity tests, exploratory tests were performed to narrow down the lethal toxicity test doses of the toxicant. During lethal toxicity tests, laboratory conditioned snails of uniform size (40 $\pm$ 2 mm in shell height) were exposed to 50 litres of test solution that contained graded, logarithmic series of concentrations of the toxicant. Ten animals were used for each test concentration of the toxicant. The experimental tanks were kept covered to minimize external disturbances. The tests were carried out at room temperature (30 $\pm$ 1.5^{o}C), and the animals were fed with

Hydrilla sp. during the test period. The test media were replenished totally every 24 hours. Appropriate triplicates and controls were invariably maintained for all experiments. The animals were examined every 12 hr, and were considered dead if they did not respond to mechanical stimulation. The dead animals were removed and the cumulative percentage mortality at every 12 hr recorded. The 96 hr LC_{50} value was calculated using probit analysis (Finney, 1971).

5.2.3.2 Sublethal toxicity studies

The aim of these tests was to find out the functional response of the animal under stress of sublethal doses of toxicant. In the present study, three sublethal concentrations of the toxicant were used, 1/2, 1/3, and 1/6 concentrations of 96 hr LC_{50} value. Desired concentrations of the toxicant were prepared by adding calculated doses of stock solution prepared fresh every day. The duration of the short term sublethal toxicity study was 48 hrs. Test medium was replenished at 24 hr and the animals were not fed during the test period.

Haemolymph samples were collected at 2, 6, 12, 24 and 48 hr post-exposure for analysis of various parameters. A batch of 8 snails was used for study at each time interval. Controls were run simultaneously, and haemolymph samples analysed corresponding to the test pattern ($n = 5$).

5.2.4 Estimation of enzyme activity

Methods of assay of Acid Phosphatase, Alkaline Phosphatase, Glutamate-Oxaloacetate Transaminase, and Glutamate-Pyruvate Transaminase enzymes are described in detail in section 2.2.2. Total haemocyte counts were performed as per the methods given in section 2.2.1.

5.3 RESULTS

5.3.1 Lethal toxicity of Dimecron^{R}

After several exploratory tests, the concentrations selected for lethal toxicity test ranged between 15.0 ppm and 60.0 ppm. Below 10.0 ppm, the mortality rate was found to be negligible even after 96 hr. 50% of the animals were killed in 45 ppm at 96 hr, and no animal survived beyond 72 hr when the concentration was raised to 75 ppm and above. In higher concentrations, mortality was found to progress quickly as a function of time. In many of the concentrations, maximum mortality was recorded between 72 and 96 hr. The 96 hr LC_{50} computed was 34.54 ppm.

5.3.2 Behavioral response to Dimecron^{R} exposure

Snails exposed to different concentrations of Dimecron^{R} (15 to 60 ppm) were observed closely to find out behavioural responses. Initially, the snails seem to be not affected by the occurrence of the pesticide in the medium. Later, the tentacles were found withdrawn. For some time, snails exhibited erratic swimming behaviour. After

6 hr of pesticide exposure, the snails appeared to have lost their balancing capacity. This response was more prominent in media containing higher doses of the pesticide. After 10 to 12 hr, the animals started to swell, the most affected part was the foot. The swollen animals failed to withdraw their visceral mass and foot into the shell even when stimulated mechanically, implying a faulty osmoregulatory mechanism in the affected snail. The swelling was also found to be more prominent in snails exposed to higher pesticide doses. During the entire period of stress, the animals were not found to feed, even though feed was provided. However, when the snails were reintroduced into well-aerated dechlorinated tap water without the toxicant, within hours they regained their earlier vitality.

5.3.3 Response to sublethal doses of Dimecron^{R}

The concentrations selected for sublethal toxicity studies were 6.0 ppm, 12.0 ppm and 18.0 ppm of Dimecron^{R} which roughly corresponded to 1/6, 1/3 and 1/2nd of the 96 hr LC_{50} value computed earlier. Behavioural response towards the sublethal concentrations were observed and found to be milder when compared with the response towards lethal doses. The loss of equilibrium as well as swelling of the exposed body parts were the characteristic features of toxic response to Dimecron^{R}. Snails were found to be active during the whole period of exposure. Response to mechanical stimulus, however, had considerably slowed down. But, during the 48 hr experimental exposure, mortality was found to be nil in all the three sublethal concentrations tested.

5.3.4 Enzyme activities under Dimecron^{R} stress

5.3.4.1 Acid phosphatase activity (Figure 1)

Table 46 gives the mean values, standard deviation, range, and level of significance of haemolymph acid phosphatase activity in Pila virens exposed to 6.0 ppm, 12.0 ppm and 18.0 ppm of Dimecron^{R}, and in the control.

When snails were exposed to 6.0 ppm of Dimecron^{R}, the acid phosphatase activity was found to be little affected except at 2 hr post-exposure when the enzyme activity registered a significant increase than the control value ($P < 0.01$). A general trend of decline in enzyme activity was observed in snails exposed to 12.0 ppm of Dimecron^{R}. At 2 and 12 hr post-exposure, no significant change in activity was observed, whereas at 6, 24 and 48 hr post-exposure, statistically significant decrease in activity was observed ($P < 0.01$, 0.05 and 0.01). ACP activity in snails exposed to 18 ppm, was found to be significantly elevated, during the early time period (2 hr) of pesticide stress on comparison with the control values ($P < 0.05$). However, the 12, 24 and 48 hr post-exposure values registered gradual decline in activity which was significantly lower at 48 hr ($P < 0.001$) when compared with control values.

Comparison of acid phosphatase activity levels at specific time periods under Dimecron^{R} toxicity in different concentrations revealed that at 2 hr post-exposure, the enzyme activity was found to be

with three sublethal concentrations of Dimecron

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	7.254	7.765	7.946	8.468	9.433
	± SD	0.727	0.636	1.285	2.018	0.800
	Range	6.527-8.344	6.698-8.364	6.527-9.649	6.811-11.976	8.911-10.841
6 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	8.706**	7.471	6.918	12.175	9.628
	± SD	0.550	1.733	0.916	3.7524	1.993
	Range	8.173-9.536	5.846-11.465	5.562-8.297	6.981-15.893	7.265-12.147
12 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	8.124	5.903**	8.812	6.340*	6.577**
	± SD	1.516	0.829	1.397	1.225	1.314
	Range	6.244-10.387	5.108-7.209	7.322-11.693	4.768-8.571	5.165-8.628
18 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	10.352*	8.081	6.300	6.783	6.244***
	± SD	3.026	1.443	1.531	1.302	0.925
	Range	6.414-15.666	6.414-9.99	4.371-8.400	4.995-9.024	4.881-7.663

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

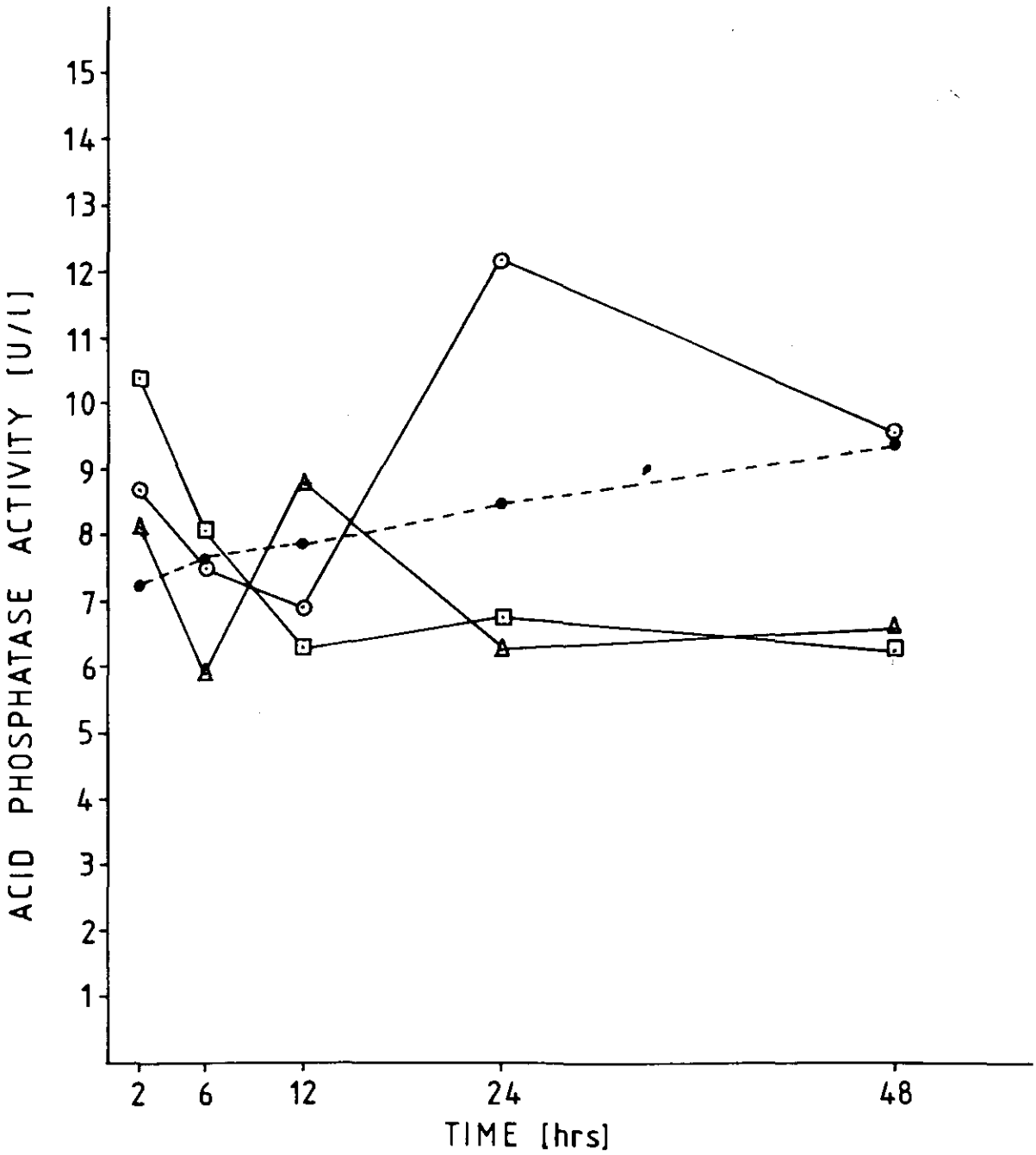

Figure 1. Haemolymph Acid Phosphatase Activity (u/l) in <u>*Pila virens*</u> *dosed with three sublethal concentrations of Dimecron*[R]*. Control (-•-), 6 ppm (⊙), 12 ppm (△), and 18 ppm (⊡).*

significantly higher at 18 ppm when compared with the activity at 12 ppm. No significant variation was observed on comparing the activity values between 6 ppm - 12 ppm, and 6 ppm - 18 ppm exposed snails. At 6 hr also, the some relationship was observed. The enzyme activity was significantly higher in 18 ppm ($\underline{P} < 0.001$) than in 12 ppm. At 12 hr, the enzyme activity was found to be significantly higher in snails exposed to 12 ppm when compared with the activities in snails exposed to 6 and 18 ppm ($\underline{P} < 0.001$). At 24 hr post-exposure, the acid phosphatase activity was found to be significantly higher in snails exposed to 6.0 ppm of Dimecron^{R} when compared with the activities in 12.0 ($\underline{P} < 0.001$) and 18.0 ppm ($P < 0.001$) exposed snails. At 48 hr post-exposure, the pattern of acid phosphatase activity was found to be similar as that observed at 24 hr post-exposure.

5.3.4.2 Alkaline phosphatase activity (Figure 2)

Table 47 gives the mean values, standard deviation, range and level of significance of alkaline phosphatase activity in the haemolymph of P. virens exposed to the three sublethal concentrations of Dimecron^{R}, and in the controls.

When the alkaline phosphatase activity in snails exposed to 6.0 ppm of Dimecron^{R} was compared with control values, no significant variation was observed at 2, 6, 12, 24 and 48 hr post-exposure. Enzyme activity in snails exposed to 12.0 ppm of pesticide was found to be significantly higher only at 24 hr post-exposure ($P < 0.001$).

Table 47. Haemolymph Alkaline Phosphatase Activity (U/l) in *Rila virens* dosed with three sublethal concentrations of Dimecron^{R}

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	4.352	3.752	5.060	4.819	3.505
	± SD	0.533	1.008	0.972	0.812	0.943
	Range	3.660-4.755	2.767-5.101	4.006-6.628	4.006-5.990	2.392-4.784
6 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	5.753	7.343	3.537	5.537	2.653
	± SD	2.753	3.968	2.055	4.671	1.733
	Range	3.747-10.548	2.507-16.080	2.017-7.118	1.383-13.804	0.951-5.620
12 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	4.604	2.867	3.747	1.380***	6.236
	± SD	1.931	1.519	1.687	0.425	4.228
	Range	2.767-8.300	0.835-4.928	1.959-6.542	0.663-1.959	1.585-13.372
18 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	7.644*	4.427	2.731*	2.244***	3.192
	± SD	2.543	1.985	1.836	0.893	2.525
	Range	3.345-11.009	2.104-8.473	1.124-5.793	1.297-3.775	0.692-7.637

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

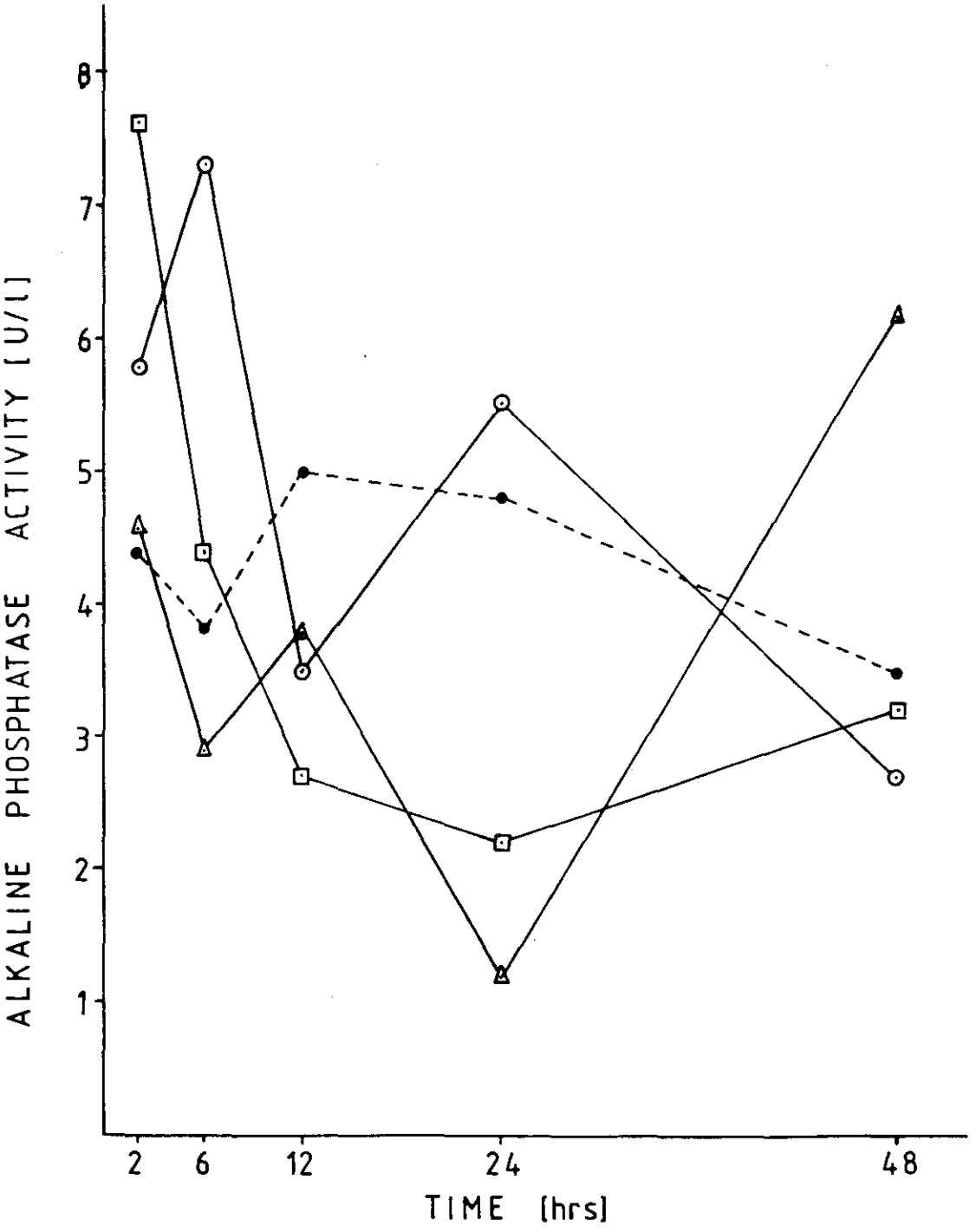

Figure 2. Haemolymph Alkaline Phosphatase Activity (u/l) in <u>*Pila virens*</u> *dosed with three sublethal concentrations of Dimecron*[R]*. Control (-•-), 6 ppm (⊙), 12 ppm (▲), and 18 ppm (⊡).*

At all the other time periods, i.e., 2, 6, 12 and 48 hr post-exposure, no significant variation was observed when compared with the control values. Statistical analysis of the values of enzyme activity in 18.0 ppm dosed snails and their controls gave the following picture. At 2 hr, the haemolymph alkaline phosphatase activity was found to be significantly higher ($\underline{P} < 0.05$). At 6 hr period no significant change was observed, while at 12 and 24 hr post-exposure periods, significant decrease in enzyme activity was observed ($\underline{P} < 0.05$ and $\underline{P} < 0.001$). No significant variation was noted at 48 hr post-exposure.

