STUDIES ON THE HAEMOLYMPH OF PENAEUS INDICUS H. MILNE EDWARDS

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P. LAXMILATHA, M. Sc.

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PROGRAMME IN MARICULTURE
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
COCHIN - 682 031

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TO MY PARENTS

CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON THE HAEMOLYMPH OF *PENAEUS INDICUS* H. MILNE EDWARDS"** is a bonafide record of the research work carried out by **Kum. P. LAXMI LATHA** under my guidance and supervision under the Post-Graduate Education and Research Programme in Mariculture, at Central Marine Fisheries Research Institute, Cochin, and that no part thereof has been presented for the award of any other Degree.

Dr.A.LAXMINARAYANA
Scientist & Research Guide
Central Institute of
Brackish Water Aquaculture
Narakkal, Cochin-682 505.

Cochin-682 505,
January 1991.

DECLARATION

I hereby declare that this thesis entitled **"STUDIES ON THE HAEMOLYMPH OF *PENAEUS INDICUS* H. MILNE EDWARDS"** has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Laxmi Latha

Cochin-682 031,
January, 1991.

P. LAXMI LATHA

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PREFACE

The role of aquaculture in the improvement of nutrition and socio-economic conditions of the fish farming communities in the rural and coastal regions, cannot be over emphasized. Shrimps, a valuable aquatic food resource high in protein, is the most favoured item among all food fish and commands a lion's share of 20% in the world seafood market. Cultured shrimp claims 22% of the total shrimp trade. The shrimp market is expanding very fast and a global race has started in shrimp farming or rather a 'Shrimp fever' has hit the South East Asian and Latin American countries.

India contributes 7.13% to the world shrimp market. The Indian white shrimp *Penaeus indicus* is a major component of the export trade in India and therefore has been the prime candidate for aquaculture in recent years. This necessitates a thorough understanding of all biological aspects of the species. The present study of the haemolymph of *Penaeus indicus* was undertaken to understand the physiological modifications manifested in the haemolymph in relation to various factors. Studies on the haemolymph provides clear indications about the physiological state and general health of the shrimp which is the most important factor in any aquaculture practice.

The objective of the study was to determine

a) The average quantity of certain biochemical constituents of the haemolymph of *Penaeus indicus* and to verify the importance

of the simple correlation between the quantity or content of the biochemical constituents in the haemolymph and the size of the species, sex, moult and reproductive stages. The biochemical constituents studied are protein, free amino acids, glucose, total lipids, cholesterol, calcium, zinc, iron and manganese.

b) Identify the species specific haemolymph protein pattern by electrophoresis and determine the qualitative variations of haemolymph proteins with respect to sex, size, moult and reproductive stages. Major protein components such as hemocyanin and female specific protein are determined with a view to understand their function.

c) Identify the circulating haemocytes with a view to understand their specific role in the various physiological functions of the species.

The thesis is presented in three chapters. Each chapter has an introduction to the particular aspect of study which includes a review of literature, methodology adopted for the study, the results obtained and discussion on the subject. The first Chapter deals with the biochemical constituents of the haemolymph, the second includes electrophoretic characterization of proteins in the haemolymph and the third Chapter deals with haemocyte identification and classification. A summary of the thesis and literature cited in the text are listed at the end.

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CHAPTER I

BIOCHEMICAL CONSTITUENTS OF THE HAEMOLYMPH

INTRODUCTION

Many fundamental features of the class crustacea are reflected in the nature of the internal medium, the haemolymph. Thus, the exoskeleton, and consequent periodic moulting, the mainly aquatic habit and ammonotelic nitrogen metabolism, all have important correlations in crustacean blood chemistry.

Shrimps live in a wide variety of environments where they are exposed to marked alterations in the physicochemical characteristics of the medium and have to adapt physiologically to all these changes. These changes are most obviously manifested in the composition of the haemolymph. During the moulting and reproductive cycles also the haemolymph constituents show remarkable variations quantitatively and qualitatively. Exposure to polluted waters can also affect the haemolymph in various ways. Thus, studies on the composition of haemolymph of shrimps will provide indications about the physiological modifications associated with moulting and reproductive cycles as well as those induced by changes in environmental conditions. It also provides valuable information on the phylogenetic status of the species.

The level of biochemical constituents in the haemolymph viz. proteins (significantly hemocyanin), free amino acids, free sugars, total lipids, and metallic ions, serve as useful indicators of physiological and

pathological condition of the particular species. Among the crustaceans, haemolymph characterization has been done in several decapods and a few isopods. While the Brachyuran crab and palinurid lobsters have received much attention, the Natantians have attracted little interest. Information on the haemolymph constituents of prawns and shrimps is limited compared to other crustaceans.

The earliest documented preliminary observations on the intraspecific variations on the levels of total protein in the sera of some decapods was by Leone (1953). The total protein in the sera of six decapods - Callinectes sapidus, Cancer magister, C. irroratus, Libinia emarginata, Homarus americanus and an arachnid Limulus polyphemus has been recorded. Variability in the haemolymph protein and copper concentrations in adult blue crabs Callinectes sapidus was investigated by Horn & Kerr (1963) Stewart et al (1967) studied the muscle - weight relationship to serum protein, haemocytes and hepatopancreas in the lobster H. americanus. Glynn (1968) conducted studies on the protein, ionic and phosphate changes associated with moult cycle of Homarus vulgaris. Stewart and Li (1969) studied the ecology of lobster H. americanus using serum protein concentration as an index. The protein and copper content in the haemolymph of Panulirus laevicauda was studied by Baztos and Bezerra (1970). Bursey and Lane (1971) recorded changes in ionic and protein concentration during moult cycle of Penaeus duorarum. Andrews (1972) studied the seasonal variations of haemolymph composition in the crayfish Orconectes limosus. Lynch & Webb (1973) studied the variation in total serum protein of the blue crab Callinectus sapidus. Baseline serum constituents of the Malaysian

prawn Macrobrachium rosenbergii and Pink shrimp Penaeus marginatus was analysed by Balazas et al. (1974). Blood, tissue constituents and water content as indices of nutritional state in the western rock lobster P. longipes has been studied by Dall (1974).

Thermal compensation in protein and RNA synthesis during the intermoult cycle of the American lobster Homarus americanus was studied by Mccarthy et al. (1976). Wong and Freeman (1976) traced the seasonal and thermal effects on the concentration of the haemolymph in the New Zealand freshwater crayfish Paranephrops zealandicus. Spindler - Barth (1976) recorded the changes in the chemical composition of the common shore crab Carcinus maenas, during the moulting cycle. Laevitt and Bayer (1979) devised a refractrometric method for determining serum protein concentration in the American lobster H. americanus. Effect of osmotic stress on the serum protein concentration of Eriocheir sinensis was studied by Gilles (1977). Hepper (1978) studied the changes in blood serum protein levels during the moulting cycle of the lobster H. gammarus. Haemolymph concentrations of total proteins, total copper and combined oxygen saturation were measured on adult crabs at various moulting stages by Truchot (1978). Kazmierczak (1978) measured the content of protein, copper and iron in the serum of A. astacus, A. leptodactylus and Orconectus limosus, occurring in Poland. Winget and Herman (1979) studied the influence of moult cycle and β-ecdysone on protein synthesis in the chelicerate arthropod Limulus polyphemus. Alyakrinskaya (1979) investigated the possible content of haemolymph of few invertebrates. Subhashini and Ravindranath (1980) assessed the quantitative methods and intra-individual variability in the

haemolymph protein concentration of Scylla serrata. Significance of variation in haemolymph copper - protein ratio in the crab Scylla serrata during different hours of the day was elucidated by Arumugam and Ravindranath (1980).

Ruczkal - Pietrzak (1981) studied the influence of eyestalk and chelae ablation on loss of haemolymph protein and weight in crayfish Orconectes limosus. Subhashini (1981) conducted extensive studies on the haemolymph proteins of Scylla serrata. Ramalingam et al. (1981) studied the effects of autotomy and bleeding stress on the haemolymph protein concentration and TCA - soluble nitrogen of the shore crab Ocypode platytarsis. Rodriguez (1981) studied the relation betweeen osmo-regulation and total serum protein of two penaeid shrimps - P. vannamei and P. stylirostris. Smith and Dall (1982) studied the blood proteein, blood volume and extracellular space relationships in two penaeids, P. esculentus and P. plebejus. Significance of periodic fluctuations in the haemolymph proteins and their catabolic products during starvation and repeated injury in Scylla serrata was traced by Subhasini and Ravindranath (1982). AQUACOP (1983) studied the use of serum protein concentration to optimise penaeid spawner quality. Protein and cation content of the haemolymph of three species of Oniscoidea, Oniscus asellus, Porcellio scaber and Cyclisticus convexus was analysed by Gondko et al. (1984). Celia et al. (1985) conducted preliminary studies on circadian variations of haemolymph protein in Penaeus japonicus. Nageswara Rao et al. (1986) studied the changes in haemolymph proteins & copper in the crab Ocypoda macrocera with respect to moult cycle. Ferraris et al. (1986) studied the effect of salinity on total protein, chloride

and calcium concentrations in the haemolymph of Penaeus monodon. Nadarajalingam and Subramaniam (1987) have investigated the light induced biochemical changes in the ovary and haemolymph of the ocypodid crabs Ocypoda platytarsis and O. macrocera. Haemolymph levels of total protein, ecdysteroids and glucose with respect to moult stages was characterized by Chan et al. (1988). Punzo (1989) studied the composition of Mygalomorph spiders.