At 2 hr post-exposure periods, haemolymph alkaline phosphatase activity registered a statistically significant increase in 18.0 ppm exposed snails when compared with the activities in snails exposed to 12.0 ppm ($\underline{P} < 0.01$) and 6.0 ppm. At 6 hr post-exposure period, significant elevation in enzyme activity was observed in snails exposed to 6.0 ppm when compared with the activities in those exposed to 12.0 ppm ($\underline{P} < 0.01$) and 18.0 ppm ($\underline{P} < 0.05$). 18.0 ppm dosed snails showed an increase in enzyme activity when compared with the activity in 12.0 ppm exposed snails ($\underline{P} < 0.05$). Significant variation was absent when enzyme activities in snails exposed to the three sublethal concentrations of Dimecron^{R} were compared at 12 hr post-exposure. At 24 hr post-exposure, snails exposed to 12 ppm toxicant registered significant fall in enzyme activity when compared with the activities in snails exposed to 6.0 ppm ($\underline{P} < 0.01$) and 18.0 ppm ($\underline{P} < 0.01$). Enzyme activity was found increased in snails exposed to 6.0 ppm

when compared with the activity in 18 ppm exposed snails ($\underline{P} < 0.05$), and the activity in 18 ppm exposed snails was significantly higher than the activity in 12.0 ppm exposed snails ($\underline{P} < 0.01$). At 48 hr post-exposure to Dimecron^{R}, significant elevation in haemolymph alkaline phosphatase activity was observed in snails exposed to 12.0 ppm, when compared with the activities in 6.0 ppm exposed ($\underline{P} < 0.01$) and 18.0 ppm exposed snails ($\underline{P} < 0.05$). The comparison of enzyme activities of 6.0 ppm and 18.0 ppm dosed snails showed no significant difference.

5.3.4.3 Glutamate Oxaloacetate Transaminase activity (Figure 3)

Table 48 gives the mean values, standard deviation, range, and level of significance of haemolymph Glutamate-Oxaloacetate Transaminase activity in the controls and in snails exposed to the three sublethal concentrations of Dimecron^{R}.

When snails were exposed to 6.0 ppm of Dimecron^{R}, the GOT activity registered a statistically significant increase during the early periods of exposure. At 2 and 6 hr the GOT activity was significantly higher when compared with the control values ($\underline{P} < 0.001$). Later, the GOT activity level reached the level of activity in the control snails. Thus at 12, 24 and 48 hr, no significant variation was observed when compared with the control values. The above pattern with slight variation was observed when the enzyme activity pattern of snails exposed to 12.0 ppm was examined. The GOT activity significantly increased at 2 and 6 hr ($\underline{P} < 0.001$ and $\underline{P} < 0.01$), later

Table 48. Haemolymph Glutamate-Oxaloacetate Transaminase Activity (U/l) in *Pila virens* dosed with three sub-lethal concentrations of Dimecron^{R}

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	13.998	14.780	14.792	16.096	16.090
	± SD	2.031	4.954	4.577	2.521	1.262
	Range	11.740-16.970	10.880-23.000	11.310-20.020	13.050-19.580	14.350-17.370
6 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	22.512***	28.068***	13.648	15.660	18.816
	± SD	1.993	3.865	4.339	4.289	5.840
	Range	19.580-24.360	23.940-36.120	8.260-23.060	8.260-22.200	13.050-26.980
12 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	27.848***	23.283*	18.593	19.195	9.089**
	± SD	4.467	3.622	6.012	4.550	3.185
	Range	21.320-36.980	17.400-27.840	11.310-30.050	13.480-24.800	2.610-12.610
18 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	32.778**	25.663**	9.943	14.085	13.428
	± SD	9.281	5.854	3.755	5.390	3.916
	Range	16.970-49.030	16.100-36.120	5.180-15.330	8.210-23.160	8.660-17.400

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

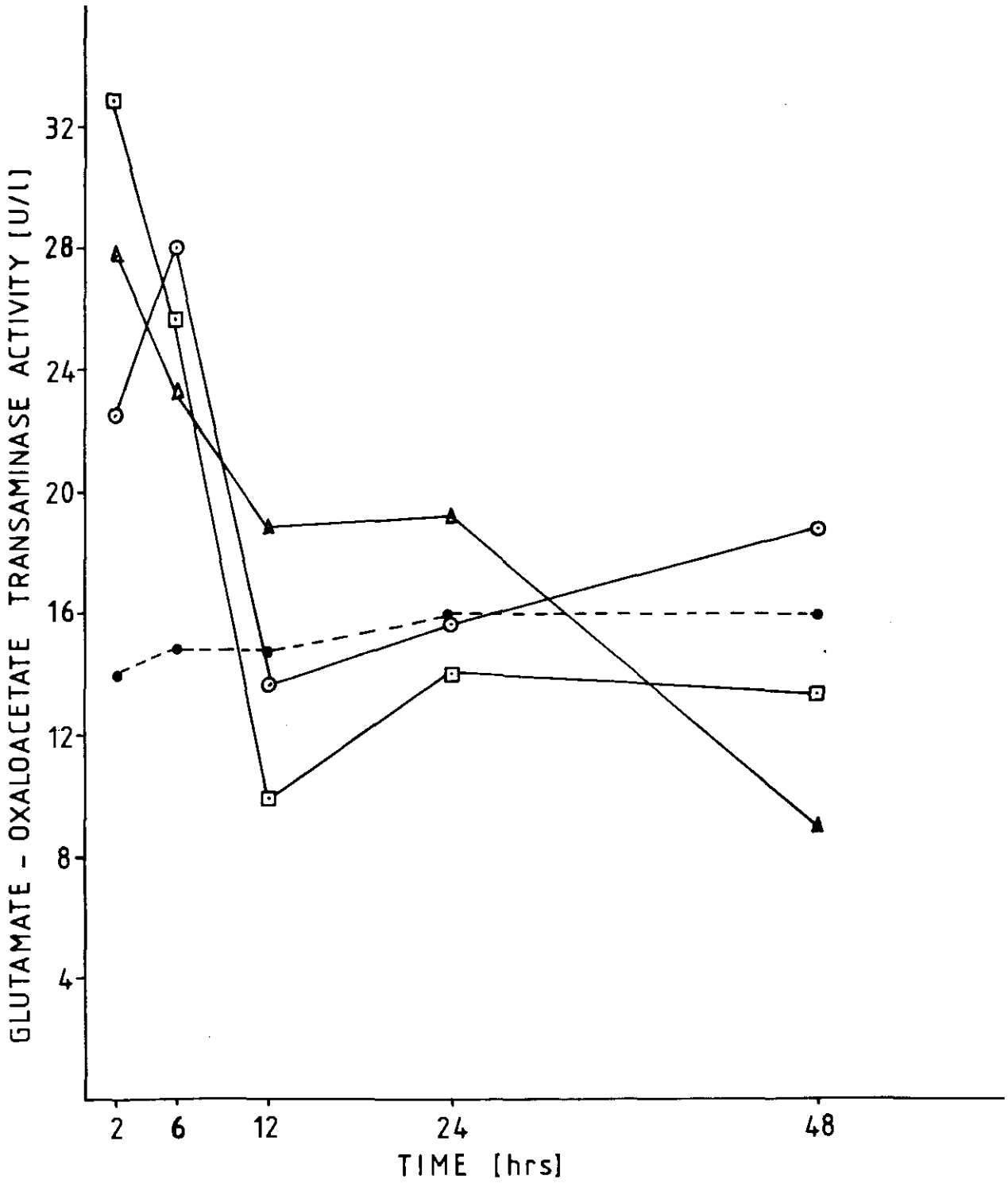

Figure 3. Haemolymph Glutamate-Oxaloacetate Transaminase Activity (u/l) in Pila virens *dosed with three sublethal concentrations of Dimecron*^{R}. *Control (-•-), 6 ppm (⊙), 12 ppm (△), and 18 ppm (⊡).*

reached near control levels at 12 and 24 hr, but at 48 hr, a significant fall in activity than the control value was observed. When the values in snails exposed to 18.0 ppm of Dimecron^{R} were examined for GOT activity, a slightly different pattern was observed. As in the two lower concentrations, increase in activity was observed at 2 and 6 hr post-exposure which was significantly higher than the control values ($\underline{P} < 0.01$). Later the level of enzyme activity fell to near control values at 24 and 48 hr but low at 12 hr post-exposure, but the decrease was not found to be statistically significant.

When the effect of different concentrations of Dimecron^{R} on the activity pattern of GOT at different time-periods was analysed statistically, the following picture emerged. At 2 hr post-exposure, the enzyme activity was found to be dose dependent. The GOT activity was found to be maximum at 18.0 ppm, significantly higher than the activity at 6.0 ppm ($\underline{P} < 0.01$). Similarly, the enzyme activity was found to be significantly higher at 12 ppm than the activity at 6.0 ppm ($\underline{P} < 0.01$). But no statistically different variation was observed between 12.0 and 18.0 ppm values. At 6 hr post-exposure, the enzyme activity was found to be significantly higher in snails exposed to 6.0 ppm than the activity at 12.0 ppm ($\underline{P} < 0.01$). At 12 hr post-exposure, the GOT activity was at its maximum in snails exposed to 12 ppm of the toxicant. In snails exposed to 18.0 ppm, the GOT activity was significantly lower than the activities in those exposed to 12 ppm ($\underline{P} < 0.001$) and 6.0 ppm ($\underline{P} < 0.05$). At 6.0 ppm,

the activity was significantly lower than the activity at 12.0 ppm ($\underline{P} < 0.05$). The maximum GOT activity at 24 hr post-exposure period was found in snails exposed to 12.0 ppm Dimecron^{R}, which was significantly higher than those at 6.0 ppm ($\underline{P} < 0.05$) and 18.0 ppm ($\underline{P} < 0.05$). When activity levels at 6.0 ppm and 18.0 ppm were compared, no significant variation was observed. At 48 hr post-exposure, the GOT activity was found to be significantly higher in snails exposed to 6.0 ppm toxicant than the activities in 12.0 ppm ($\underline{P} < 0.001$) and 18.0 ppm ($\underline{P} < 0.05$) dosed snails. The lowest enzyme activity was found in snails exposed to 12.0 ppm of Dimecron^{R} when compared with the activities in 6.0 ppm ($\underline{P} < 0.001$), and 18.0 ppm dosed ($\underline{P} < 0.01$) snails.

5.3.4.4 Glutamate Pyruvate Transaminase activity (Figure 4)

Table 49 gives the mean values, standard deviation, range, and level of significance of the activity of Glutamate Pyruvate Transaminase in the haemolymph of snails exposed to the three concentrations of Dimecron^{R}, and the controls.

Statistically significant increase in the activity of GPT was observed in snails exposed to 6.0 ppm of Dimecron^{R} at 2 hr post exposure, when compared with the control value ($\underline{P} < 0.01$). After the initial rise in activity, no significant variation was observed in enzyme activity except at 24 hr post-exposure, when a significant decline in activity was observed ($\underline{P} < 0.05$). In 12.0 ppm test medium

Table 49. Haemolymph Glutamate-Pyruvate Transaminase Activity (U/l) in Pila virens dosed with three sublethal concentrations of Dimecron^{R}

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	2.978	3.298	2.738	3.596	3.772
	± SD	0.685	0.598	0.783	0.890	1.080
	Range	2.217-3.669	3.020-4.328	2.058-4.045	2.642-4.431	3.292-5.670
6 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	3.291**	3.168	3.111	2.198*	3.712
	± SD	1.309	2.141	1.373	1.026	0.587
	Range	0.810-4.907	0.792-7.431	1.583-5.889	0.633-3.585	2.728-4.313
12 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	7.909***	2.597	4.449	2.653	4.002
	± SD	1.948	1.669	2.080	1.065	1.268
	Range	4.421-10.69	0.570-5.174	2.058-7.973	1.583-4.313	2.317-6.371
18 ppm of Dimecron^{R} dosed	N	8	8	8	8	8
	Mean value	9.222***	2.479	4.158	2.577	3.012
	± SD	2.257	1.372	1.260	1.291	1.240
	Range	6.331-13.180	0.633-4.045	2.642-6.437	0.891-4.421	0.891-4.798

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

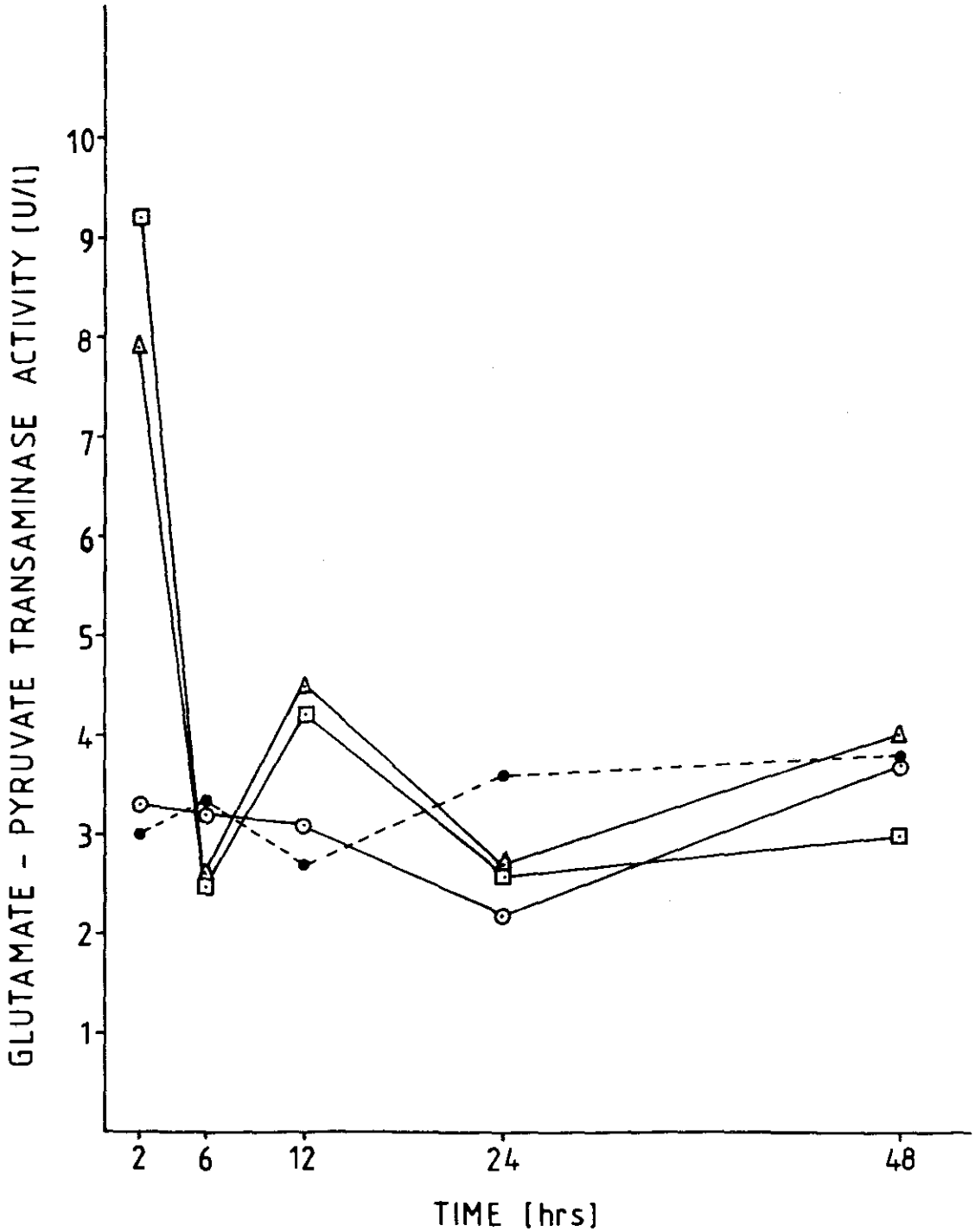

Figure 4. Haemolymph Glutamate-Pyruvate Transaminase Activity (u/l) in <u>*Pila virens*</u> *dosed with three sublethal concentrations of Dimecron*[R]*. Control (-•-), 6 ppm (⊙), 12 ppm (△), and 18 ppm (⊡).*

also, the initial response was the same as that seen in 6.0 ppm dosed ones. The activity was found to be significantly higher than that of control at 2 hr post-exposure. Following this initial elevation, the activity remained more or less static up to 48 hrs exhibiting no significant variation from the control values. When the response of snails at 18.0 ppm toxicant was analysed, comparable results were obtained. At 2 hr, significant rise in GPT activity was observed, but at 6, 12, 24 and 48 hr post-exposure, no significant variation was observed on comparison with the control values.

An interesting picture emerged when the response of snails, in terms of GPT activity towards different concentrations of the toxicant at different time periods was analysed statistically. At 2 hr post-exposure, snails exposed to all the three sublethal concentrations of Dimecron^{R} registered increased level of GPT activity than their respective controls. When the increase in activities was compared statistically, the response was found to be dose-dependent. Snails exposed to 12.0 ppm showed a significant rise in GPT activity than those exposed to 6.0 ppm ($\underline{P} < 0.001$). Similarly, the enzyme activity was more in snails exposed to 18.0 ppm than in those exposed to 6.0 ppm ($\underline{P} < 0.001$) and 12.0 ppm ($\underline{P} < 0.001$). At 6, 12, 24 and 48 hr post-exposure, the activity pattern was not found to be influenced by the concentration of the toxicant in the test medium.

5.3.4.5 GOT:GPT Ratio

Table 50 gives the mean values, range, standard deviation and the level of significance of GOT:GPT ratios in snails exposed to the three sublethal concentrations of Dimecron^{R}, and in the controls.

Both time and concentration dependent variations were observed when the GOT:GPT ratios of the toxicant exposed snails were compared with those of the controls. A fairly high ratio was observed at 6 and 24 hr post-exposure periods, (which was significantly higher at 6 hr in 18 ppm dosed snails ($P < 0.05$)) in snails exposed to the three sublethal concentrations of the pesticide, whereas at other time periods (2, 12 and 48 hr post-exposure) the values were found comparable or lower to those of the control values. The concentration of the toxicant appeared to be an influencing factor in GOT:GPT ratio. The ratios were found to be low in snails exposed to higher concentrations (12 and 18 ppm) and high in lower concentration (6 ppm) at all time periods except at 6 hr post-exposure.

5.3.5 Total haemocyte number (Figure 5)

Table 51 gives the mean values, standard deviation, range and level of significance of variations in the total haemocyte counts in P. virens exposed to three sublethal concentrations of Dimecron^{R} and in the control.

When the snails were exposed to 6.0 ppm of Dimecron^{R}, an increase in total haemocyte number was noted than the control values.

Table 50. GOT:GPT ratio in Pila virens dosed with three sublethal concentrations of Dimecron®

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
Control	N	5	5	5	5	5
	Mean value	4.880	4.651	5.423	4.734	4.013
	± SD	1.210	2.061	1.042	1.612	1.062
	Range	3.432-6.380	3.302-8.150	4.440-7.172	3.234-7.412	2.930-5.472
6 ppm of Dimecron® dosed	N	8	8	8	8	8
	Mean value	8.772	12.581	4.800	9.791	5.262
	± SD	5.924	8.430	1.722	7.041	2.222
	Range	4.523-22.000	4.863-31.310	3.082-8.240	2.730-22.660	3.082-9.882
12 ppm of Dimecron® dosed	N	8	8	8	8	8
	Mean value	3.651	13.880	4.590	8.054	2.302*
	± SD	1.022	10.750	1.532	3.432	0.760
	Range	2.531-5.610	4.012-36.630	2.794-7.162	4.842-15.381	1.134-3.320
18 ppm of Dimecron® dosed	N	8	8	8	8	8
	Mean value	3.642	13.090*	2.503**	5.980	4.283
	± SD	1.021	6.290	1.182	1.740	2.144
	Range	2.244-5.003	6.662-25.430	1.412-5.250	4.024-9.270	2.111-17.570

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

The increase noted during the early hours (2 and 6 hr) was, however, not significant statistically. At 12 hr post-exposure, a significant decline in total number of haemocytes was observed ($\underline{P} < 0.05$). At 24 and 48 hr post-exposure, the variations observed were not significant. In snails exposed to 12.0 ppm of Dimecron^{R}, the total number of haemocytes showed no significant variation at 2, 6, 12 and 48 hr post-exposure. However, at 24 hr post-exposure, the total number of haemocytes registered a significant decrease than the control value ($\underline{P} < 0.01$). A general trend of reduction in total counts was observed when snails were exposed to 18.0 ppm of Dimecron^{R}. After a period of significant increase at 2 hr post-exposure ($\underline{P} < 0.05$), and non-significant increase at 6 hr, the total number of haemocytes registered significant fall when compared with the controls. At 12, ($\underline{P} < 0.05$) 24 ($\underline{P} < 0.05$), and 48 hr ($\underline{P} < 0.001$), the decrease in total haemocyte number was found to be statistically significant.

The following picture emerged when the effect of different sublethal concentrations of Dimecron^{R} at specific time periods on total haemocyte number of $\underline{P.\ virens}$ was analysed statistically.

At2 hr post-exposure, there was a general trend for increase in the total number of haemocytes in $\underline{P.\ virens}$ exposed to the three concentrations of Dimecron^{R} but the variations were not statistically significant. At 6 hr post-exposure also there is a general increase in haemocyte number except in snails exposed to 12.0 ppm. The total number of haemocytes was found to be significantly lower in snails

Table 51. Total haemocytes/mm^3 in *Pila virens* dosed with three sublethal concentrations of Dimecron^R

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	5994.00	6792.00	5618.00	6620.00	6024.00
Control	± SD	1850.00	2070.11	1331.68	1847.24	1212.10
	Range	4580-9070	4510-10440	4510-7590	4830-9290	4630-7650
	N	8	8	8	8	8
6 ppm of	Mean value	7846.00	8501.00	3573.00*	7200.00	5334.00
Dimecron^R	± SD	2914.23	3313.11	1455.33	2445.93	2124.36
dosed	Range	5020-15470	5010-15260	1990-6120	4190-12630	3050-9150
	N	8	8	8	8	8
12 ppm of	Mean value	6809.00	5353.00	5632.00	3248.00**	5088.00
Dimecron^R	± SD	1088.68	1273.08	2503.02	1497.06	1638.90
dosed	Range	5150-8390	3530-7160	1920-9120	700-5620	2190-7730
	N	8	8	8	8	8
18 ppm of	Mean value	7767.00*	7320.00	3140.00*	4317.00*	2628.00***
Dimecron^R	± SD	3084.03	3328.34	1981.70	1654.17	1209.92
dosed	Range	4630-13960	3310-14960	1410-7520	1930-6740	1790-5350

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

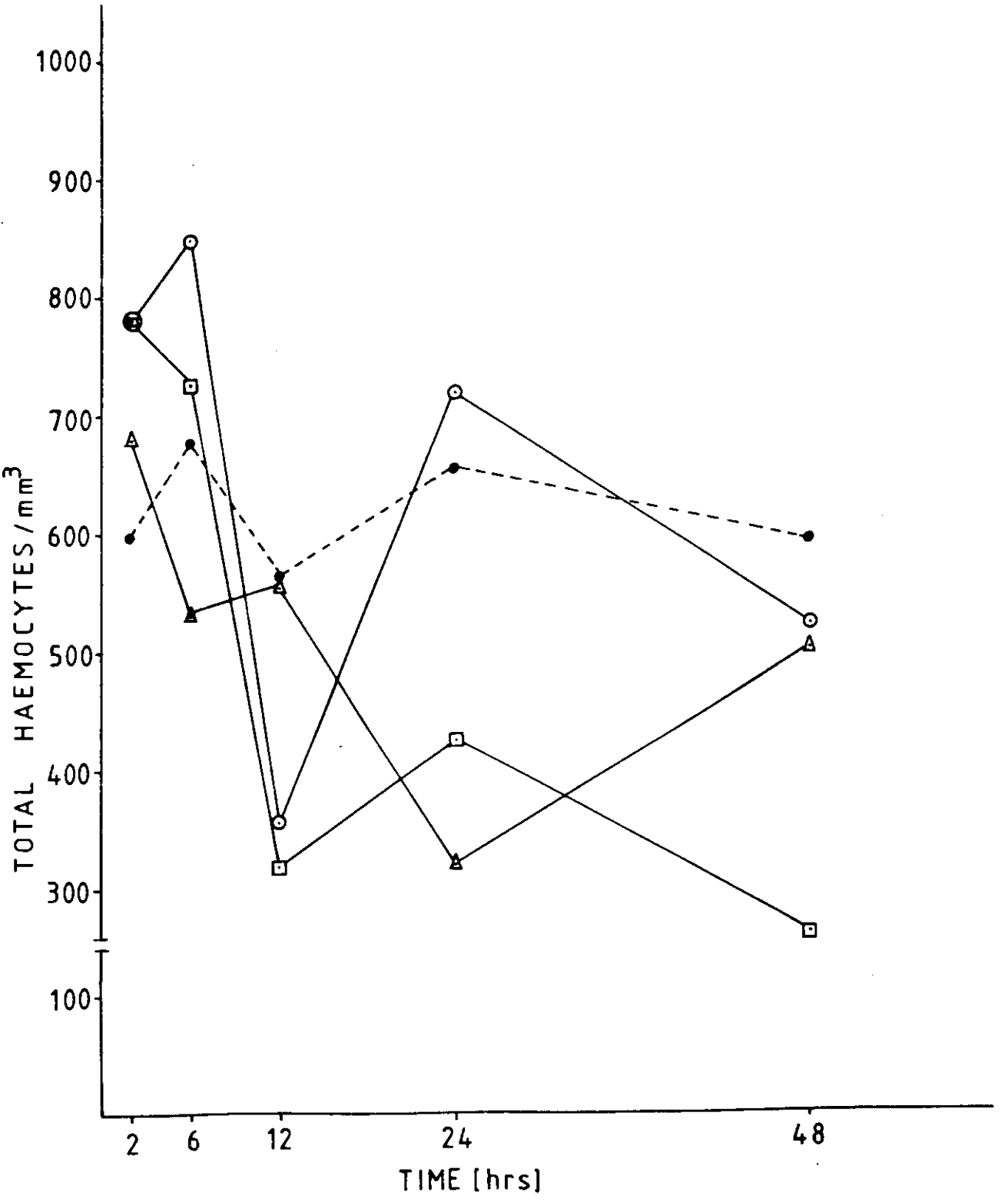

Figure 5. Total Haemocytes/mm^3 in Pila virens dosed with three sublethal concentrations of Dimecron^R. Control (-•-), 6 ppm (⊙), 12 ppm (△), and 18 ppm (⊡).

exposed at this concentration ($\underline{P} < 0.01$) than in those exposed to 6.0 ppm of Dimecron^{R}. No significant variation was observed in the counts in snails exposed to 6 and 18 ppm. At 12 hr post-exposure, the total number of haemocytes in snails exposed to 6.0 and 18.0 ppm was found to be significantly lower than the total count in snails exposed to 12.0 ppm of Dimecron^{R} ($\underline{P} < 0.05$). A comparison of total haemocyte number in snails exposed to different sublethal concentrations of Dimecron^{R} at 24 hr post-exposure yielded interesting results. The highest value was observed in snails exposed to the lowest concentration (6.0 ppm). The lowest number was seen in snails exposed to 12 ppm which was significantly lower than that of 6.0 ppm ($\underline{P} < 0.001$) exposed ones. In snails exposed to 18.0 ppm also, the total number was found to be significantly lower than that of snails exposed to 6.0 ppm ($\underline{P} < 0.01$). At 48 hr post-exposure, the haemocyte number registered a dose dependent decrease. The total number was maximum in snails exposed to the lowest concentration (6.0 ppm). The decrease in total haemocyte number in 18.0 ppm exposed snails was found to be statistically significant when compared with the counts in those exposed to 6.0 ($\underline{P} < 0.01$) and 12.0 ppm ($\underline{P} < 0.001$) of Dimecron^{R}.

5.4 DISCUSSION

The importance of conducting sublethal pollution tests to delineate the effects on biochemical and physiological mechanisms of the exposed organisms especially as they relate to respiration, osmoregulation, ionic regulations, the composition of body fluids,

enzyme systems, bioaccumulation, development of tolerance, and predisposition to diseases and parasites was emphasized by Perkins (1979). The stress of a pollutant measured on a laboratory animal may be a little more than the adaptation response exhibited by an organism in adjusting to normal environmental changes. Such acclimation capacity may be essential for survival of the species through the various stressful conditions encountered in its life cycle. Adaptive physiological response can be distinguished from physiological response in the ultimate expressions of biological performances which constitute to survival, growth and reproduction of the species (Waldichuk, 1979).