While extensive work has been done on the protein content in the haemolymph of decapods little attention has been paid to the amino acid composition of the haemolymph. Free as well as bound amino acids play a significant role in osmoregulation and acid-base regulation of the species. Torres (1972) has studied the variation of free amino acids in Penaeus kerathurus during the period between months and during periods of fasting. Lynch and Webb (1973) studied the variations in serum free amino acids and total ninhydrin positive substances of the blue crab Callinectes sapidus. Free amino acids of the haemolymph of Ligia exotica, Porcellio laevis, Armadillidium vulgare and Armadillo officinalis was estimated by Sevilla and Lagarrigue (1974). Charmantier et al. (1975) studied the level of free amino acids in the haemolymph during the puberty molt and senescence of isopod Sphaerona serratum. While working on a purified diet for prawn, Deshimaru (1975) studied the changes in free amino acids in the haemolymph of P. japonicus after feeding. The retention of amino acids in the haemolymph during diuresis in Rhodnius was studied by Maddrell et al. (1980). Fair and Sick (1980) evaluated the serum amino acid concentrations during starvation in the prawn, Macrobrachium rosenbergii

as an indicator of metabolic requirements. Amino acid composition of haemolymph of the crab, Paratelphusa levis during healthy and pathogenic conditions was elucidated by Kumar Bhagwati and Raghu Varman (1981). The relationship between plasma proteins and intra-cellular free amino acids during osmotic regulation in C. maenas was studied by Zatta (1987).

Blood sugars in the haemolymph of crustaceans has been least studied although the slightest handling, environmental or pathological stress is reflected by the altered level of sugars in the haemolymph. Kleinholz and Little (1949) conducted experiments to study the regulation of blood sugar concentration in Libinia emarginata. Riegal (1960) studied the blood glucose in crayfish in relation to moulting and handling. Meenakshi and Scheer (1961) traced the metabolism of glucose in the crabs C. magister and Hemigrapsus nudus. Dall (1964) evaluated the carbohydrates in the haemolymph of Metapenaeus mastersii. Variations in the blood glucose level of Callinectes sapidus and Uca pugilator was studied by Dean and Vernberg (1965). Telford (1968) identified and measured sugars in the blood of three species of Atlantic crabs. Carbohydrate metabolism of two crustaceans during starvation, Emerita asiatica and Ligia exotica was studied by Parvathy (1971a). The blood sugar metabolism during molting in the isopod Ligia exotica and glycogen storage in relation to the moult cycle in E. asiatica and L. exotica was also studied by Parvathy (1971,b,c). Sevilla and Lagarrigue (1973) conducted preliminary studies on the glucose in the haemolymph of four oniscoides, Ligia italica, Porcellio laevis, Armadillidium vulgare and Armadillo officinalis. Lynch and Webb (1973) evaluated the variations in serum glucose levels in natural

populations of mature blue crab Callinectes sapidus. Romestand and Trilles (1974) studied the variations in glucide content of the haemolymph and hepatopancreas of Minertia oestroides. Hamann (1974) studied the relationship between sinus gland and neuroendocrine control of circadian blood sugar variations in the crayfish. Quantitative changes in the blood sugar levels of Crangon vulgaris was studied by Poolsanguan and Uglow (1974). Spindler et al (1974) traced cyclic nucleotides and crustacean blood glucose levels. Carbohydrate levels in the freshwater field crab Paratelphusa hydrodromous in relation to sex, size and nutritional state was evaluated by Ramamurthi and Veerabhadrachari (1975). William and Lutz (1975) examined the role of haemolymph in the carbohydrate metabolism of Carcinus maenas. Telford (1975a,b) studied the blood glucose variation in crayfish with respect to moulting and source of glucose and role of eyestalk factor in hyperglycemia of Cambaras robustus. Quilter (1977) studied the effect of optic nerve section on blood glucose levels in Paranephrops zealandicus Saravanan (1979) conducted studies on the haemolymph carbohydrates in Scylla serrata. Saravanan and Ravindranath (1981) studied the significance of periodic fluctuations in the haemolymph carbohydrates of Scylla serrata during starvation and repeated injury. Nagabhushanam and Kulkarni (1979,1980) investigated the neuroendocrine regulation of blood glucose as well as variation in relation to ovarian maturation in Parapenaeopsis hardwickii. Stettan (1980) studied the metabolism of glucose and glycogen in Limulus polyphemus in vivo. Effect of the variation of salinity on the glycemic levels of Calinectes danae was investigated by Lacerda and Sawaya (1983). Blood glucose regulation in an intertidal crab, Chasmagnathus granulata

was investigated by Dos santos et al.(1986). Spaargaren and Haefner, Jr., (1987) studied the effect of environmental osmotic conditions on blood and tissue glucose levels in the brown shrimp Crangon crangon. Aspects of entrainment of Crustacean Hyperglycemic Hormone (CHH) cell activity and haemolymph glucose levels in crayfish Astacus leptodactylus was studied by Kallen et al (1988).

Lipid and fatty acid constituents of the haemolymph of crustaceans have been studied with respect to dietary requirements. Zandee (1962) studied the lipid metabolism in Asticus asticus, Morris (1973) conducted preliminary investigation on the changes in the lipid composition of Acanthephyra purpurea during its duirual migration. Castell et al.(1975) conducted feeding trials on juvenile American lobster H. americanus to study the cholesterol requirements. Teshima et al.(1975) investigated the bioconversion of desmosterol to cholesterol at various stages of moulting cycle in Palaemon serratus. Sevilla and Lagarrigue (1975) conducted preliminary studies on the total lipids in haemolymph of four species of Oniscoides Ligia italica, Porcelli laevis, Armadillidium vulgare and Armadillo officinalis. Seasonal variations of the fatty acid composition of Penaeus japonicus was elucidated by Guary et al (1975). Lautier and Lagarrigue (1976) investigated the variations in total lipids, phospholipids, triglycerides and cholesterol content in the ovary, hepatopancreas and haemolymph of female crab Pachygraspsus marmoratus. Teshima et al.(1976) investigated the sterol biosynthesis from acetate and fate of dietary cholesterol and desmosterol in crabs. Kanazawa et al.(1976) traced the variation of lipids and cholesterol content of prawn tissue (Penaeus japonicus) during

the moulting cycle. Teshima and Kanazawa (1976) studied the variation in lipid classes during the moulting cycle of freshwater shrimp palaemon serratus. Pezalla and Cerman (1977) conducted quantitative and qualitative analysis on the midgut gland and haemolymph lipids in the chelicerate arthropod Limulus polyphemus. Lee and Puppione (1978) isolated and analysed the haemolymph lipoproteins in the spiny lobster, Panulirus interruptus. Teshima and Kanazawa (1978) investigated the release and transport of lipids into the haemolymph of Penaeus japonicus. Morajkar and Nagabushanam (1981) traced the variation in cholesterol content in the haemolymph of female prawn, Macrobrachium kistensis during the reproductive cycle. Serrazanetti et al. (1982) investigated the sterol constituents of the isopod, Idotea balthica. Cholesterol turnover and ecdysone content in tissues of normal and de-eyestalked crabs (Cancer antennarius) was investigated by Vensel et al. (1984). Spaargaren and Mors (1985) measured the total lipid, β-lipoprotein, triglyceride, cholesterol and free fatty acid concentrations in the blood of shore crab, Carcinas maenas, experimentally exposed to various environmental osmotic conditions. Spaziani (1988) studied the annual profile of concentrations of seerum high density lipoproteins in Canceer antennarius. Kanazawa et al. (1988) investigated the tissue uptake of radioactive cholesterol in the prawn, Penaeus japonicus during induced ovarian maturation.