In this chapter results of the study on the activity pattern of enzymes, specifically, acid phosphatase, alkaline phosphatase, glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase in the haemolymph of P. virens exposed to the three sublethal concentrations of an organophosphate pesticide, Dimecron^{R} are discussed. An initial elevation in acid phosphatase activity which was significantly higher at 2 hr post-exposure in snails exposed to the sublethal concentrations, 6 and 18 ppm, was the immediate response. As the period of exposure progressed, the activity level was found to fall to the level of activity in the controls, or significantly below the control values. The initial increase in ACP activity can be attributed to the release of enzymes from lysosomes. A similar increase in ACP activity was observed in the freshwater mussel,

Lamellidens marginalis exposed to sublethal concentrations of Monocrotophos (Vijayendrababu and Vasudev, 1984). The increase in activity was found to be more when the pesticide concentration was higher (Reddy et al., 1987). The initial increase in ACP activity was found to be normalised gradually and one of the reasons for this could be the interaction of the enzyme molecule with the toxicant, forming conjugants, leading to a fall in titre of the enzyme to reach the control level. In crabs exposed to Sumithion (an organophosphate pesticide), the ACP activity was reported to be higher during early period, but later the level decreased. The elevation in ACP level was attributed to the tissue damage in exposed crabs (Reddy et al., 1984). The higher the concentration of sumithion, the greater was the tissue damage observed. ACP activity was found to increase along with the increase in intensity of tissue damage. More enzyme was needed for the removal of the damaged cells. A tissue damage of the same kind in gastropods exposed to pesticides, leading to the increase in level of the marker enzymes, cannot be ruled out. Several mechanisms have been suggested for the release of hydrolases from lysosomes. These include, (i) toxicant induced alterations in haemocytes resulting in more production and release of ACP (Suresh and Mohandas,1990a), (ii) proliferation of smooth endoplasmic reticulam in the parenchymatous cells, that leads to more production and release of microsomal enzymes resulting in increased activity (Hart and Fouts, 1965), (iii) peroxidation of lysosomal membrane leading to membrane

breakdown, or increase in permeability of lysosomal membrane, or both, resulting in the liberation of ACP thereby causing increased level (Novikoff, 1961), or (iv) degeneration and necrosis induced in tissued (Reddy, 1986).

In snails exposed to higher concentrations (12 and 18 ppm), the ACP activity was found to be lower after 12 hrs of exposure. Since the fall in enzyme activity was found to be consistent after 12 hrs, one of the reasons could be the injury to the mitochondrial system caused by the toxicant in high concentrations to the extent that the enzymes are blocked, and the other could be the depression of cellular oxidation, or the uncoupling of oxidative phosphorylation. However, significant decline in ACP activity was not observed in snails exposed to low concentration (6 ppm) indicating no damage to the mitochondrial system because of the low concentration of the toxicant.

In this connection it may be noted that there is a close correlation between the ACP activity level and the total haemocyte count at given periods. Whenever the activity level was high, the total count was high and when the level was low, the count was also low. Correlating these two, the following explanation is given for the variation in the activity levels of ACP. The initial increase (at 2 hr) in the activity level in snails exposed to 6, 12, and 18 ppm of the toxicant may be either due to (i) hypersynthesis of ACP by the lysosomes in haemocytes normally present in circulation,

or (ii) normal synthesis of ACP by haemocytes mobilized into the haemolymph from other sources, and also by those normally present in the circulation. In the case of Pila, the second explanation seems to be more reasonable as there was substantial increase in the total count when the enzyme activity level was high. At 6 ppm; the high enzyme level at the early time period was sufficient enough to destroy the toxicant till 24 hr post-exposure when there was further release of enzyme into circulation and hence the enzyme value is high, though not significant. At 12 ppm, although the concentration was high the elevated enzyme level at 2 hr post-exposure was not sufficient to meet the requirement, and hence at 6 hr post-exposure the activity level was low but by 12 hr post-exposure there was further release of enzyme into the haemolymph and this was immediately used to detoxify the toxicant and hence the enzyme values at 24 and 48 hr post-exposure was low. At 18 ppm, the significantly elevated level at 2 hr post-exposure was sufficient enough upto 24 hr post-exposure, but at 48 hr post-exposure this was not sufficient and there was no subsequent release as well and hence the enzyme level at this time period was significantly lower. At 6 and 12 ppm as mentioned earlier; there was further release of enzyme at 24 and 12 hr post-exposure, respectively, because compared to the elevated level at 18 ppm at 2 hr post-exposure, the levels at this time period in the other two concentrations were not very high; to inactivate the toxicant, and hence further release was required. When the enzyme activity was

low, there was corresponding decrease in total count as well. The low levels of enzyme activity at 24 and 48 hr post-exposure at 12 ppm, and at 12, 24 and 48 hr at 18 ppm, and the low haemocyte count at these time periods, point to the presumption that the low activity levels at later time periods were more due to transmigration of toxicant-laden haemocytes and/or cell death than to the inhibition of enzyme synthesis of blocking of enzyme action, although these are not ruled out. There are several situations, including pollution stress, where haemocytes undergo transmigration across epithelial surfaces (see Suresh and Mohandas, 1990b), and haemocyte mortality was reported by Pickwell and Steinert (1984). So, both these eventualities can happen.

Alkaline phosphatase was observed to follow a slightly different pattern of activity when compared with the acid phosphatase activity pattern. The haemolymph ALP activity also showed higher values at 2 hr post-exposure in the experimentals (significantly higher at 18 ppm) than the controls. At 12 ppm concentration, 24 hr post-exposure value, and 18 ppm concentration, 24 and 48 hr post-exposure values post-exposure values were significantly lower; and in general a low level of activity was noticed at later time periods. A similar instance of ALP elevation during organophosphate pesticide toxicity was reported by Ntiforo and Stein (1967). Malathion, accordingly, stimulates the liberation of cellular enzymes, or induces complex changes of the molecular architecture of membrane bound enzyme

systems, releasing the enzymes. An increase followed by decrease in serum ALP activity in Salmo gairdneri exposed to synthetic tararyl phosphate was reported by Lockhart et al. (1975). The decline in enzyme activity may be due either to inhibition of oxidative phosphorylation or the injury of mitochondrial system. Desiah (1978) has suggested that the inhibition of oxidative phosphorylation was the main cause of the inhibition of phosphatases during pentachlorophenol toxicity. Simon (1953) stated that concentration of toxic substance higher than those needed to prevent oxidative phosphorylation injured the mitochondrial system so greatly as to block the action of enzymes concerned with oxidative metabolism. The mitochondrial damage theory is more convincing in the present study because the fall in activity is more prominent in snails exposed to the highest concentration of the toxicant.

When the pattern of activity of ALP and the total haemocytes number in snails exposed to Dimecron^{R} were compared, a remarkable degree of parallelism can be observed at different concentrations. The explantion given earlier correlating ACP activity pattern and the total haemocyte count is equally applicable in the case of ALP activity pattern and the total count. Although Ciro et al. (1975) treats ALP as membrane enzyme, Cheng and Rodrick (1975), and Huffman and Tripp (1982) consider this as lysosomal enzyme, At this stage it is difficult to pinpoint the origin of ALP because the source is haemocyte (having membrane and lysosome) and the enzyme activity is linked with cell number. However, a comparable relationship was found

missing in the activity patterns of ACP and ALP at different time periods in snails exposed to the same concentration of the toxicant. This may be due to the fact that (i) lysosomes even in single cell types are variable in their enzyme content (Dean, 1977), and (ii) different subpopulations of haemocytes, with functional compartmentalization occur in cells (Yoshino and Granath, 1985 ; Cheng and Downs, 1988). Thus even if there is lysosomal membrane destabilization, it need not result in the simultaneous release of ACP and ALP (Suresh, 1988).

The possible reasons suggested by others for the decrease in phosphatase activity in test animals are (i) toxicants prevent orderly formation of energy rich compounds prior to synthesis of ATP, thus acting as uncoupling agents that hinder phosphorylation that normally accompany oxidation (Mitchell, 1961; Kelly and Syrett 1964; Weinbach and Garbus, 1969; Yap et al., 1975), (ii) injury to the mitochondrial system caused by higher concentration of toxicants block the action of enzymes (Simon, 1953), (iii) toxicants interact with the enzyme to form conjugants leading to enzyme inhibition (Hilmy et al ., 1981), (iv) removal of cofactors of enzyme activity (Ikehara (1978) showed that the EDTA inhibition on ALP activity was due to the removal of Zinc ions from the enzyme protein and this inactivation was overcome by the addition of excess of Magnesium ions), and (v) membrane destabilization.

Induced stress in gastropods stimulates gross physiological adjustments including metabolic shifts (Livingstone and Zwaan, 1983).

To compensate the rapid dimunition in carbohydrate metabolites, the snails may depend on non-carbohydrate precursors for energy release. The synthesis of glucose from non-carbohydrate precursor has been demonstrated in whole tissue of the snails B. alexandrina and B. truncatus from the substrates pyruvate, lactate, ketoglutarate, L-alanine, L-aspartic acid and L-glutamate (Ishak et al., 1975; Sharaf et al., 1975). Transaminases are widely reported in gastropod tissues (Sollock et al., 1979) including P.globosa (Mohan and Babu, 1976). The following discussion gives a detailed account of the activity pattern of haemolymph Glutamate Oxaloacetate Transaminase (GOT), and Glutamate Pyruvate Transaminase (GPT) in P. virens exposed to the three sublethal concentrations of Dimecron^{R}.

During early periods of pesticide exposure (2 and 6 hr post-exposure), the GOT activity level was found to be significantly higher than the control values. This can be considered as a stress induced immediate reaction of the organism to compensate the drain in metabolites. A sudden shift from carbohydrate to amino acid precursors for energy release is suggested during lindane stress in O.mossambicus (Murthy et al., 1985). Rao et al.(1987) reported increased proteolytic activity leading to increase in free amino acids in S.mossambicus exposed to Benthicarb. Further, in the present study, initial increase in GOT activity (2 hr post-exposure) was found to be dose dependent. A sudden rise in free amino acids in haemolymph along with the rise in aminotransferase enzymes is a clear

indication of gluconeogenesis, a well known metabolic adjustment to meet the increased energy demand created by the toxic stress.

As the time of exposure progressed, the GOT activity was found to become normal to the level of control snails. The normalization observed at 12 hr post-exposure and later could be explained as the conditioning of the snail to the toxic stress or acclimation to the presence of the pesticide. At this stag it is difficult to explain the low activity level noticed at 48 hr post-exposure at 12 ppm concentration.

The initial metabolic shifting from aerobic to anaerobic mode at the instance of pesticide stress was further supported by the work of Ahamed et al.(1978) in L.marginalis . Sublethal toxicity studies with malathion in freshwater mussels proved beyond doubt that there exists a mechanism of metabolic shifting during pesticide stress in snails also (Ahamed et al., 1978), as explained in fishes (Storer, 1967; Dange, 1986).

The elevation in GPT activity during exposure to Dimecron^{R} was found to be less persistent than that of GOT, in the sense that the dose dependent rise in activity was noticed only at the early time period (2 hr post-exposure). The increase in activity noted was very high indicating a sudden but prominent utilization of alanine amino acid to counteract the stress imposed by the high concentration of dimecron. Donaldson (1981) proposed the occurrence of a functional hypothalamic-pituitory inter-renal axis which is sensitive

and responsive to stresses. Increased production of cortiosteroids and catecholamines (Mazeaud et al., 1977) is reported in fishes during stress. The corticosteroids and catecholamines thus released were believed to play a very important role in toxicant induced gluconeogenesis. Such a primary neuroendocrine response in gastropods stimulating the sudden increase in aminotransferase activity is yet to be proved; although hormonal regulation for carbohydrate metabolism is reported in gastropods (see Joosse and Geraerts, 1983).

The increase in GPT activity was found to return to normal level of activity within a short time (at 6 hr post-exposure). The immediate recovery could be due to the fact that at this time period, the GOT activity was still higher, and thereby compensating the GPT activity. The activity levels of both these normalised by 12 hr post-exposure, indicating normalisation. From the GOT:GPT ratio it is clear that the high ratio at 6 hr post-exposure was more due to high GOT activity and normal GPT activity.

The variations in the total number of circulating haemocytes were considered to depend on the physiological state of clams (Feng, 1965 a,b; Foley and Cheng, 1974; Thompson et al., 1978) and gastropods (Muller, 1956; Sminia, 1972, 1983). Variations in haemocyte number has been attributed to the effects of biotic as well as abiotic factors on the physiology of molluscs.

Total number of haemocytes in circulation in snails exposed to three sublethal concentrations of Dimecron ^{R} was found to increase

at early time periods particularly at higher concentrations, but decline at later time periods. The presence of pesticide molecules in the haemolymph stimulates the immune system to translocate tissue haemocytes into haemolymph or induces their origin from amoebocyte producing organ (Sminia, 1981). The cells thus released may discharge their immune function at cellular level, or at humoral level by releasing the lysosomal enzymes into the haemolymph.

The initial increase in haemocyte number is followed by decrease later. This change can be attributed to the detoxification mechanism suggested by George et al.(1976). Several reports indicate that gastropods eliminate foreign materials by means of special mechanism in which amoebocytes laden with phagocytosed foreign particles leave the animal by active migration to the external environment via epithelia of the kidney, mantle, gut, reproductive tract, and foot (Tripp, 1961; Brown and Brown, 1965; Brown, 1967; Cheng et al., 1970). The total number of haemocyte in snails exposed to low concentration, however, returns to the level of the control indicating complete controlof the situation at low concentration of the toxicant. At higher concentrations, the decrease was found to be somewhat dose dependent. This decrease may be due either to transmigration of haemocytes, and/or cell death.

C H A P T E R - VI

EFFECT OF ENDOCEL 35 EC ON THE ACTIVITY PATTERN OF HAEMOLYMPH ACID PHOSPHATASE, ALKALINE PHOSPHATASE, GLUTAMATE OXALOACETATE TRANSAMINASE AND GLUTAMATE PYRUVATE TRANSAMINASE OF PILA VIRENS

6.1 INTRODUCTION

Endocel 35 EC is a widely used insecticide in India for diverse agricultural practices. Endosulfan (6, 7, 8, 9, 10, 10,-hexa chloro-1, 5, 5a, 8, 9, 9a, - hexahydro -6, 9-methano-2, 4, 3, -benzo dioxathiepin -3-oxide), a polycyclic chlorinated hydrocarbon of cyclodiene group is the active ingredient of Endocel 35 EC. Endosulfan is a mixture of alpha and beta isomers having a ratio of 70:30. WHO (1984) classified endosulfan in the category of technical products that are moderately hazardous. The physical, chemical, as well as toxicological effects of endosulfan ~~are~~ in experimental animals have been reported by various workers (WHO, 1984).

The effect of endosulfan on aquatic organisms is of special interest because it is highly toxic to some species of fish, and is extensively used for the protection of rice against rice stem borer in situations which involve a complex relationship between plant and aquatic life. Endosulfan, like other organochloride pesticides, possesses high chemical stability and strong lipophilic properties. Aquatic systems can accumulate organochloride pesticide residues directly from water through their respiratory processes, and also from food. It remains accumulated in fat deposits (Teran

and Sierra, 1987). In mammalian and avian tissues, endosulfan is converted into diol precursors and thereby makes it less toxic than cyclodiene pesticides (Shankland and Schroeder, 1973). It is not known whether the same mechanism of metabolism is present in molluscs.