Haemolymph studies in Arthropoda have concerned various biochemical parameters such as those mentioned above. From the view point of biochemistry of respiratory proteins, haemolymph studies are interesting in species where oxygen is transported by hemocyanin. Numerous reports

indicate that some components such as calcium, zinc and magnesium in the haemolymph and other environmental factors affect significantly, the structure and function of this protein. The role of these metallic ions in relation to acid base regulation has also initiated profound interest.

The role of calcium in moulting and calcium metabolism has been the focus of haemolymph studies. Robertson (1977) initiated studies on calcium metabolism of the shore crab, Carcinus maenas. He also studied ionic regulation in relation to moulting cycle in the same species (1960). Bryan (1964) studied the mechanism of zinc regulation in the lobster, Homarus americanus. The absorption and loss of radioactive and nonradioactive manganese by the lobster Homarus vulgaris was traced by Bryan and Ward (1965). Bryan (1968) measured the concentration of zinc and copper in 18 species of decapod crustaceans, ranging from freshwater to purely marine species. Knauer (1970) determined the content of magnesium, manganese, iron, copper and zinc in the tissue of two penaeids, Penaeus aztecus and P. duorarum. Robertson (1970) measured the principal osmotic constituents in the blood of Limulus polyphemus to study the osmotic and ionic regulation in the horseshoe crab.

Spaargaren and Kraay (1973) studied the contribution of chloride to the electrolyte regulation in blood and tissue of the shrimp Crangon crangon. Bittner and Kopanda (1973) investigated the factors influencing moulting in the crayfish, Procambarus clarki. Lynch and Webb (1973) quantified the serum chloride and osmotic constituents of the blue crab Callinectes sapidus. Variations in the major cations in the serum of blue

crab C. sapidus was studied by Colvoceresses and Lynch (1973). Greenaway (1974, 1976) estimated the total blood calcium and haemolymph calcium concentrations as well the calcium balance at premoult stage of the fresh-water crayfish, Austropotamobius pallipes. He also studied the calcium balance at postmoult stage (1974). Major cation variations, sodium, magnesium, calcium and potassium in the serum of C. sapidus was investigated by Colvoceresses et al (1974).

Colvoceresses and Lynch (1975) studied the variations in serum copper and zinc of the blue crab, C. sapidus Hecht (1975) attempted to investigate the origin of calcium used in the moulting process of the crab Seserma catenata. Martin (1975) studied the variations of copper and zinc during the inter moult cycle of Cancer irroratus.

Greenaway (1976) studied the regulation of haemolymph calcium concentrations of the crab Carcinus maenas acclimated to different salinities. Bohm and Eibisch (1976) conducted studies on the mineral metabolism of Orconectes limosus and Armadillidium nasatum in the premoulting stage. Wright (1977) studied the uptake of cadmium in the haemolymph of the shore crab, C. maenas and its relationship with copper and other divalent cations. Dejours and Beekenkamp (1978) investigated the changes in acid-base balance of the haemolymph during the premoult, moult and postmoult stages of the crayfish, Astacus leptodactylus. Truchot (1978) investigated the variations of blood concentration of functional hemocyanin during the moult cycle in the crab, C. maenas. Boone and Schoffeniels (1979) evaluated the possible osmoregulatory function of hemocyanin during

hypo-osmotic stress in the shore crab, C. maenes. Wright (1979, 1980) studied the calcium regulation in intermoult,premoult and postmoult Gammarus pulex. Regulation of blood ions in Carcinus maenas was worked out by Zanders (1980).

Roer (1980) investigated the mechanisms of resorption and deposition of calcium in the carapace of the crab C. maenas. Gondko et al.(1981) compared the haemolymph content of protein and cations (Ca, Mg, Na, & K), clotting time and pH in three crayfish species Astacus astacus, A. leptodactylus and Orconectes limosus. Rao et al.(1982) studied the fluctuation in calcium levels in the exoskeleton, muscle and haemolymph of Penaeus indicus cultured in brackishwater pond. White and Rainbow (1982) investigated the regulation and accumulation of copper, zinc and cadmium by the shrimp Palaemon elegans. Greenaway (1983) examined the dynamics of calcium transport in two widely euryhaline marine crabs C. sapidus and C. maenas. Nikinmaa et al.(1983) investigated the effects of hypoxia and acidification on the haemolymph pH values and ion concentrations in the freshwater crayfish, Astacus astacus. Zatta et al. (1983) worked out the role of hemocyanin in the metal transport in Carcinus maenas blood.

Sparkes and Greenaway (1984) identified the haemolymph as the storage site for cuticular ions during premoult in the freshwater land crab, Holothuisana transversa. Fieber and Lutz (1984) traced the magnesium and calcium metabolism during moulting in the freshwater prawn, Macrobrachium rosenbergii. Zatta (1984) investigated zinc transport in

the haemolymph of C. maenas. White and Rainbow (1984) studied the zinc flux, effects of temperature, zinc concentrations as well as zinc regulation during moulting. Bryan et al (1984) traced zinc regulation pathways in the lobster, Homarus gammarus. Calcium balance and moulting in crustacea was elucidated by Greenaway (1985). Timing and mechanism of postmoult calcification in the blue crab C. sapidus was elucidated by Cameron (1989) Nugegoda and Rainbow (1989) studied the effects of salinity changes on zinc uptake and regulation by decapod crustaceans Palaemon elegans and Palaemonetes varians.

From the foregoing survey of literature, it is clear that haemolymph studies on penaeid species is rather limited. The present study involves the evaluation of certain organic and metallic constituents of the haemolymph of *Penaeus indicus* with respect to sex, size groups, moult and reproductive cycle. The biochemical constituents studied are total protein, total free amino acids, glucose, total lipid, total free cholesterol, copper, calcium, iron, zinc and manganese.

MATERIALS AND METHODS

1.1. Collection of specimens:

Penaeus indicus used for the present study was collected from the growout ponds of Marine Prawn Hatchery Laboratory of Central Institute of Brackishwater Aquaculture and the Brackishwater Farm of Matsyafed at Narakkal. The prawns were transported to the laboratory at Central Marine Fisheries Research Institute, Cochin and maintained in 23-25‰ seawater in 1 ton fibreglass tanks provided with continuous aeration. After a period of 12-16 hour acclimation, the haemolymph was extracted.

1.2. Haemolymph Extraction:

Haemolymph was extracted by inserting a 22 or 23 guage needle attached to a 1 ml syringe, below the cephalothorax, into the heart. The specimen was blotted dry before extraction to prevent water from being mixed with the haemolymph. The total length, total weight, sex and moult stage was recorded. The syringe was rinsed with 3% sodium citrate solution before extraction to prevent clotting of the haemolymph. The haemolymph samples from individual specimens were transferred into separate glass tubes. The samples collected were either used immediately for analyses or stored at 4°C for subsequent analyses.

The study was undertaken under threee main aspects:

1. Biochemical
2. Electrophoretic
3. Cytological

1.3. **Biochemical studies:**

Variations in biochemical constituents in the haemolymph of in relation to the following was studied.

a) Sex (Male & Female)

b) Size groups (Group I, (60-80 mm Group II, 80-100 mm Group III, 100-120 mm Group IV 120-140 mm)

c) Moulting cycle according to Drach and Tchernigovtzeff (1967)

A	-	Early postmoult
B	-	Late postmoult
C	-	Intermoult
$D_{0,}$	-	Early premoult
D_1	-	Mid premoult
D_1'''	-	Late premoult
D_{2-3}	-	Premoult

d) Maturity stages (Ist, IInd, IIIrd, IVth (fully mature) and Vth (spent))

The biochemical constituents in the haemolymph estimated are total protein, total free amino acids, glucose, total lipids, cholesterol, copper, calcium, zinc, iron and manganese. Haemolymph samples from specimens of similar moult stages and sex was pooled together for all estimations for the smaller size group i.e. 60-80 mm.

Standard methods were followed for all biochemical estimations with necessary modifications. Standard graphs were plotted with the concentration of each biochemical parameter in different dilutions of the working standard solution, in the X-axis and the optical density in the Y-axis. The concentration of different parameters in the samples was compared and

calculated from the graph and also by the following formula:

$$\text{Amount of the bio-chemical constituent present in the sample} = \frac{\text{OD of the Sample}}{\text{OD of the Standard}} \times \frac{\text{Concentration of the standard}}{\text{Weight of the sample}}$$

The total optical density or the colour developed for total proteins, total free amino acids, glucose, total lipids and cholesterol was measured using a senior spectrophotometer (ECIL G686 5D). The samples were taken in quartz cuvetts. The copper calcium, iron, zinc and manganese content in the haemolymph was estimated using Perkin-Elmer 2380 Atomic Absorption Spectrophotometer with appropriate standards. Only Analar grade, extrapure chemicals were used for all estimations.