Toxic concentrations of endosulfan in water to various species of freshwater fishes are (24 hr LC 100 ppm)-Trout (Salmo gairdneri) 0.01; Pike (Esox lucius) 0.005, Carp (Cyprinus carpio) 0.01, 48 hr, Gold fish (Carassius auratus) 0.01, and Guppy (Lebistes reticulatus) 0.009 (Brooks, 1974). Gold fish exposed for 5 days to a daily change of water containing 0.001 ppm of endosulfan acquired an average tissue residue of 0.35 ppm, and when exposure ceased 98% of the initial tissue endosulfan was excreted within 14 days as free diol (Brooks, 1974). For various insect larvae, the 24 hr LC_{100} lies between 0.015 and 1.5 ppm, and for crustacea such as Daphnia, Cyclops and Asillus sp. between 0.1 and 1.5 ppm (Brooks, 1974). The 96 hr LC_{50} for endosulfan in sea water tests was found to be 0.2 µg/l (McLeese and Metcalf, 1980). The LC_{50} value in Barbus stigma was found to be 0.0043 ppm (Manoharan and Subbiah, 1982).

The effect of endosulfan exposure on fish metabolism has been extensively investigated. Ramalingam and Ramalingam (1982) opined that pesticidal stress influences the conversion of tissue protein into soluble fractions reaching to the blood for utilization. It is suggested that during initial pesticide exposure, the proteins are less used and this results in anaerobic phase. In the long

term pesticidal exposure, much of energy must have been used to compensate the stress, hence the depletion of protein (Indrani, 1980). Praveen et al. (1987) reported considerable decrease of protein content in muscle, liver, and kidney of freshwater fish, Clarias batrachus exposed to sublethal concentration of endosulfan. Kidney and liver seem to be the main sites of degradation and detoxification of endosulfan in fishers (Murthy and Devi, 1982). Earlier, Bouck and Ball (1968) had described liver damage in animals exposed to chlorinated pesticides. Bhatnagar et al. (1987) reported gross histopathological alterations in liver of Channa gachua exposed to endosulfan. Dechlorination is another method of detoxifying organochloride pesticides, for example, DDT is converted into DDD by microsomal enzymes in the presence of NADPH or NADH in liver and kidney (Parke, 1968).

Radhaiah et al. (1987) made detailed investigations on the biochemical response of Tilapia mossambica exposed to organochloride pesticide. They observed an increase in the level of total lipid content (lipogenesis under pesticide intoxication), in the activity of LDH and GDH, and decrease in the activity of SDH (indicating a shift to anaerobic metabolism). Earlier, Gupta and Chandra (1977) had reported increase in liver weight in endosulfan exposed rats, and attributed this to proliferation of smooth endoplasmic reticulum. Marked decline in cell mediated and antibody mediated immunity was noted in rats exposed to endosulfan (Banerjee and Hussain, 1987).

Endosulfan toxicity studies in invertebrates are few and information regarding the impact on enzyme activity is meagre. In cockroaches, treated with endosulfan, respiratory rate was found to be high. No effect was found on central nervous system. Narahashi (1971) gave a detailed study on the impact of endosulfan on isolated nerve cords of insects. Excessive release of acetylcholine in nerve cord and blood was also seen (Shankland and Schroeder, 1973). The biodegradation and concentration of endosulfan in house flies were investigated by Brooks (1974). Vijayakumari et al. (1987) reported significant increase in haemolymph copper concentration upon sublethal exposure, and significant decrease upon lethal exposure indicating an increase in haemocyanin concentration upon sublethal and decrease upon lethal exposure.

Increased enzyme activities were reported by Gillette et al. (1969) in organisms exposed to organochlorine pesticides. Lane and Scura (1970) described the enhancement of serum glutamate-oxaloacetate transaminase activity in sailfin mollie exposed to low levels of dieldrin (an organochlorine pesticide). However, very little work has been done in the field of enzyme analysis in response to endosulfan toxicity especially with respect to molluscs.

Since very little is known about the toxic effects of organochloride pesticides in invertebrates in general, and more particularly on the activity pattern of enzymes, it was thought worthwhile to investigate on this aspect. This chapter includes

observations on the activity pattern of haemolymph phosphatases and transaminases in P. virens exposed to sublethal concentrations of Endocel 35 EC, which is commonly used in paddy fields.

6.2 MATHERIALS AND METHODS

Methods of collection of test animal, laboratory conditioning, selection of size group, mode of collection of haemolymph, and the statistical analysis of the data are explained in section 2.2.

6.2.1 Test Medium

Dechlorinated tapwater, aerated to full saturation, was taken as the test medium. The pH of the test medium was between 7.0 and 7.5. The addition to toxicant did not bring about any appreciable variation in pH. All experiments were carried out at laboratory temperature of 30 $\pm$ 1.5^{o}C.

6.2.2 The Toxicant : Endocel 35 EC

Endocel 35 EC is an organochlorine pesticide manufactured and marketed by Excel Industries Limited, Bombay, India. It is a broad spectrum insecticide and acaricide extensively used in paddy fields, an in other crops against insects. It is a stomach and contact poison of target organism.

Endocel 35 EC is a dark-brown liquid consisting of Endosulfan technical : 35% W/W; solvents, emulsifier; stabilisers; 65% W/W. Active ingredient is Endosulfan or 6, 7, 8, 9, 10, 10;-Hexachloro-1, 5, 5a, 8, 9, 9a-hexahydro - 6, 9-methano - 2, 4, 3 - benzo

dioxathiepin - 3 - oxide. Stock solutions were prepared fresh for each set of experiment.

6.2.2.1 Toxicant concentration

Initially, stock solutions of the toxicant were prepared by mixing known, calculated quantities of the commercial formulation of Endocel 35 EC in distilled water. At the time of dosing, media with desired ppm were prepared by pipetting calculated volume of stock solution into the test medium. Fresh toxicant stock solutions were prepared at the time of each experimental dosing.

6.2.3 Toxicity studies

6.2.3.1 Lethal toxicity studies

Before conducting acute toxicity tests, exploratory tests were performed to narrow down the lethal toxicity test doses of the toxicant. During lethal toxicity tests, laboratory conditioned snails of uniform size (40 $\pm$ 2 mm in shell height) were exposed to 50 litres of test solution that contained graded, logarithmic series of concentration of Endocel 35 EC. Ten snails were used for each test concentration of the toxicant. The experimental tanks were kept covered to minimize external disturbances. The tests were carried out at room temperature (30 $\pm$ 1.5^{o}C), and the animals were fed with Hydrilla plants during the test period. The media were replenished totally every 24 hrs. Appropriate triplicates and controls were invariably run for all experiments.

The animals were checked every 12 hr, and were considered dead if they did not respond to mechanical stimulation. The dead animals were removed and cumulative percentage mortality at every 12 hr recorded. The 96 hr LC_{50} value and the 95% confidence limit were calculated using Probit Analysis (Finney, 1971).

6.2.3.2 Short-term sublethal toxicity studies

Short-term sublethal toxicity studies were conducted to find out the functional response of the snails under stress of sublethal doses of the toxicant. Three sublethal concentrations of Endocel 35 EC were used for short-term sublethal toxicity studies, i.e., 1/6, 1/3 and 1/2 concentrations of the 96 hr LC_{50} value. Desired concentrations were made by adding calculated doses of stock solution prepared afresh. The duration of the short-term sublethal toxicity study was 48 hrs. The media were replenished at 24 hrs, and the animals were not fed during the period of test.

Haemolymph samples were analysed at 2, 6, 12, 24 and 48 hr post-exposure for various study aspects. A batch of 8 animals was used for study at each time period. Controls were run simultaneously, and data analysed corresponding to the test pattern ($\underline{n}$ = 5). The various aspects studied during short-term sublethal toxicity studies were: (i) Variations in total haemocyte number at 2, 6, 12, 24 and 48 hr post-exposure to the three sublethal concentrations of Endocel 35 EC., (ii) Activity pattern of two phosphatases, acid phosphatase

and alkaline phosphatase during the study intervals, and (iii) Activity pattern of two transaminases, glutamate oxaloacetate transaminase and Glutamate-Pyruvate transaminase during the study intervals. The enzymes were analysed following the methodologies given in detail in section 2.2.6; and total counts were performed as per the methods given in section 2.2.5.

6.3 RESULTS

6.3.1 Behavioural response to Endocel 35 EC exposure

Snails were found to be more sensitive to Endocel 35 EC than to Dimecron®. They showed a sudden response to the exposure. They immediately closed the operculam, later gradually opened, extruding large amount of mucus. The mucus secretion was very high in snails exposed to comparatively high doses. Snails seem to have lost their balancing capacity and floated aimlessly. One of the interesting features of endocel toxicity was the gradual atrophy of the tentacles. The atrophy was also found to be more severe in higher doses. The animals failed to take food even when exposed to very low concentrations of the toxicant. The mortality rate was found to be very high in exploratory tests.

6.3.2 Lethal toxicity of Endocel 35 EC

Through several exploratory tests, the concentrations for LC_{50} studies were brought down to the range of 0.8 ppm to 2.0 ppm. Below 0.8 ppm, mortality was found to be 10% during the 96 hr exposure

period. 50% of the snails were killed in 1.6 ppm, and no animal was found to survive after 48 hr when the concentrations were raised above 2.2 ppm. In higher concentrations, mortality was found to progress quickly as a function of time. The 96 hr LC_{50} computed was 1.225 ppm. The period of LC_{50} showed decrease with increase in concentration of toxicant.

6.3.3 Sublethal toxic response

The sublethal concentrations of Endocel 35 EC selected for sublethal toxicity studies were 0.2, 0.4 and 0.6 ppm, which are approximately 1/6, 1/3, and 1/2nd of the LC_{50} value of Endocel 35 EC to P. virens. Behavioural response towards the sublethal concentrations included the release of large quantities of mucus. Erratic swimming and vigorous tentacle beating were also observed. Mortality rate was found to be nil during the entire exposure period of 48 hrs.

6.3.4 Enzyme activities under toxicant stress

6.3.4.1 Acid phosphatase activity (Table 52 ; Figure 6)

The acid phosphatase activity in snails exposed to 0.2 ppm of Endocel 35 EC for 48 hr was found to be low at all time periods (2, 6, 12, 24 and 48 hr post-exposure) when compared with the levels of enzyme activity in control snails. The decline noted, however, was not statistically significant. Snails exposed to 0.4 ppm of the toxicant exhibited the same pattern of enzyme activity as shown

Table 52. Haemolymph Acid Phosphatase Activity (U/l) in Pila virens dosed with three sublethal concentrations of Endocel 35 EC

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	9.116	9.468	8.734	9.195	8.946
Control	± SD	1.257	1.324	1.823	0.292	1.285
	Range	7.436-10.841	7.436-10.898	7.606-11.976	8.911-9.649	6.527-9.649
	N	8	8	8	8	8
0.2 ppm of	Mean value	7.677	7.131	7.201	9.153	6.783
Endocel 35 EC	± SD	2.239	2.147	1.471	3.137	2.141
dosed	Range	6.414-13.112	5.392-12.203	5.223-9.763	6.868-16.574	4.825-10.841
	N	8	8	8	8	8
0.4 ppm of	Mean value	8.571	6.620*	7.322	8.032	7.074
Endocel 35 EC	± SD	1.917	1.891	1.800	1.574	1.486
dosed	Range	6.641-11.295	4.711-10.274	4.654-10.728	6.244-10.955	5.676-9.252
	N	8	8	8	8	8
0.6 ppm of	Mean value	8.649	6.165**	7.329	6.605**	5.768**
Endocel 35 EC	± SD	1.810	1.362	1.236	1.310	0.670
dosed	Range	6.641-11.409	5.165-9.138	5.790-9.763	5.335-9.138	4.825-6.754

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

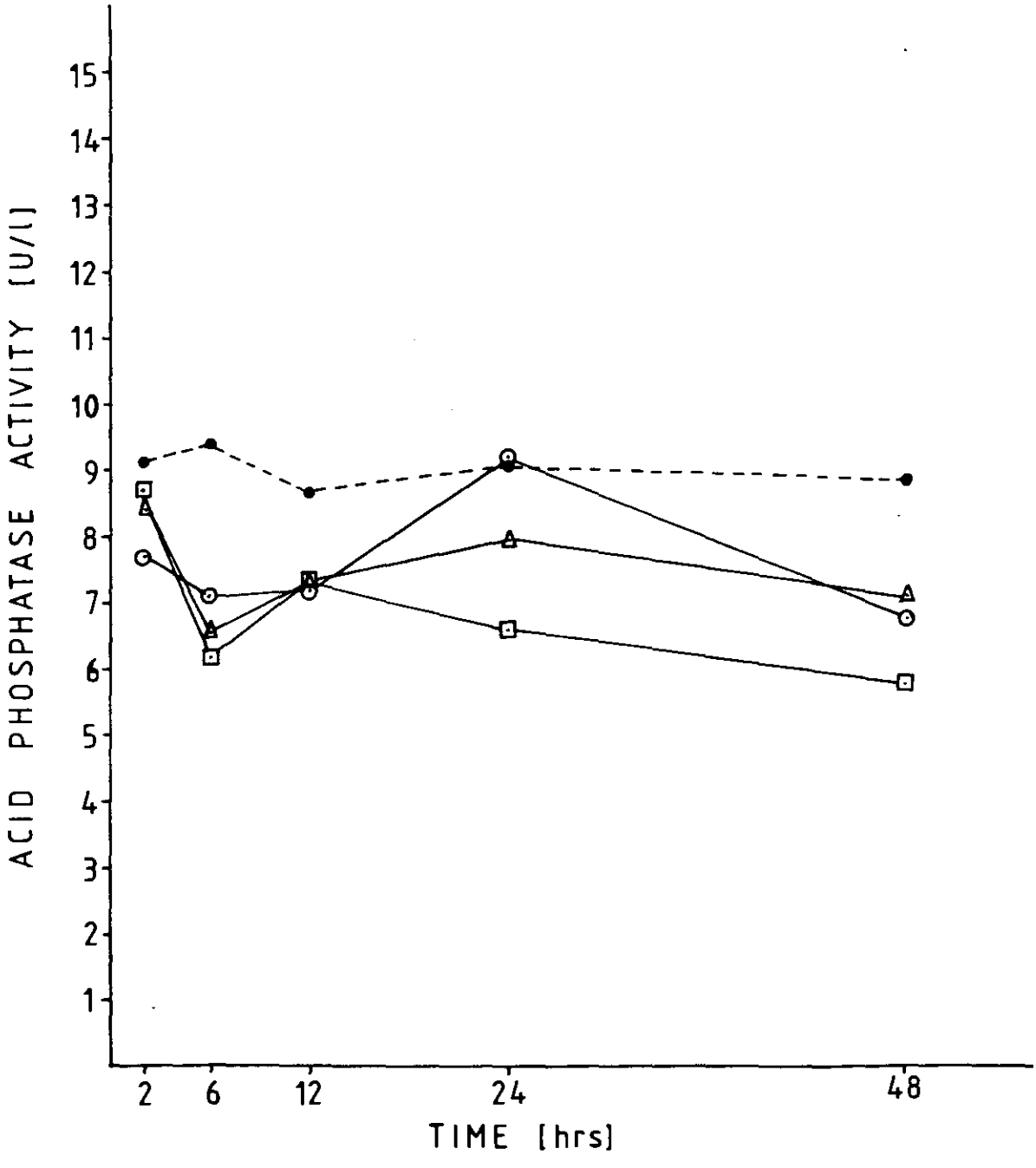

Figure 6. Haemolymph Acid Phosphatase Activity (u/l) in <u>Pila virens</u> dosed with three sublethal concentrations of Endocel 35 EC. Control (-•-), 0.2 ppm (⊙), 0.4 ppm (△), and 0.6 ppm (⊡).

by 0.2 ppm exposed snails except at 6 hr post-exposure, when the fall in activity was significant when compared with the control ($\underline{P} < 0.05$). The decrease in the level of activity of ACP in Endocel dosed snails was found to be more prominant when the toxicant concentration was the highest (0.6 ppm). The fall in activity was found to be statistically significant at 6, 24 and 48 hr post-exposure period, when compared with the control values ($\underline{P} < 0.01$).