1.3. **Total Protein:**

The Biuret method (Gornall et al., 1949) was adopted for estimation of protein in the haemolymph. Bovine Serum Albumin Fraction V crystals from Sigma co. was used as standard. A standard stock solution was prepared at a concentration of 25 mg/5 ml 1 N NaoH. Different dilutions in the range of 0.5-5 mg/ml was prepared from the stock solution to which the alkaline and Biuret reagent was added as in the case of haemolymph samples. The procedure for the haemolymph sample was as follows:

1) (0.05 ml) 5 µl of haemolymph was taken in a centrifuge tube using a fine calibrated micropipette, to which 1 ml of deproteinizing agent (80% ethanol) was added.

2) Centrifuged at 3000 rpm for 5 minutes, decanted the supernatant (used for total free amino acid estimation); 2 ml of 1 N NaoH was added to dissolve the precipitate.

3) After 10 minutes, added 8 ml of Biuret reagent, mixed well and allowed to stand at room temperature for 30 minutes.

4) Blank having 2 ml 1 N NaoH and 8 ml Biuret reagent was set up simultaneously.

5) After 30 minutes the optical density of the blue colour developed was measured against the blank at 540 nm.

1.3.2. **Total free amino acids:**

The Ninhydrin method (Yemm & Cocking, 1955) was adopted for estimation of total free amino acids in the haemolymph. A combined standard of Glycine and Glutamic acid is prepared, 1 ml of which contains 0.006 mg amino nitrogen, which is equivalent to 0.04758 mg AA per ml. The standard and haemolymph sample was estimated as follows:

1) 50 µl haemolymph, 1 ml deproteinizing agent is added. (80% ethanol)

2) Centrifuged at 5000 rpm for 5 mins and the supernatant is decanted.

3) To the supernatant (1 ml) 0.5 ml citrate buffer pH 5.0 (0.2 M) is added.

4) Blank (1 ml distilled water) and standard (1 ml) are set up simultaneously.

5) To each of the above, 1.2 ml of 'solution C' is added.

6. Heated in a boiling water bath for 15 minutes.

7) Cooled in running tap water for 5 minutes.

8) To each, added 2.3 ml of 60% ethanol

9) The optical density of the purple colour developed was measured at 570 nm against the blank.

1.3.3. Glucose:

Nelson-Somogyi method (1944) was adopted for estimation of glucose in the haemolymph with slight modifications. A standard stock solution was prepared using D-glucose in saturated Benzoic acid (20 mg/100 ml). Different dilutions of the working solution with concentration of glucose ranging from 10-100 µl/ml was prepared and the procedure adopted given below was followed:-

1. To 100 µl of haemolymph, 1.5 ml deionized water was added, to which 1 ml 5% $ZnSO_4$ solution was added.
2. Centrifuged for 20 minutes at 5000 rpm. 1 ml of the supernatant was used for estimation.
3. To (1 ml) the supernatant, 1 ml tartaric reagent was added.
4. Blank (1 ml deionized water) was set up simultaneously to which 1 ml tartaric reagent was added.
5. Mixed well, heat for 20 minutes in a boiling water bath.
6. Cooled, added 1 ml Arsenomolybdate reagent. Mixed well.
7. Diluted to 5 ml (i.e. 2 ml D.W). Mixed well.
8. The optical density of the blue colour developed was measured at 520 nm against the blank.

1.3.4. Cholesterol:

Total cholesterol was determined by Henley's (1957) method as given by Varley (1975). Standard stock solution was prepared using 100 mg of cholesterol in 100 ml of glacial acetic acid to get a concentration of 1 mg/ml. Working solution was prepared fresh by mixing 1 ml of the stock solution with 24 ml of ferric chloride-acetic acid reagent. Different dilutions of this solution with concentration ranging from 20-200 µg/5ml were treated as in the case of haemolymph samples.

1) To 100 µl of haemolymph, 5 ml of ferric chloride - acetic acid reagent was added, and left overnight in the refrigerator for the proteins to flocculate.

2) Centrifuged for 20 minutes at 3000 rpm and the supernatant was transferred to a clean, dry glass tube.

3) 3 ml of Con. H_2SO_4 was added and thoroughly mixed.

4) 5 ml ferric chloride acetic acid reagent was treated as the blank, to which 3 ml H_2SO_4 was added.

5) After 30 minutes the optical density of the brown colour developed was measured at 560 nm against the blank.

1.3.5. **Total lipids:**

Total lipids was determined by sulphophospho-vanillin method of Barnes & Blackstock (1978). Standard stock solution was prepared fresh by dissolving 80 mg of cholesterol in 100 ml of chloroform-methanol (2:1 v/v) mixture which is equivalent to 100 mg of total lipid in 100 ml 2:1 v/v chloroform-methanol mixture. Working solutions of different concentrations were prepared from the stock solution in the range 50-500 mg/0.5 ml and procedure adopted for the haemolymph was followed. 0.5 ml chloroform-methanol mixture is treated as blank.

1) To 100 µl of haemolymph, 1 ml of (2:1 v/v) chloroform-methanol mixture was added and left overnight in the refrigerator.

2) 0.5 ml of this lipid extract (supernatant) was taken in clean, dry glass tubes and dried <u>in vacuo</u> over silica gel in a desiccator.

3) To the dried sample,0.5ml of Con.H_2SO_4 was added and shaken well.

4) The tube was plugged with non-absorbant cotton and heated at 100°C in a boiling water bath for 10 minutes and cooled under running tap water.

5) 0.1 ml of the acid digest is transferred to a clean dry glass tube and 2.5 ml of sulphophospho-vanillin reagent was added and mixed thoroughly in a cyclo-mixer.

6) After 30 minutes, the optical density of the pinkish red colour developed is measured at 520 nm against the blank.

1.3.6. **Copper, Zinc, Iron, Manganese and Calcium:**

100 µl of haemolymph sample was taken in clean dry digestion tubes. 2 ml of extrapure concentrated nitric acid and 1 ml perchloric acid was added and digested for 2 hours at 150°C in digestion chamber, until a clear solution was formed. The digested samples were diluted to 5 ml with deionized water. For calcium estimation, the samples were diluted with 0.1%(w/v) Lanthanum Chloride solution in deionized water to eliminate interferring elements.

The samples were analysed in Atomic Absorption Spectrophotometer using air acetylene flame at 324.5 nm for copper, 213 nm for zinc, 279 nm for manganese, 248.3 nm for iron and 422.7 nm for calcium, against the blank.

The standard stock solution for Copper was prepared by dissolving 1.000 g of copper metal in a minimum volume of concentrated nitric acid and diluting to 1 litre with 1% (v/v) concentrated nitric acid, to get a final concentration of 1000 mg/l. Different working standard solutions (1 ppm, 2 ppm, 3 ppm) were prepared fresh by diluting with deionized

water. Denionized water (100 ml) was used as blank.

The standard stock solution for Zinc was prepared by dissolving 0.500 g of zinc metal in a minimum volume of concentrated hydrochloric acid and diluting to 1 litre with 1% (v/v) hydrochloric acid. Different dilutions were prepared as in the case of copper, to give a final concentration of 500 mg/l.

The standard stock solution for Iron was prepared by dissolving 1.000 g of iron wire in 50 ml of concentrated nitric acid and diluting to 1 litre with deionized water to get a final concentration of 1000 mg/l. Different dilutions were prepared as in the case of copper.

The standard stock solution for Manganese was prepared by dissolving 1.000 g of manganese metal in a minimum volume of concentrated nitric acid and diluting to 1 litre with 1% (v/v) nitric acid to get a final concentration of 1000 mg/l. Different working standard solutions were prepared as in the case of copper.

The standard stock solution for Calcium was prepared for 4 hours at 120°C, in 50 ml deionized water, to which 10 ml hydrochloric acid was added, and diluted to 1 litre, to get a final concentration of 500 mg/l. Different working standard solutions were prepared as in the case of copper. 0.1% Lanthanum Chloride is used as blank.

Statistical Analyses:

Mean and standard deviation for each of the biochemical parameter was calculated. Analysis of variance (ANOVA-1, programmed in BASICA) and T-test for comparison of means were performed for each parameter to test significant difference,

a) between the four size groups

b) between sexes in each size group, and

c) between the moult stages in size group II (80 - 100 mm) except for glucose for which size group III (100- 120 mm) was used.

RESULTS

Total Protein

The mean total protein content recorded in the haemolymph in the four size groups was 46.79 ± 9.2 mg/ml in group I (60-80 mm), 49.72 ± 12.3 mg/ml in group II (80-100 mm), 66.32 ± 14.54 mg/ml in group III (100-120 mm) and 72.56 ± 15.33 mg/ml in group IV (120-140 mm) (Table 1a). The total protein content in the haemolymph ranged fom 20 mg/ml to 130 mg/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in the level of total protein in the haemolymph among the four size groups. There is significant increase in protein content with increase in size of the species.

The mean total protein content in male and female in each group in indicated in Table 1b. There was no significant difference in the protein content in the haemolymph of males and females, in any of the groups. In group I, the mean protein content in the males was 46.62 ± 9.62 mg/ml and in females 46.98 ± 9.59 mg/ml. In group II, the mean protein content in males was 48.412 ± 12.95 mg/ml and in females 51.027 ± 12.6 mg/ml. In group III, the mean protein content in males and females was 65.84 ± 15.513 mg/ml and 66.81 ± 14.72 mg/ml respectively and in group IV 71.68 ± 16.16 mg/ml and 73.45 ± 15.69 mg/ml in males and females respectively.

The protein content in the haemolymph of *P. indicus* during the moult cycle indicated a cyclic pattern. In all size groups, the protein content of the haemolymph was minimum during the period just after ecdysis

(stage A), and increased gradually thereafter and reached a peak in premoult stage (D_1''') and sudden decline just before ecdysis (D_{2-3}). (Figs 1a, 2a, 3a, 4a). Analysis of variance in group II (80-100 mm) indicated that the differences in protein content in the haemolymph between the different moult stages was highly significant (Table 1c). The total mean protein content in the haemolymph recorded in the different moult stages are 31.19 mg/ml (Early postmoult or stage A), 39.89 mg/ml (Late postmoult or stage B) 45.28 mg/ml (Intermoult or stage C), 52.14 mg/ml (Early premoult or D_0) 58.33 mg/ml (Mid premoult or D_1'), 70.83 mg/ml (Late premoult or D_1'''), and 46.10 mg/ml (premoult or D_{2-3}).

Total free amino acids

The total free amino acids (TFAA) in the haemolymph indicated pattern similar to total protein. The mean total free amino acids in the group I (60.80 mm) was 1.63 ± 0.35 mg/ml in group II (80-100 mm) 2.71 ± 0.55 mg/ml, in group III 3.56 ± 0.73 mg/ml and in group IV 3.893 ± 0.85 mg/ml. The total free amino acids in the haemolymph ranged from 0.5 mg/ml to 9.7 mg/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in level of total free amino acids between the four size groups (Table 2a). The TFAA content in the haemolymph registered progressive increase with size group.

The total free amino acids in the haemolymph of males and females in each group is indicated in Table 2b. There was no significant difference in total free amino acid content in the haemolymph of males and females in any size group. In group I the mean TFAA content recorded was 1.632 ± 0.403 mg/ml in males and 1.647 ± 0.313 mg/ml in females. In group II,

the mean TFFA content in males is 2.681 ± 0.572 mg/ml and 2.71 ± 0.563 mg/ml in females. In group III the TFAA content recorded is 3.528 ± 0.718 ad 3.589 ± 0.802 mg/ml in males and females respectively and in group IV, it was 3.295 ± 0.927 mg/ml and 3.862 ± 0.927 mg/ml respectively.

The total free amino acid content in the haemolymph during the moult cycle indicated a cyclic pattern. In all size groups, the total free amino acid content was minimum during the period just after ecdysis (stage A) and gradually increased thereafter and reached a peak in early and mid premoult stages (upto D_1''') and declined suddenly during premoult (D_{2-3}) (Figs. 1a, 2a, 3a, 4a). Analysis of variance of the total free amino acid content in the haemolymph in the size group II (80-100 mm), indicated that the difference of TFAA content in the different moult stages was highly significant (Table 2c). The mean TFAA content in the haemolymph recorded during stage A was 1.81 mg/ml, stage B 2.13 mg/ml, stage C 2.58 mg/ml, stage D_0 2.97 mg/ml, stage D_1' 3.15 mg/ml, stage D_1''' 3.27 mg/ml and stage D_{2-3} 3.08 mg/ml.

Total lipid:

The mean total lipid content in the haemolymph in the four size groups in indicated in Table 3a. In group I, the total lipid content was 0.907 ± 0.18 mg/ml, in group II 1.34 ± 0.34 mg/ml, group III 1.49 ± 0.33 mg/ml and group IV 1.67 ± 0.42 mg/ml. The total lipid content in the haemolymph ranged from 0.56 mg/ml to 3.8 mg/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in the level of total lipid in the haemolymph among the four size groups (Table 3a).

The total lipid content in the haemolymph of males and females in each group is indicated in Table 3b. There was no significant difference in total lipid content in the haemolymph of males and females in any size group. In group I the mean total lipid content in the haemolymph was 0.875 ± 0.164 mg/ml and0.938 ± 0.20 mg/ml, in group II 1.351 ± 0.349 mg/ml and 1.326 ± 0.358 mg/ml, group III 1.493 ± 0.344 mg/ml and 1.502 ± 0.333 mg/ml and group IV 1.661 ± 0.445 mg/ml and 1.690 ± 0.421 mg/ml in males and females respectively.

The total lipid content in the haemolymph during the moult cycle indicated a cyclic pattern. In all size groups, the total lipid content was minimum during the period just after ecdysis (stage A) and gradually increased thereafter and reached a peak in early and mid premoult stages and declined suddenly durig premoult (Fig. 3 1a, 2a, 3a, 4a). Analysis of variance of lipid content in the haemolymph in size group II (80-100 mm) indicated that the difference in total lipid content in the different moult stages was highly significant (Table 3c). The mean total lipid content in the haemolymph recorded during early postmoult was 0.905 mg/ml, late postmoult - 0.926 mg/ml, intermoult - 1.114 mg/ml early premoult - 1.497 mg/ml, mid premoult - 1.639 mg/ml, late premoult - 1.73 mg/ml and premoult - 1.605 mg/ml.

Cholesterol:

The mean total free cholesterol content recorded in the haemolymph in the four size groups was 0.434 ± 0.05 mg/ml (group I) 0.5917 ± 0.14 mg/ml (group II) 0.637 ± 0.17 mg/ml (group III) and 0.99 ± 0.25 mg/ml (group IV).

The total free cholesterol content in the haemolymph ranged from 0.10 mg/ml to 2.36 mg/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in level of cholesterol among the four size groups (Table 4a). The cholesterol content in the haemolymph increased with increase in size.

The total cholesterol content in the haemolymph in the males and females of each size group is indicated in Table 4b. There was no significant difference in the cholesterol content in the haemolymph of male and female in any size group. In group I, the mean cholesterol content in the haemolymph was 0.4301 ± 0.048 mg/ml and 0.438 ± 0.56 mg/ml, in group II 0.5922 ± 0.151 mg/ml and 0.5911 ± 0.1504 mg/ml, group III 0.6295 ± 0.1807 mg/ml and 0.653 ± 0.168 mg/ml and group IV 1.02 ± 0.237 mg/ml and 1.00 ± 0.263 mg/ml in males and females respectively.

The cholesterol content in the haemolymph during the moult cycle exhibited a cyclic trend. In all size groups, the cholesterol content during the period just after ecdysis was minimum and gradually increased upto the late premoult stage and declined just before ecdysis (premoult) (Figs. 1a, 2a, 3a, 4a). Analysis of variance of cholesterol content in the haemolymph in size group II (80 - 100 mm) indicated that the difference in cholesterol content between the different moult stages was highly significant (Table 4c). The mean cholesterol content in the haemolymph recorded during early postmoult is 0.398 mg/ml, late postmoult - 0.453 mg/ml, intermoult - 0.488 mg/ml, early premoult - 0.597 mg/ml, midpremoult - 0.751 mg/ml, late premoult - 0.727 mg/ml and premoult - 0.686 mg/ml.

Glucose:

The mean glucose content recorded in the haemolymph in the four size groups was 0.267 ± 0.02 mg/ml (group I), 0.369 ± 0.03 mg/ml (group II), 0.504 ± 0.08 mg/ml (group III), and 0.592 ± 0.12 mg/ml (group IV). The glucose content in the haemolymph ranged from 0.1 to 1.1 mg/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in the level of glucose among the four size groups (Table 5a). Glucose content in haemolymph increased with size of the species.