To find out the influence of concentrations of the toxicant on ACP activity, hour-wise comparisons also were made. Up to and including 12 hr of Endocel 35 EC exposure, the acid phosphatase activity was found to be not significantly affected by the concentration of the toxicant. At 24 hr post-exposure period, statistically significant decrease in activity was found in snails exposed to 0.6 ppm on comparing the activities in snails exposed to 0.4 ppm ($\underline{P} < 0.05$) and 0.2 ppm ($\underline{P} < 0.05$). At 48 hr post-exposure also, significant decrease in enzyme activity was found in snails exposed to 0.6 ppm when compared with the activities in snails exposed to 0.4 ppm of Endocel 35 EC ($\underline{P} < 0.05$).

6.3.4.2 Alkaline phosphatase activity (Table 53 ; Figure 7)

A uniform pattern of alkaline phosphatase activity without much variation was observed when the values in snails exposed to 0.2, 0.4 and 0.6 ppm of the toxicant were compared with the respective control values. However, a generalised trend of decline in activities was observed at all time periods of exposure, which was significantly

Table 53. Haemolymph Alkaline Phosphatase Activity (U/l) in Pila virens dosed with three sublethal concentrations of Endocel 35 EC

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	4.680	4.853	4.352	4.669	4.433
Control	± SD	2.151	2.037	1.153	1.657	1.569
	Range	1.960-6.974	2.824-7.920	3.228-5.995	2.046-6.290	2.767-6.139
	N	8	8	8	8	8
0.2 ppm of	Mean value	3.945	4.226	3.228	3.538	4.237
Endocel 35 EC	± SD	1.689	3.416	1.430	1.220	3.388
dosed	Range	1.700-6.830	1.470-9.280	1.470-5.592	2.277-5.995	1.326-11.211
	N	8	8	8	8	8
0.4 ppm of	Mean value	2.994	2.248*	2.688	5.109	3.613
Endocel 35 EC	± SD	2.176	1.834	1.403	2.451	2.903
dosed	Range	0.721-5.793	0.202-5.245	0.721-5.044	1.153-8.156	1.816-10.663
	N	8	8	8	8	8
0.6 ppm of	Mean value	5.443	2.223*	4.345	3.192	3.351
Endocel 35 EC	± SD	1.589	1.360	2.252	1.979	1.134
dosed	Range	3.113-7.320	0.0692-4.726	2.190-8.358	0.634-6.023	1.816-5.361

Significance Level : * $P < 0.05$

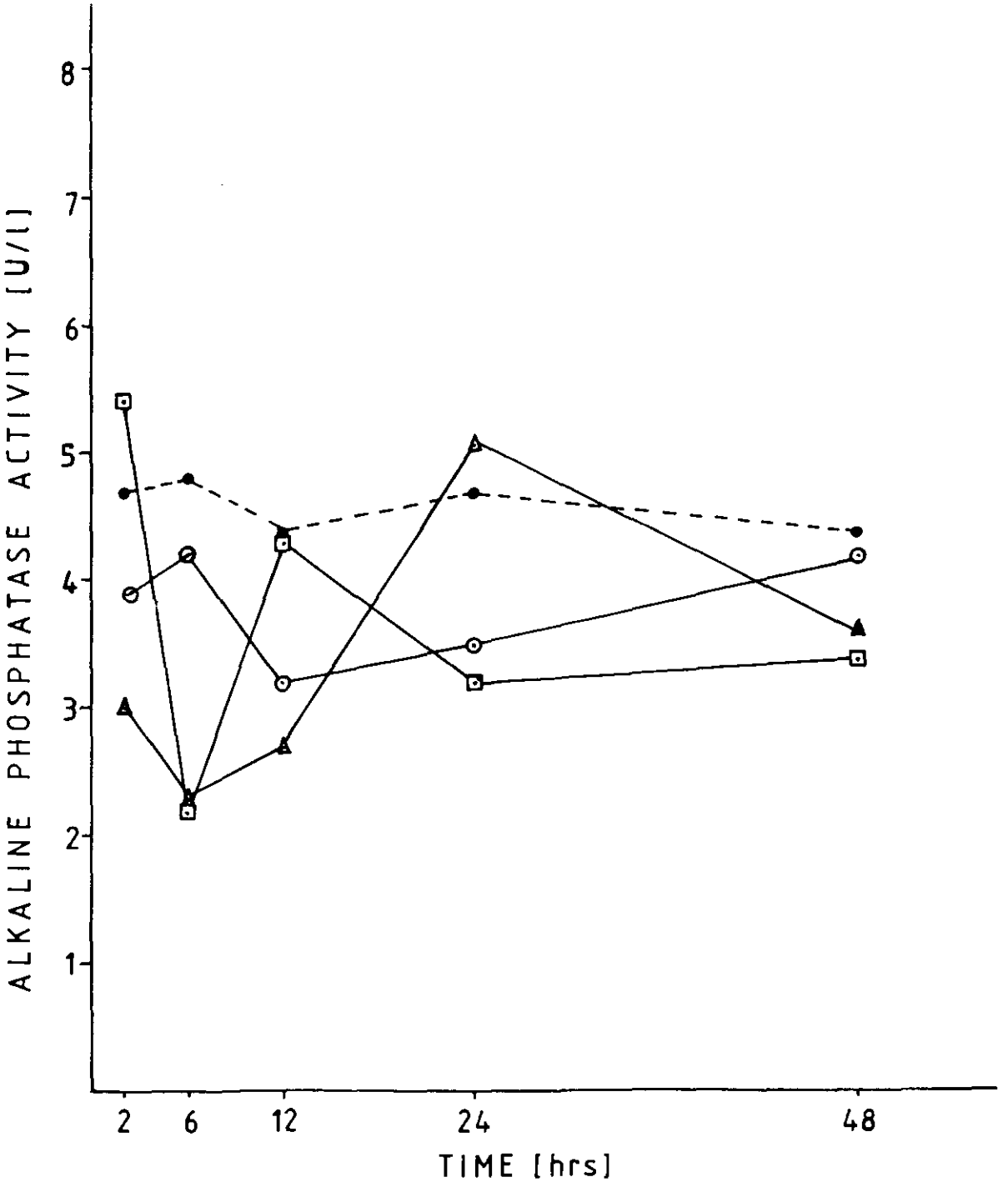

Figure 7. Haemolymph Alkaline Phosphatase Activity (u/l) in <u>*Pila virens*</u> *dosed with three sublethal concentrations of Endocel 35 EC. Control (-·-), 0.2 ppm (⊙), 0.4 ppm (△), and 0.6 ppm (⊡).*

less than the control values at 6 hr in higher concentrations ($\underline{P} < 0.05$).

When the activity pattern of alkaline phosphatase in different concentrations of the toxicant at specific time periods was compared, the following picture emerged. At 2 hr post-exposure, the decrease in enzyme activity was significantly lower in snails exposed to 0.2 ppm when compared with the activity in those exposed to 0.6 ppm ($\underline{P} < 0.05$). At 6 and 12 hr post-exposure, the trend in decrease of enzyme activity was more or less similar except in snails exposed to 0.4 ppm where the level of activity was significantly lower than the activity in those exposed to 0.6 ppm ($\underline{P} < 0.05$) at 12 hr. When the enzyme activity at 24 hr post-exposure was compared among snails exposed to different concentrations of Endocel 35 EC, enzyme activity was found to be significantly lower in snails exposed to 0.2 ppm and 0.6 ppm when compared with the activity in those exposed to 0.4 ppm ($\underline{P} < 0.05$). At 48 hr exposure, the enzyme activity levels in snails exposed to the three sublethal concentrations of endocel were found to be uniformly low as the concentrations increased, but not statistically significant.

6.3.4.3 Glutamate-Oxaloacetate Transaminase activity (Table 54 ; Figure 8)

When snails were exposed to 0.2 ppm of the toxicant, an initial fall in GOT activity was observed at 2 hr post-exposure, which was

Table 54. Haemolymph Glutamate-Oxaloacetate Transaminase Activity (U/l) in Pila virens dosed with three sublethal concentrations of Endocel 35 EC

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	16.683	16.167	15.492	15.839	15.384
Control	± SD	3.628	2.075	4.159	4.518	4.188
	Range	13.488-22.600	14.358-19.382	11.314-20.020	11.314-21.320	11.314-20.020
	N	8	8	8	8	8
0.2 ppm of	Mean value	7.281**	15.373	11.089	13.880	20.006
Endocel 35 EC	± SD	4.724	3.600	5.733	5.435	6.748
dosed	Range	0.436-14.790	11.310-22.200	2.610-17.400	6.900-23.440	13.050-25.240
	N	8	8	8	8	8
0.4 ppm of	Mean value	7.284*	20.171	11.800	12.038	19.085
Endocel 35 EC	± SD	5.639	4.623	5.190	5.070	8.840
dosed	Range	1.300-17.840	14.350-25.680	6.090-20.020	7.520-23.940	9.570-37.780
	N	8	8	8	8	8
0.6 ppm of	Mean value	6.251**	14.080	9.948	13.376	14.789
Endocel 35 EC	± SD	4.202	3.129	4.792	4.550	2.879
dosed	Range	1.740-11.740	6.520-16.530	4.780-18.710	6.960-20.880	10.440-17.400

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

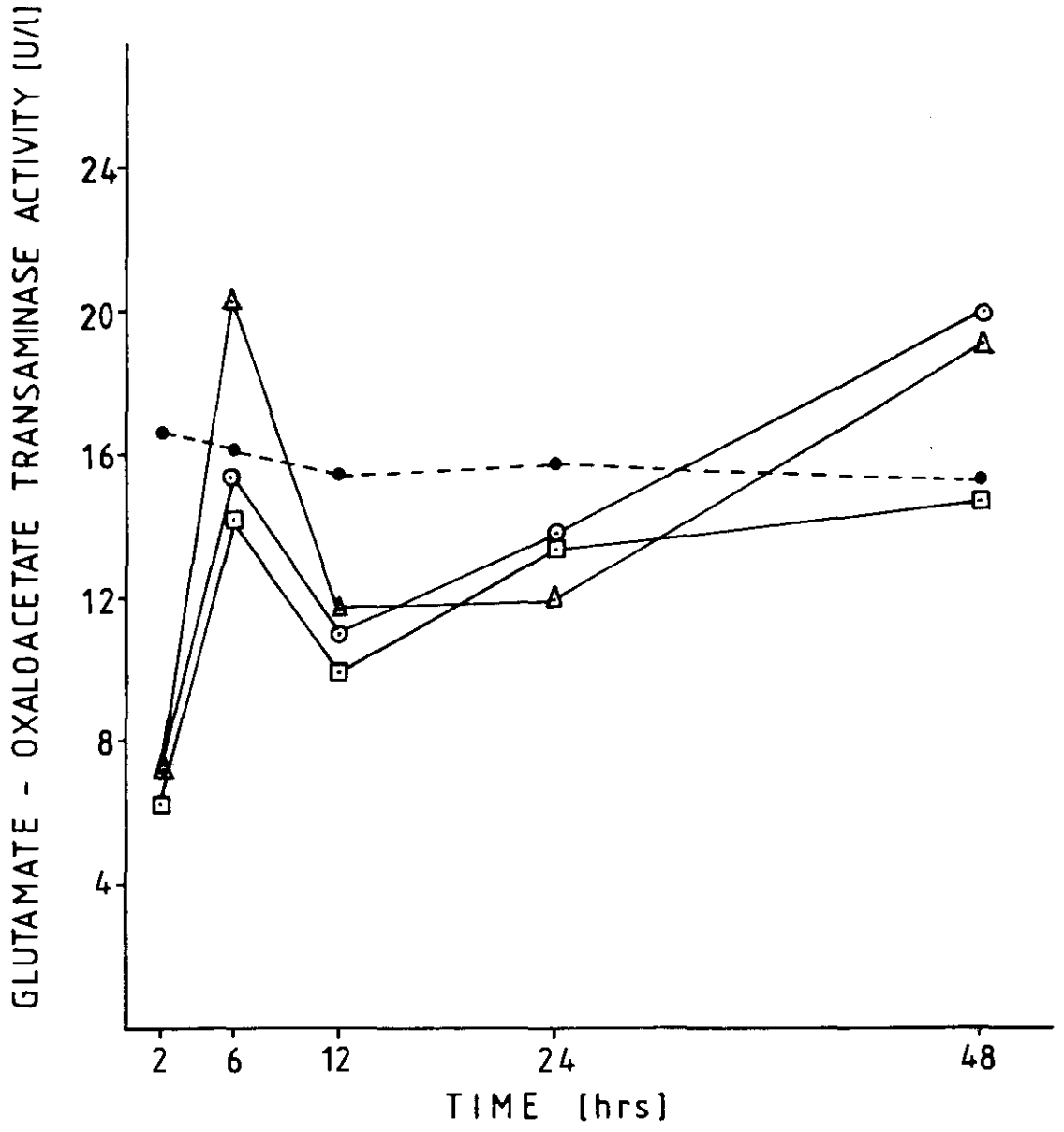

Figure 8. Haemolymph Glutamate-Oxaloacetate Transaminase Activity (u/l) in Pila virens *dosed with three sublethal concentrations of Endocel 35 EC. Control (—•—), 0.2 ppm (⊙), 0.4 ppm (△), and 0.6 ppm (⊡).*

statistically significant when compared with the control value ($P < 0.01$). No significant variation in enzyme activity was observed at 6, 12, 24 and 48 hr periods of exposure on comparison with control values. The activity pattern of enzyme in snails exposed to 0.4 ppm of endocel was comparable to the activity pattern of 0.2 ppm exposed snails. At 2 hr, there was significant decrease in enzyme activity ($P < 0.05$). At 6, 12, 24 and 48 hr, the enzyme activity values were found to be not significantly different from the control values. GOT activity in snails exposed to 0.6 ppm of Endocel 35 EC also registered the same pattern seen in those exposed to the other concentrations; i.e., significant fall at 2 hr, and no change at other time periods on comparing with the control values. However, the fall in enzyme activity in 0.6 ppm ($P < 0.01$) was more prominent than the one in 0.4 ppm ($P < 0.05$).

When the GOT activity levels in snails exposed to the different concentrations of the toxicant were compared at specific time periods, the following pattern emerged. No significant variation in values was observed among the snails exposed to different doses at 2, 12, 24 and 48 hr post-exposure, but at 6 hr the value in 0.04 ppm dosed snails was significantly higher than those in 0.02 and 0.06 ppm ($P < 0.01$).