The mean glucose content in the haemolymph in the males and females in each size group is indicated in Table 5b. There was no significant difference in the glucose content in the haemolymph of males and females in any size groups. In group I, the mean cholesterol content in the haemolymph was 0.265 ± 0.027 mg/ml and 0.268 ± 0.022 mg/ml, in group II, 0.365 ± 0.033 mg/ml, and 0.366 ± 0.049 mg/ml in group III, 0.503 ± 0.099 mg/ml and 0.504 ± 0.082 mg/ml and in group IV, 0.590 ± 0.127 mg/ml and 0.594 ± 0.125 mg/ml in males and females respectively.

The glucose content in the haemolymph during the moult cycle exhibited a cyclic trend. In all size groups, the glucose content in the haemolymph during the period just after ecdysis was minimum and gradually increased upto the late premoult and declined suddenly just before ecdysis (premoult) (Figs. 1a, 2a, 3a, 4a). Analysis of variance of glucose content in the haemolymph in size group III (100 - 120 mm) indicated that the difference in glucose content between the different moult stages was highly

significant (Table 5c). The mean glucose content in haemolymph recorded during early postmoult was 0.378 mg/ml, late postmoult - 0.410 mg/ml, intermoult - 0.460 mg/ml, early premoult 0.518 mg/ml, mid premoult 0.563 mg/ml, late premoult - 0.614 mg/ml and premoult - 6.585 mg/ml.

Copper:

The mean total copper content recorded in the haemolymph in the four size groups was 270.57 ± 52.21 /ug/ml (group I), 347.38 ± 55.53 /ug/ml (group II), 454.27 ± 91.68 /ug/ml (group III) and 619.97 ± 177.41 /ug/ml (group IV). The total copper content in the haemolymph ranged from 135 /ug/ml to 1225 /ug/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in the level of copper among the four size groups (Table 6a).

The mean total copper content in the haemolymph in the males and females in each size group is indicated in Table 6b. There was no significant difference in the copper content in the haemolymph of male and female in any size group. In group I, the mean copper content in the haemolymph was 272.61 ± 53.89 /ug/ml and 268.52 ± 54.69 /ug/ml, in group II 340.16 ± 62.88 /ug/ml and 354.59 ± 51.04 /ug/ml, group III 466.85 ± 75.70 /ug/ml and 518.83 ± 104.45 /ug/ml and group IV 583.16 ± 146.23 and 565.32 ± 209.11 /ug/ml in the males and females respectively.

The copper content in the haemolymph exhibited a cyclic trend similar to total protein during the moult cycle. In all size groups, the copper content was minimum during the period just after ecdysis and

gradually increased upto the late premoult stage and declined just prior to ecdysis (premoult) (Figs. 1a, 2a, 3a and 4a). Analysis of variance of copper content in the haemolymph in size group II (80-100 mm) indicated that the difference in the copper content between the different moult stages was highly significant (Table 6c). The mean copper content in the haemolymph recorded during early postmoult is 267.92 /ug/ml, late postmoult 290.83 /ug/ml, intermoult 338.17 /ug/ml,early premoult 375.42 /ug/ml, mid premoult 397.16 /ug/ml, late premoult 425.42 /ug/ml and premoult 336.75 /ug/ml.

Iron:

The mean total iron content recorded in thee haemolymph in the four size groups was 364.16 ± 12.33 /ug/ml in group I, 391.01 ± 17.79 /ug/ml in group II, 495.12 ± 40.27 /ug/ml in group III and 713.38 ± 58.16 /ug/ml in group IV. The total iron content in the haemolymph ranged from 290 to 1740 /ug/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in level of iron among the four size groups. The iron content in the haemolymph increased with increase in size of the species (Table 7a).

The mean total iron content in males and females in each group is indicated in Table 7b. There was no significant difference in the iron content in the haemolymph of males and females in any of the size groups. In group I, the mean iron content was 363.92 ± 12.52 /ug/ml and 364.40 ± 13.13 /ug/ml, in group II 388.44 ± 19.37 /ug/ml and 393.57 ± 17.17 /ug/ml, in group III 481.9 ± 32.83 /ug/ml and 499.74 ± 46.09 /ug/ml and in group IV 713.33 ± 64.99 /ug/ml and 713.44 ± 55.72 /ug/ml in males and females respectively.

The iron content in the haemolymph exhibited a cyclic trend during the moult cycle but the differences between the moult stages was not significant (Table 7c). In all size groups, the iron content during the period just after ecdysis (early postmoult) was minimum and increased gradually upto late premoult and declined suddenly just prior to ecdysis (premoult) (Figs. 1a, 2a, 3a, 4a). The mean iron content in the haemolymph in size group II (80 - 100mm) recorded during early postmoult was 364.58 /ug/ml late postmoult 372.08 /ug/ml, intermoult 387.91 /ug/ml early premoult 398.75 /ug/ml, mid premoult 407.58 /ug/ml, late premoult 414.58 /ug/ml and premoult (ecdysis) 392.08 /ug/ml.

Zinc:

The mean total zinc content recorded in the haemolymph in the four size groups was 193.22 ± 13.69 /ug/ml in group I, 201.62 ± 15.5 /ug/ml in group II, 237.85 ± 32.85 /ug/ml in group III and 266.60 ± 59.69 /ug/ml in group IV. The total zinc content in the haemolymph ranged from 60 to 455 /ug/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in the level of zinc among the four size groups (Table 8a). The zinc content in the haemolymph registered an increase with increase in size of the species.

The mean total zinc content in the haemolymph in the males and females in each size group is indicated in Table 8b. There is no significant difference in the zinc content in the haemolymph of male and female in any size group. In group I the mean zinc content in the haemolymph is 191.80 ± 15.02 /ug/ml and 194.64 ± 13.18 /ug/ml, group II 199.63 ± 13.61

/ug/ml and 203.59 ± 18.06 /ug/ml, group III 285.71 ± 34.44 /ug/ml and 239.99 ± 33.79 /ug/ml and group IV 255.47 ± 54.68 /ug/ml and 277.73 ± 47.83 /ug/ml in males and females respectively

The zinc content in the haemolymph exhibited a cyclic trend during the moult stages but the differences between the moult stages was not significant (Table 8c). In all size groups, the zinc groups, the zinc content in the haemolymph was minimum during the period just after ecdysis and gradually increased upto late premoult and then declined suddenly prior to ecdysis (Figs. 1a, 2a, 3a, 4a). The mean zinc content in the haemolymph in size group II recorded during early postmoult was 179.16 /ug/ml, late postmoult was 188.75 /ug/ml, intermoult 197.92 /ug/ml, early premoult 210.41 /ug/ml, mid premoult 216.83 /ug/ml, late premoult 223.33 /ug/ml and premoult (ecdysis) - 196.25 /ug/ml.

Manganese:

The mean total manganese content recorded in the haemolymph in the four size groups was 7.14 ± 0.62 /ug/ml in group I, 9.28 ± 0.65 /ug/ml in group II, 16.35 ± 1.4 /ug/ml in group III and 16.96 ± 4.23 /ug/ml in group IV. The manganese content in the haemolymph ranged from 65 to 25 /ug/ml between the four size groups. Analysis of variance between the four size groups indicated significant difference in the level of manganese among the four size groups (Table 9a). The manganese content in the haemolymph registered an increase with increase in size of the species.

The mean total manganese content in the haemolymph in the males and females in each size group is indicated in Table 9b. There is

no significant difference in the manganese content in the haemolymph of males and females in any size group. In group I, the mean manganese content in the haemolymph is 7.02 ± 0.657 /ug/ml and 7.25 ± 0.623 /ug/ml, in group II 9.154 ± 0.694 /ug/ml and 9.397 ± 0.641 /ug/ml, in group III 164 ± 0.929 /ug/ml and 16.30 ± 1.85 mg/ml and group IV 16.307 ± 4.18 mg/ml and 17.6 ± 3.983 /ug/ml in males and females respectively.

The manganese content in the haemolymph exhibited differences during the moult stages but a definite cyclic trend was not obvious and also the differences between the moult stages was not significant (Table 9c). In all size groups, while the manganese content in the haemolymph was minimum in early postmoult and maximum in late premoult, a definite pattern was lacking. The mean manganese content in the haemolymph in size group II recorded during early postmoult is 8.33 /ug/ml, late postmoult 8.75 /ug/ml, intermoult and early premoult 9.16 /ug/ml, mid and late premoult 10 /ug/ml and premoult 9.58 /ug/ml.

Calcium:

The mean total calcium content recorded in the haemolymph in the four size groups was 986.11 ± 72.3 /ug/ml in group I, 1258.57 ± 329 /ug/ml in group II, 1887.19 ± 182.27 /ug/ml in group III and 2134.32 ± 290.81 /ug/ml in group IV. The total calcium content in the haemolymph ranged from 625 /ug/ml to 3770 /ug/ml between the four size groups. Analysis of variance between the four size groups indicated significant differences in the level of calcium among the four size groups (Table 10a). The calcium content in the haemolymph registered an increase with increase in size of the species.

The mean total calcium content in the haemolymph in the males and females in each size group is indicated in table 10b. There is no significant difference in the calcium content in the haemolymph of male and female in any size group. In group I, the mean calcium content in haemolymph is 987.49 ± 58.49 /ug/ml and 108.21 ± 87.53 /ug/ml, in group II 12151.30 ± 344.49 /ug/ml and 1265.83 ± 340.23 /ug/ml, group III 1875.63 ± 196.76 /ug/ml and 1898.44 ± 181.38 /ug/ml and group IV 2125.06 ± 296.43 /ug/ml and 2142.13 ± 306.64 /ug/ml, in males and females respectively.

The calcium content in the haemolymph exhibited a cyclic trend during the moult cycle. In all size groups, the calcium content in the haemolymph was maximum just prior to ecdysis and minimum during intermoult and early premoult. The calcium content again rose sharply prior to ecdysis. The calcium content during early postmoult was significantly higher than the intermoult and premoult stages (Figs. 1a, 2a, 3a, 4a). Analysis of variance of calcium content in the haemolymph in size group II indicated that the difference in calcium content between the moult stages was highly significant (Table 10c). The mean calcium content in the haemolymph recorded during early postmoult is 1474.17 /ug/ml, late postmoult 1227.91 /ug/ml, intermoult 1050 /ug/ml, early premoult 893.75 /ug/ml, mid premoult 855.83 /ug/ml, late premoult 1612.08 /ug/ml and premoult (ecdysis) 1693.76 /ug/ml).

Maturity stages:

Protein content in the haemolymph during ovarian development increased from stage I to stage IV ie. fully mature stage and declined

immediately after spawning (spent stage). The mean total protein content in the haemolymph in stage I was 90.55 ± 39.74 mg/ml, 94.99 ± 18.63 mg/ml in the stage II, 105.55 ± 32.58 mg/ml in stage III, 122.21 ± 31.6 mg/ml in the fully mature stage and 93.88 ± 26.20 mg/ml in the spent stage (Table 11, Fig. 5a).

The total free amino acid content recorded in the haemolymph during ovarian development exhibited a similar pattern. The mean total free amino acid in the haemolymph in stage I was 2.16 ± 0.99 mg/ml, 2.276 ± 2.04 mg/ml in stage II, 2.869 ± 1.05 mg/ml in stage III, 3.12 ± 1.52 mg/ml, in fully mature stage and 2.24 ± 1.03 mg/ml in spent stage (Table 11, Fig. 5a).

The total lipid content in the haemolymph also registered a similar pattern during ovarian development. The mean total lipid in the haemolymph recorded in stage I was 0.96 ± 3.14 mg/ml, 1.238 ± 0.69 mg/ml in stage II, 1.577 ± 0.843 mg/ml in stage III, 1.794 ± 0.96 mg/ml in fully mature stage and 1.40 ± 0.779 mg/ml in spent stage (Table 11, Fig. 5a).

The mean cholesterol content in the haemolymph recorded was 0.811 ± 0.613 mg/ml in stage I, 0.862 ± 0.843 mg/ml in stage II, 1.25 ± 0.64 mg/ml in stage III, 1.347 ± 0.72 mg/ml in fully mature stage and 0.798 ± 0.204 mg/ml in spent stage (Table 11, Fig. 5a).

The mean glucose content in the haemolymph recorded in the maturity stages was 0.56 ± 0.192 mg/ml in stage I, 0.63 ± 0.247 mg/ml in stage II, 0.811 ± 0.408 mg/ml in stage III, 0.89 ± 0.44 mg/ml in mature

stage and 0.66 ± 0.20 mg/ml in spent stage (Table 11, Fig. 5a).

The mean copper content recorded during the development stages in the haemolymph was 399.16 ± 84.62 /ug/ml in stage I, 404.16 ± 84.43 /ug/ml in stage II, 439.16 ± 107.48 /ug/ml in stage III, 534.16 ± 58.76 /ug/ml in mature stage and 456 ± 134.20 /ug/ml in spent stage (Table 11, Fig. 5b).

The mean calcium content recorded during the developmental stages in the haemolymph was 610 ± 371.30 /ug/ml in stage I, 808.33 ± 109.4 /ug/ml in stage II, 1108.33 ± 362.14 /ug/ml in stage III, 1211.6 ± 294.38 /ug/ml in fully mature stage and 974.16 ± 91.49 /ug/ml in spent stage (Table 11, Fig.5b)

The mean zinc content in the haemolymph recorded during the developmental stages was 85 ± 27.08 /ug/ml in stage I, 117.5 ± 52.45 /ug/ml in stage II, 144.16 ± 57.83 /ug/ml in stage III, 153.33 ± 29.81 /ug/ml in fully mature stage and 140 ± 62.38 /ug/ml in spent stage (Table 11, Fig. 5b).

The mean iron content in the haemolymph recorded during the developmental stages was 370.83 ± 44.5 /ug/ml in stage I, 387.5 ± 44.5 /ug/ml in stage II, 434.16 ± 71.49 /ug/ml in stage III, 480.83 ± 109.19 /ug/ml in fully mature stage and 439.16 ± 51.99 /ug/ml in spent stage (Table 11, Fig. 5b).

The mean manganese content in the haemolymph recorded during the development stages was 5.83 ± 1.86 /ug/ml in stage I, 5.83 ± 1.86 /ug/ml in spent stage. There is no definite increase in manganese content in the haemolymph during the different maturity stages (Table 11, Fig. 5b).