6.3.4.4 Glutamate Pyruvate Transaminase activity (Table 55;Figure 9)

GPT activity in snails exposed to 0.2 ppm of pesticide showed no significant variation up to and including 24 hr post-exposure

when compared with the control values. However, at 48 hr post-exposure, the enzyme activity was found to be significantly elevated than the control values ($\underline{P} < 0.01$). Snails exposed to 0.4 ppm of the toxicant showed elevated level of enzyme activity at 2 hr post-exposure. No significant variation was found at 6 and 12 hr post-exposure, while the GPT activity was found to increase significantly at 24 hr post-exposure ($\underline{P} < 0.05$). This trend of significant increase in enzyme activity was maintained at 48 hr post-exposure also, when compared with the control values ($\underline{P} < 0.05$). When the snails were exposed to 0.6 ppm of Endocel 35 EC, statistically significant increase was found at 2, 24 and 48 hr exposure periods on comparison with the control values ($\underline{P} < 0.05$, 0.05 and 0.01 respectively). At 6 and 12 hr post-exposure, the GPT activity was found to be not significantly different from the control values.

Statistical analysis of the data of GPT activity in snails exposed to the three sublethal concentrations of Endocel 35 EC with respect to different time periods gave the following results. At 2 hr post-exposure when the GPT activity levels at 0.2 and 0.4 ppms were compared, significant elevation was noted ($\underline{P} < 0.001$) in snails exposed to higher concentration of Endocel 35 EC. At 6 hr post-exposure, no significant change in activity level was observed in snails exposed to lower concentrations i.e., 0.2 and 0.4 ppm, while in snails exposed to the highest concentration the enzyme

Table 55. Haemolymph Glutamate-Pyruvate Transaminase Activity (U/l) in *Pila virens* dosed with three sublethal concentrations of Endocel 35 EC

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	2.799	2.774	3.849	2.329	2.485
Control	± SD	1.179	1.034	2.092	0.991	0.692
	Range	0.950-3.392	1.583-3.669	2.317-7.315	1.583-4.045	1.742-3.392
	N	8	8	8	8	8
0.2 ppm of	Mean value	4.228	2.765	3.603	4.381	5.667**
Endocel 35 EC	± SD	1.637	1.206	1.529	1.853	1.688
dosed	Range	2.284-7.315	0.475-4.045	1.267-5.670	2.375-7.431	2.916-8.193
	N	8	8	8	8	8
0.4 ppm of	Mean value	6.567***	3.919	2.342	4.827*	6.155*
Endocel 35 EC	± SD	1.464	1.934	1.225	1.525	2.398
dosed	Range	4.313-7.876	2.540-8.311	0.950-4.609	2.317-7.211	3.936-10.940
	N	8	8	8	8	8
0.6 ppm of	Mean value	5.634*	1.900	3.289	4.742*	4.473**
Endocel 35 EC	± SD	2.356	1.191	1.678	2.363	0.957
dosed	Range	2.317-8.413	0.792-4.328	1.583-5.778	1.108-9.061	2.916-5.670

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

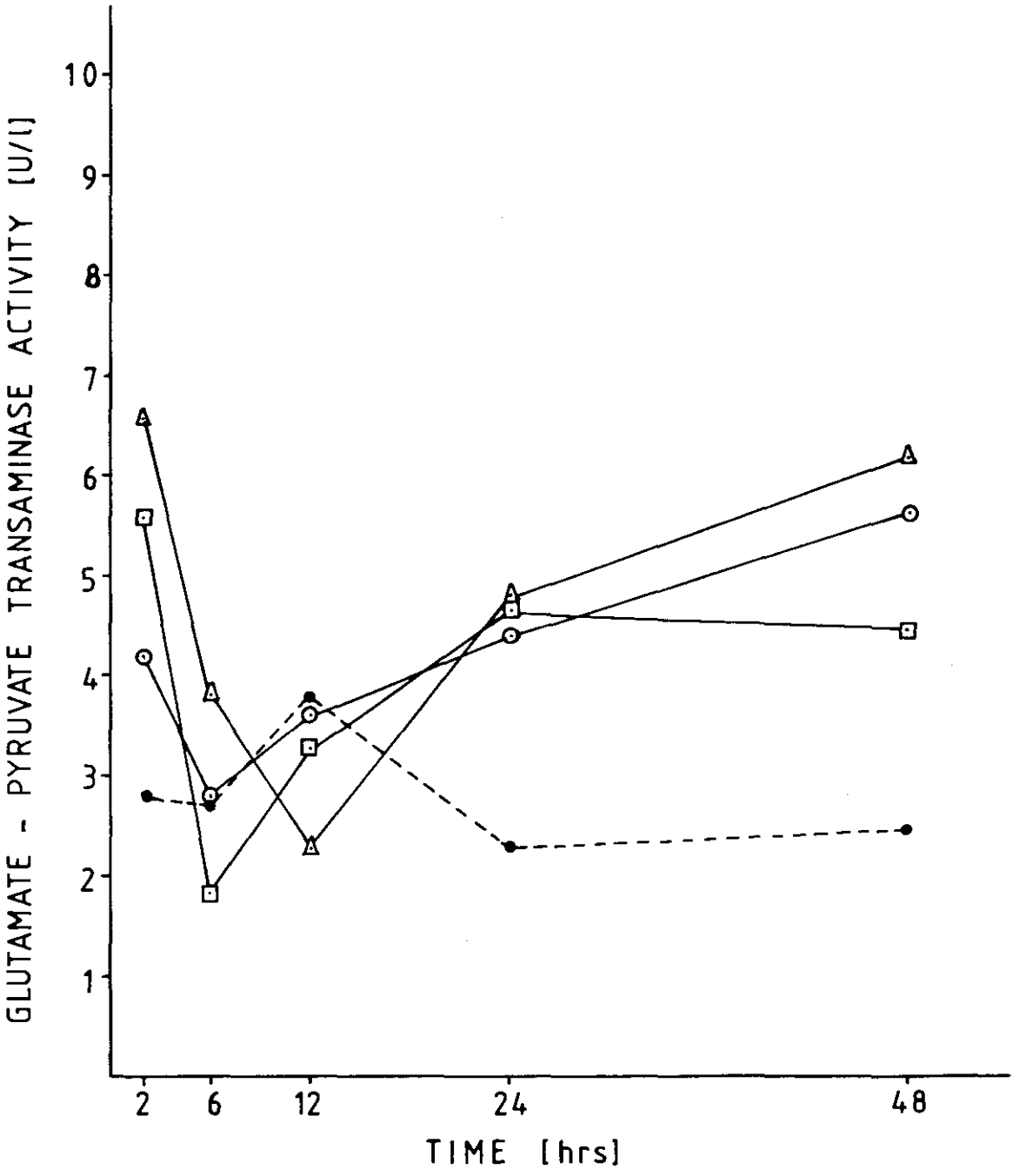

Figure 9. Haemolymph Glutamate-Pyruvate Transaminase Activity (u/l) in <u>*Pila virens*</u> *dosed with three sublethal concentrations of Endocel 35 EC. Control (—•—), 0.2 ppm (⊙), 0.4 ppm (⧋), and 0.6 ppm (⊡).*

activity was significantly lower than that of 0.4 ppm ($\underline{P} < 0.01$). At 12 hr post-exposure, the enzyme activity in 0.4 ppm dosed snails was found to be significantly lower than that of 0.2 ppm dosed ones ($\underline{P} < 0.05$). At 24 and 48 hr post-exposure, the activity of enzyme in snails exposed to 0.2, 0.4 and 0.6 ppm of pesticide failed to register any significant variation.

6.3.4.5 GOT:GPT Ratio (Table 56)

A clear time dependent variation in GOT:GPT ratio was observed when values of snails exposed to the three sublethal concentrations of the pesticide were compared with that of the controls. At 2 hr post-exposure, GOT:GPT ratio was found to be significantly lower ($\underline{P} < 0.01$) in snails exposed to all the three sublethal concentrations, while at 6 and 12 hr post-exposure, the values were more or less the same as that of the controls. At 24 hr post-exposure, the decrease in ratio was found to be less ($\underline{P} < 0.05$) in lower concentration (0.2 ppm) than in those exposed to higher concentrations ($\underline{P} < 0.01$) when compared with the control values. At 48 hr post-exposure also, a uniform decline in ratio was observed. Analysis of the ratios illustrates a clear pattern of initial decrease (2 hr post-exposure) followed by normalization towards control values (6 and 12 hr post-exposure) but again a decrease at later time periods (24 and 48 hr post-exposure) at all the three concentrations, confirming a time dependent shift in the activities of GOT and GPT during Endocel 35 EC toxicity.

Table 56. GOT:GPT ratio in Pila virens dosed with three sublethal concentrations of Endocel 35 EC

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	7.291	6.852	4.973	7.641	6.532
Control	± SD	4.410	3.472	2.571	3.723	2.662
	Range	4.262-15.010	3.923-11.250	1.602-7.583	3.762-13.460	4.720-11.230
	N	8	8	8	8	8
0.2 ppm of	Mean value	1.780**	5.090	3.710	3.370*	3.540*
Endocel 35 EC	± SD	1.080	1.070	2.770	1.360	0.780
dosed	Range	0.120-3.260	3.610-6.940	0.840-9.930	1.960-5.840	2.440-4.360
	N	8	8	8	8	8
0.4 ppm of	Mean value	1.080**	5.580	5.490	2.612**	3.173*
Endocel 35 EC	± SD	0.755	1.710	1.830	0.991	1.041
dosed	Range	0.240-2.260	3.090-8.000	2.950-8.260	1.802-4.504	1.682-4.663
	N	8	8	8	8	8
0.6 ppm of	Mean value	1.111**	9.371	3.341	2.702**	3.483*
Endocel 35 EC	± SD	0.563	4.862	1.911	0.882	1.213
dosed	Range	0.220-2.083	3.812-18.150	2.133-7.960	1.513-3.582	1.840-5.812

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

6.3.5 Total Haemocyte Count (Table 57 ; Figure 10)

Variations in haemocyte number in snails exposed to 0.2, 0.4 and 0.6 ppm of Endocel 35 EC follow a clear pattern. During the initial hours of exposure (2 and 6 hr), there was marked increase in total haemocytes number in general in all the three concentrations; especially a significant increase in snails exposed to the highest concentration of 0.6 ppm ($\underline{P} < 0.05$). Later, at 12 and 24 hr post-exposure, the total haemocyte number was more or less close to that of the controls. During the final phase, i.e., at 48 hr post-exposure, there was marked reduction in total haemocyte number when compared with the respective controls. The fall in haemocyte number was found to be statistically significant in snails exposed to 0.2 ppm and 0.4 ppm of the toxicant ($\underline{P}< 0.01$, $\underline{P}< 0.05$).

When the total haemocyte number at 2 hr post-exposure period in snails exposed to the three toxicant concentrations was compared, it was found that the increase was significantly higher in 0.6 ppm exposed snails than in those exposed to 0.2 ppm ($\underline{P} < 0.05$). At 6 hr post-exposure the number was found to be significantly higher in 0.2 ppm exposed snails than in those exposed to 0.6 ppm of the pesticide ($\underline{P} <0.01$).

Table 57. Total haemocytes/mm^3 in *Pila virens* dosed with three sublethal concentrations of Endocel 35 EC

	Hours	2 hrs	6 hrs	12 hrs	24 hrs	48 hrs
	N	5	5	5	5	5
	Mean value	5010.00	5658.00	5080.00	5792.00	5448.00
Control	± SD	1259.56	1762.73	753.82	1668.10	1174.85
	Range	4240-6920	4120-8380	4510-6370	4510-8460	3900-7070
	N	8	8	8	8	8
0.2 ppm of	Mean value	6431.00	7164.00	7869.00	5318.00	2748.00**
Endocel 35 EC	± SD	3449.65	1675.63	3959.49	1722.93	1317.74
dosed	Range	3410-12090	5500-9980	2590-13430	4090-10050	1100-4840
	N	8	8	8	8	8
0.4 ppm of	Mean value	7857.00	7128.00	5994.00	5766.00	3297.00*
Endocel 35 EC	± SD	3573.67	3971.38	2302.76	1901.69	1885.85
dosed	Range	4650-16360	3820-16600	2970-9000	3250-8590	1330-6010
	N	8	8	8	8	8
0.6 ppm of	Mean value	9028.00*	5021.00	5258.00	5776.00	3922.00
Endocel 35 EC	± SD	2484.36	1190.29	4003.19	1534.18	1708.11
dosed	Range	4730-16290	3260-7580	2110-15200	4580-9480	1560-6000

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

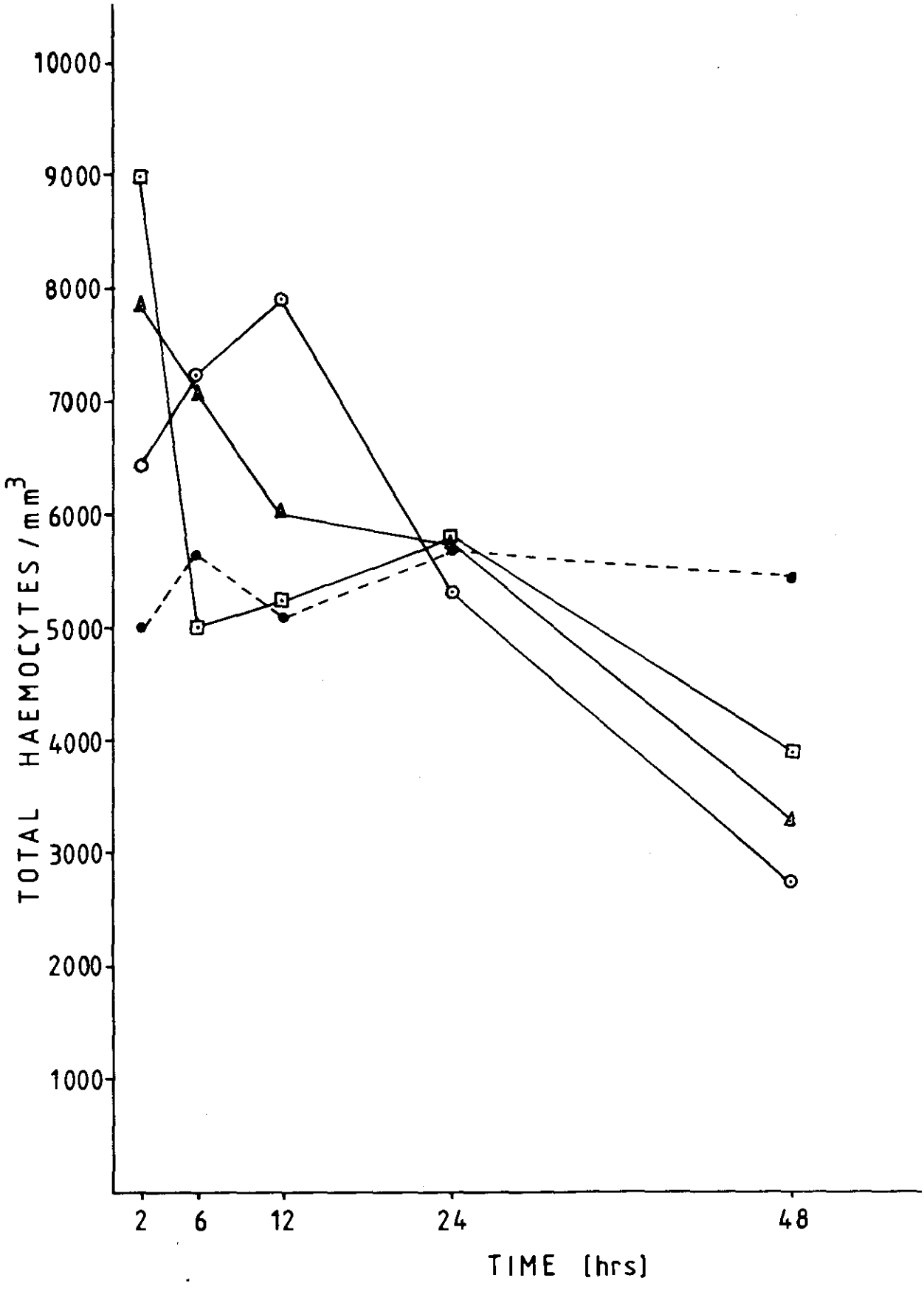

Figure 10. Total Haemocytes/mm^3 in Pila virens *dosed with three sublethal concentrations of Endocel 35 EC. Control (-·-), 0.2 ppm (⊙), 0.4 ppm (△), and 0.6 ppm (⊡).*

6.4 DISCUSSION

A more or less uniform pattern of decrease in the activity levels of acid and alkaline phosphatases (which was significantly lower at 6 hr post-exposure at 0.4 and 0.6 ppm concentrations) was observed in the haemolymph of snails exposed to the three sublethal concentrations of Endocel 35 EC. In the case of ACP, the activity levels at 24 and 48 hr post-exposure at 0.6 ppm were also significantly lower. Interestingly in Dimecron^{R} exposed snails there was conspicuous increase in the level of activities of both ACP and ALP at early time period but in Endocel exposed snails at no time period such elevation was observed. In Endocel exposed snails same sort of parallelism could be drawn in the activity pattern of both the enzymes but in Dimecron exposed snails this was not conspicuous. The present study indicates that the level of ACP activity is seriously affected at the highest concentration of the toxicant.

Inhibition of protein synthesis is a well known effect of endosulfan on aquatic organisms (Indrani, 1980; Murthy and Devi, 1982; Ramalingam and Ramalingam, 1982; Nammalwar, 1984). The general decrease in the activity levels of both ACP and ALP may be attributed to the failure of protein synthesis. Endosulfan (the active ingredient) being a highly lipophilic pesticide easily penetrates the membrane systems, including mitochondria. The overall depression in phosphatases activity can also be attributed to the injury elicited to the mitrochondrial system. In 0.2 ppm exposed snails although

the activity levels of both the enzymes were low, there was no significant statistical difference in values between the experimentals and the controls. Two reasons can be attributed to this, (i) at low concentration, the pesticide could not cause significeant damage to the mitochondrial system, and (ii) the conversion of low amounts of endosulfan into less toxic diol precursor takes place (Shankland and Schroeder, 1973). At higher concentration, the conversion is not much successful, leading to persistence of toxic endosulfan resulting in injury to the oxidative system. A similar pattern of dose dependent suppression of humoral and cell mediated immunity response was reported in rats exposed to endosulfan (Banerjee and Hussain, 1987).

Aspartate and alanine aminotransferases are known to play a strategic role in mobilising L-aminoacids for gluconeogenesis and function as links between carbohydrate and protein metabolism under altered physiological, pathological, and induced environmental stress conditions. In the present study of the GOT activity was found to exhibit a significant decrease during the initial period of exposure in all the three sublethal concentrations. The fall in enzyme activity was found to be maximum in the highest concentration. According to Chow and Pond (1972), the decrease in activity of GOT could be due to (i) damage caused to mitochondrial membrane, loss of matrix and swelling of mitochondrion, or (ii) a decreased availability of precursor aminoacid. Tissue damages, especially liver, in endosulfan exposed fishes are reported by many

investigators (Bouck and Ball, 1968; Hutterner et al., 1968; Manoharan and Subbiah, 1982; Bhatnagar et al., 1987).

The decrease in GOT activity level at 2 hr post-exposure was found to return to the normal level immediately, and later the level of activity remained as that of the control up to 48 hr of exposure in all the three sublethal concentrations. Since the initial fall in activity was normalized immediately, the reason for decrease in activity cannot be attributed to mitochondrial damage although damage may be there. It could be due more to lesser availability of aspartate precursor due to heavy utilization of aminoacids to counter the stress as indicated by Chow and Pond (1978). It could also be due to inhibition of proteolytic enzymes by the toxicant.

The initial response in Endocel 35 EC exposed snails regarding GPT activity was found to be quite opposite to that of GOT activity. In higher concentrations, the GPT registered significant rise in activity than the controls. As the time of exposure continued, the GPT activity was found to return to the normal level but by 24 hr post-exposure, the GPT activity showed significant increase again in snails exposed to the three sublethal concentrations. This increase in GPT activity level might be to compensate the reduction in GOT activity. In gastropods, the GOT level tends to be quite a bit higher than the GPT level in tissues (Swami and Reddy, 1978; Sollock et al., 1979). Catabolism of alanine in most gastropods involve transamination to pyruvate (Livingstone and Zwaan,

1983). While studying the detoxification mechanism of ammonia, in the fish T. mossambica, Chetty et al. (1980) found a similar type of antagonistic pattern of behaviour of GOT and GPT. The increase in GPT was suggested to compensate the loss of GOT activity (Cheng et al., 1980), or due to the increased pyruvate availability (Chetty et al., 1980). Vasanthi et al. (1987), observed a sudden shift to anaerobic respiration in S. mossambicus exposed to Endosulfan. The initial high elevation in GPT activity can be considered as a compensatory mechanism to the low GOT level present at that time period. When the GOT activity reaches the normal level later, the GPT level also registered corresponding decline in activity to bring it to normal levels. Increase in activity of both transaminases during the final phase of the experimental can be attributed to the general increase in gluconeogenic activity which is characteristic of pesticide induced anaerobic metabolism. In the GOT:GPT ratio, it is obvious that the ratio was significantly lower at 2, 24 and 48 hr post-exposure at all concentrations, whereas in Dimecron exposed snails the ratio was lower only at two time periods, at higher concentrations. The reason for the low ratio in Endocel exposed snails is more due to the high activity level of GPT than to the low GOT activity level.

An increase in total haemocyte number was observed in snails exposed to all the three sublethal concentrations of Endocel at the early time period. The increase was found to be dose dependent with

significant increase in the highest concentration. As suggested by Sminia et al. (1983), the increase in cell number can be traced back to the release of circulating haemocytes from connective tissue reservoir compartments due to the presence of pesticide molecules or the production from the amoebocyte producing organ (Jeong et al., 1983). In addition to these, the mitotic activity of the existing cells also will help to maintain the high number of cell for prolonged period (Sminia et al., 1983). At 48 hr exposure, there was a well marked, and significant decrease (at 0.2 and 0.4 ppm) in cell number in all the three concentrations, indicating mass mortality of cells (Pickwell and Steinert, 1984) after encounter with the toxicant molecules, or largescale migration of pesticide-laden haemocytes to the excretory route (George et al., 1976; Coombs 1977; Suresh, 1988). But no correlation could be seen between the low enzyme activity pattern and the total haemocyte number. When the enzyme activity levels were low, there was no corresponding decrease in total count and when there were significantly higher or low counts, there was no corresponding variation in enzyme activity levels. This indicates that the toxicant has adversely affected the enzyme system. However, at this stage it is difficult to say whether the toxicant has adversely affected the release of the enzymes or the synthesis of enzymes. However, had there been damage to the haemocytes the bound enzymes should have been released and there would have been increase in the enzyme activity levels in the haemolymph. But

at no time period an increase was noticed. On the contrary, the decrease in the levels of activity noticed at certain time periods where the toxicant concentrations were high indicates that the toxicant has affected enzyme synthesis.

C H A P T E R - VII

SUMMARY

The present study is an attempt to understand the physiological responses of a freshwater gastropod, in terms of haematological parameters, in normal conditions as well as in various natural and man made altered conditions of the environment. Pila virens, a freshwater prosobranch, commonly found in paddy fields, ponds, and streams of Kerala is selected as the test animal for the present investigation. Various haemolymph constituents such as total carbohydrate, glycogen, total protein, total lipid, urea, ammonia, sodium, potassium, calcium, and chloride which are directly involved in the control and maintenance of different physiological systems, were analysed in the present study. Selected haematological parameters like total haemocyte number, and packed cell volume were also determined. Besides, the activity pattern of selected haemolymph enzymes such as acid phosphatase, (ACP), alkaline phosphatase (ALP), glutamate-oxaloacetate transaminase (GOT), and glutamate-pyruvate transaminase (GPT), all having diagnostic value in terms of internal defence system and metabolism of the organism, were also studied.

The thesis consists of seven chapters followed by the list of references. The first chapter is a general introduction illustrating the importance of the present study.

The introductory part of the second chapter gives detailed information on the present status of the haematological studies in prosobranchs especially to those parameters pertaining to the present

study. Results of the comparative studies among the three selected age/size groups of snails are given in detail in this chapter. The total number of haemocytes in the intermediate group snails was found to be very high than that of the other two size groups. Concentrations of haemolymph glycogen, urea, and chloride were found to be more or less stable in all the three size groups of snails, while the activity levels of total carbohydrate, total protein, total lipid, ammonia, sodium, potassium and, calcium were found to vary in different size/age group snails. Study of the activity pattern of selected haemolymph enzymes also showed that the levels of activities vary among snails of the three size groups. ALP activity was found to be more or less consistent in all the three size groups. ACP activity showed an increasing trend, while both GOT and GPT showed decreasing trend in activity with increase in shell size of snails. The possible influence of age, sex, metabolic rate etc., on various physiological activities of snails is indicated and discussed in detail.

The third chapter deals with the study on the effects of starvation on haemolymph constituents, and activity pattern of selected haemolymph enzymes in the common size group (intermediate size group) snails. In the introductory part, an update information on literature in the field of molluscan starvation studies is given. Results of the three sets of experiments, i.e, 10, 20, and 30 days of starvation, are given in this chapter. Total haemocyte number,

and glycogen levels were found to be little affected even at 30 days of starvation. Haemolymph total carbohydrate, protein, lipid, sodium, potassium, calcium, and chloride concentrations were found to decrease considerably with progress in days of starvation. Haemolymph urea registered increasing trend in 10, 20, and 30 day starved snails, while ammonia was higher in 10 and 20 day starved, but considerably lower in 30 day starved snails than the control values. Analysis of haemolymph enzyme activity levels showed interesting patterns. ACP and ALP activities were found lower in 10 and 20 day starved snails while GOT and GPT activities were significantly lower in 30 day starved snails. Considerable fluctuations in the concentrations of various haemolymph consituents give an insight into the various adaptive measures devised by snails to overcome periods of prolonged unavailability of food. Correlating the pattern of variations in different constituents with metabolic adjustments gives indication of a time dependent shift in metabolite preference, and the significance of this metabolic adaptation is discussed.

Many freshwater prosobranchs including P. virens aestivate during summer when freshwater ponds and paddy fields get dried up. In the fourth chapter, the effects of aestivation at different time periods (maximum 3 months) on various haemolymph constituents, total haemocyte number and on the activity pattern of selected haemolymph enzymes are reported. An initial significant increase followed

by linear decrease in total haemocyte number was noted in aestivating snails. Haemolymph glycogen and total carbohydrate concentrations were found to fluctuate, the lowest concentrations being in two month aestivated snails, while protein and lipid concentrations were found to decrease with progress in time of dormancy. Minimum haemolymph glycogen level was always maintained as there was no significant drop. Haemolymph Na^+, K^+, Ca^{2+}, Cl^-, and ammonia levels were found to be significantly higher in aestivating snails at all the time periods studied. Urea concentration was found to decrease in two month aestivated snails, while a sharp increase was noted in three month aestivated snails. Activity levels of ACP and GOT were generally low in aestivating snails, but ALP levels was found to be higher than that of active snails. ALP activity was very high in one month aestivated snails. GPT activity showed significant increase in two month aestivated snails. Variations in concentration of haemolymph metabolities, end products, haemocyte number, and in enzyme activity levels seem to be correlated in specific direction for the metabolic adaptations which help the snails to survive in an inactive form for prolonged periods without taking food and water. Physiological adaptations as indicated in haematological characters are analysed and interpreted in the discussion part.

Chapters five and six deal with toxicity studies of pesticides on P. virens. Being the dominant group of freshwater molluscs in paddy fields of Kerala, P. virens is exposed to various

types of pesticides routinely used for rice cultivation. Physiological responses of snails exposed to pesticides, reflected in the form of different haematological changes are analysed in detail, especially with respect to enzymes which are active participiants in body defence as well as in metabolic reaction.

A detailed review of literature on the influence of various toxicants, especially of pesticides, on the activity pattern of selected enzymes is given in the introductory part of the fifth chapter. The impact of toxicity of one of the widely used organophosphate pesticides, Dimecron^{R}, on P. virens forms the subject matter of this chapter. Behavioural changes appearing in snails during lethal and sublethal exposure to Dimecron^{R}, which have direct impact on the survival capacity of the organism as well as on the community structure of freshwater habitat, are reported in this chapter. The 96 hr LC_{50} value of P. virens computed for Dimecron^{R} was 34.54 ppm. Activity patterns of ACP, ALP, GOT, GPT, and total haemocyte number were analysed in snails exposed to three sublethal concentrations of Dimecron^{R}. ACP and ALP showed a general trend of increase in activity levels during early time periods of exposure, while the activity levels gradually declined with increase in exposure time. The activity pattern of phosphatases was found to be generally dose-dependent. Total haemocyte number also showed increasing trend during early time periods but later came down below the control level at 48 hr post-exposure. The parallel behaviour of phosphatases

and haemocyte number is correlated, and interpreted in terms of their combined role in responding to pesticide challenge. Both GOT and GPT activity levels were also found to be higher in haemolymph of exposed snails during early time periods but later almost normalised to the control levels. Higher activity levels of GOT were found to be maintained for longer periods when compared with the activity levels of GPT. Probable changes in metabolic activities induced by pesticide stress, in the light of the variations in activity pattern of enzymes are discussed.

In the sixth chapter, the impact of the organochloride pesticide Endocel 35 EC on P. virens, on behavioural responses, as well as on selected haematological characters, are studied. The 96 h LC_{50} value computed for Endocel 35 EC for P. virens was 1.225 ppm. Behavioural changes in snails exposed to different lethal and sublethal concentrations are reported. An increase in total haemocyte count was found during early exposure time period, which was found to be more in higher doses of the three sublethal concentrations studied. The haemocyte number declined with progress in exposure time particularly at later time periods, and at 48 hr post-exposure the decrease was found to be more prominent in snails exposed to lower concentrations than in those exposed to the higher concentration. The activity patterns of phosphatases in control and in those exposed to three sublethal concentrations of pesticide were compared, and the variations analysed. Analysis of the activity pattern of GOT and GPT yielded interesting results. GOT activity

was found to be significantly lower in the initial period of exposure, unlike the results obtained during Dimecron^{R} exposure, while GPT was found to be significantly higher, particularly at higher concentrations. At later time periods also i.e., at 24 and 48 hr, GPT activity was markedly elevated than the activity in control snails. The changes in activity levels of GOT and GPT tend to change GOT:GPT ratio drastically from that observed in control snails as well as from those exposed to three sublethal concentrations of Dimecron^{R}. The probable metabolic changes and compensatory activities of enzymes are discussed in the light of the findings. Physiological responses of P. virens, as reflected in selected haematological characters, to the two classes of pesticides (organochloride and organophosphate), were found to differ considerably as observed in the present study.

In conclusion it can be said that age brings changes in the haemolymph constituents, in the total haemocyte counts, as well as in the activity pattern of selected phosphatases and transferases. Considerable fluctuations noticed in the haemolymph constituents and in other parameters during starvation, aestivation and during sublethal exposure to pesticides give an indication of the various physiological and metabolic adaptive measures devised by the organism to overcome the situation. This study emphasises the fact that in molluscs haemolymph can be taken as an organ system to measure stress induced alterations in the organism.

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