GROUP - I

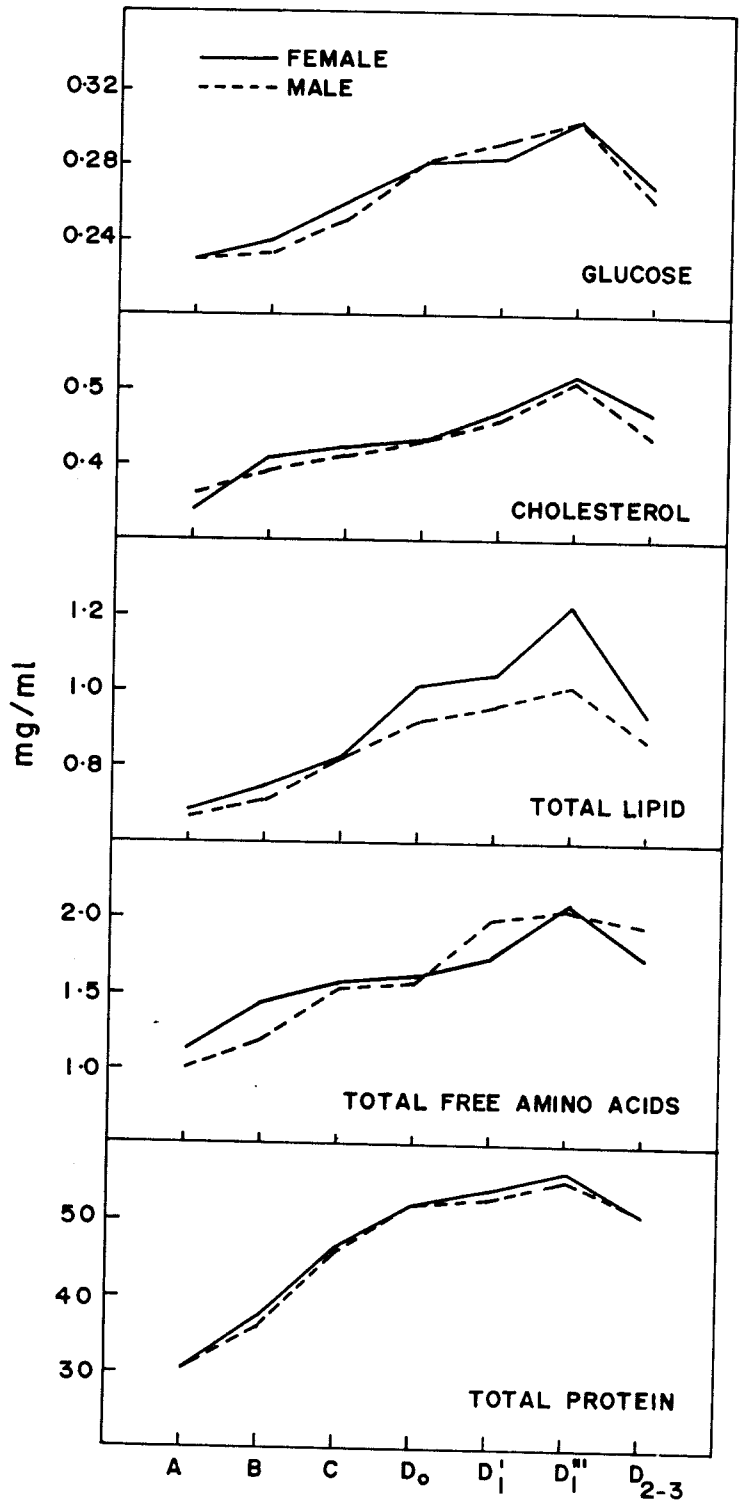

Fig. 1a

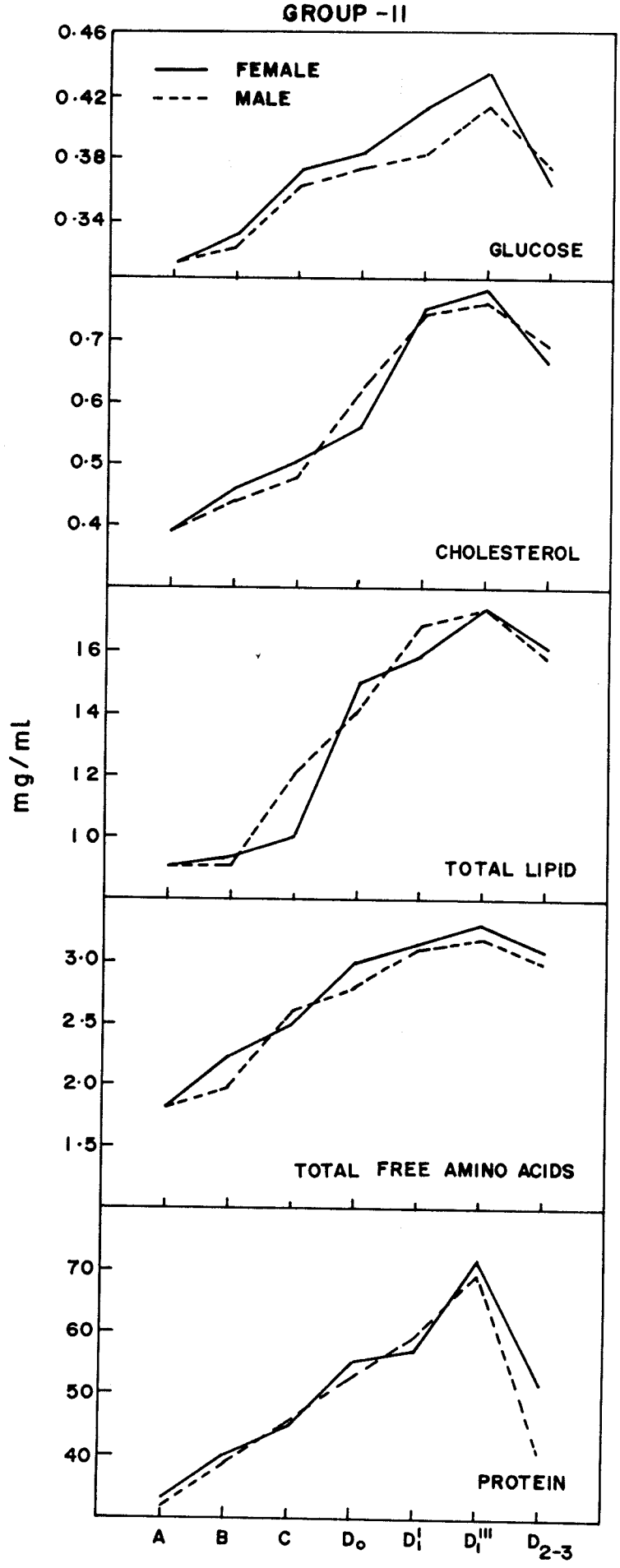
GROUP - II
FEMALE
MALE
GLUCOSE
CHOLESTEROL
TOTAL LIPID
TOTAL FREE AMINO ACIDS
PROTEIN
mg/ml
A
B
C
Do
DI
DIII
D2-3

Fig. 2a

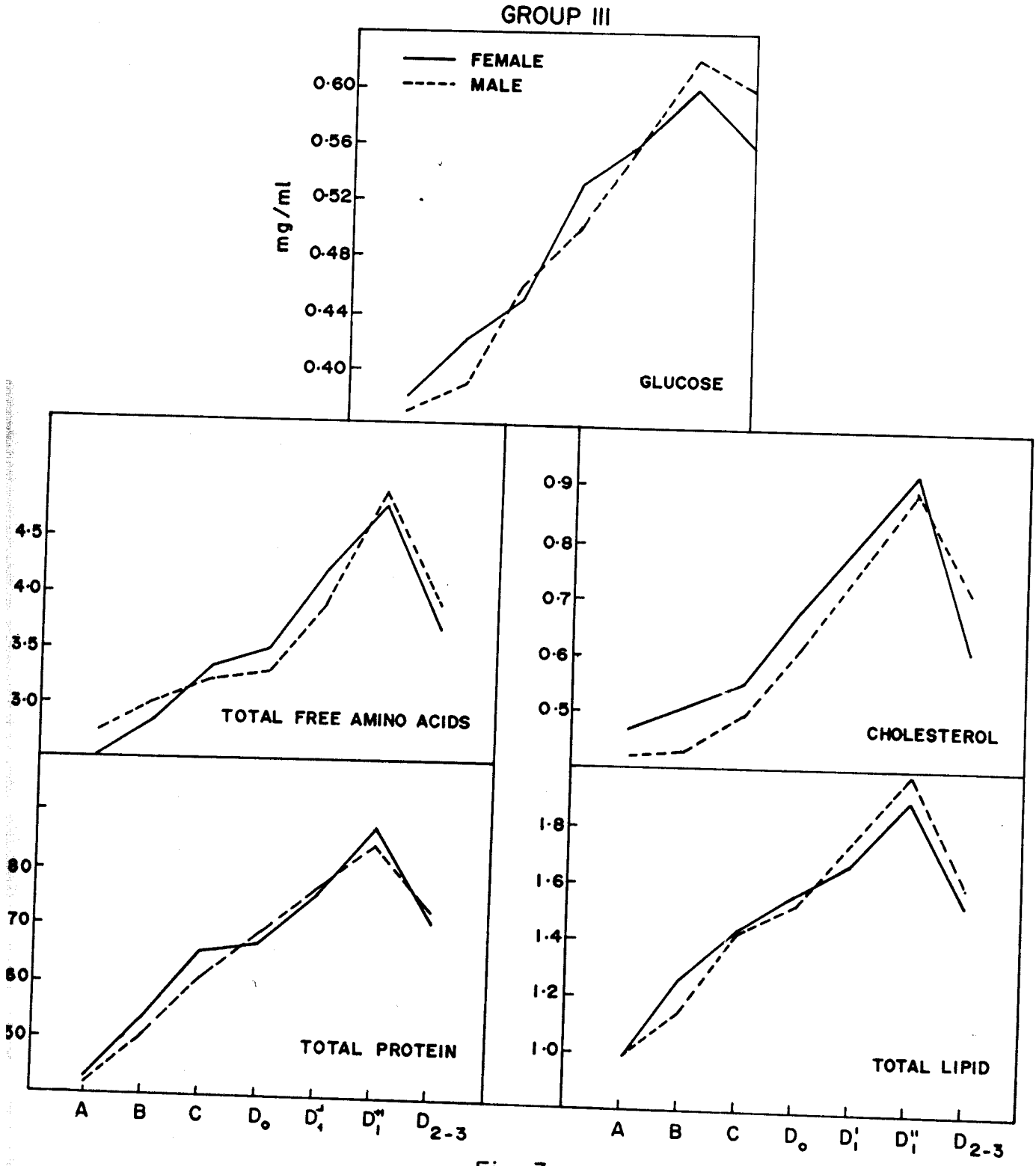
GROUP III
FEMALE
MALE
mg/ml
0·60
0·56
0·52
0·48
0·44
0·40
GLUCOSE
4·5
4·0
3·5
3·0
TOTAL FREE AMINO ACIDS
0·9
0·8
0·7
0·6
0·5
CHOLESTEROL
80
70
60
50
TOTAL PROTEIN
1·8
1·6
1·4
1·2
1·0
TOTAL LIPID
A
B
C
D0
D1'
D1''
D2-3

Fig. 3 a

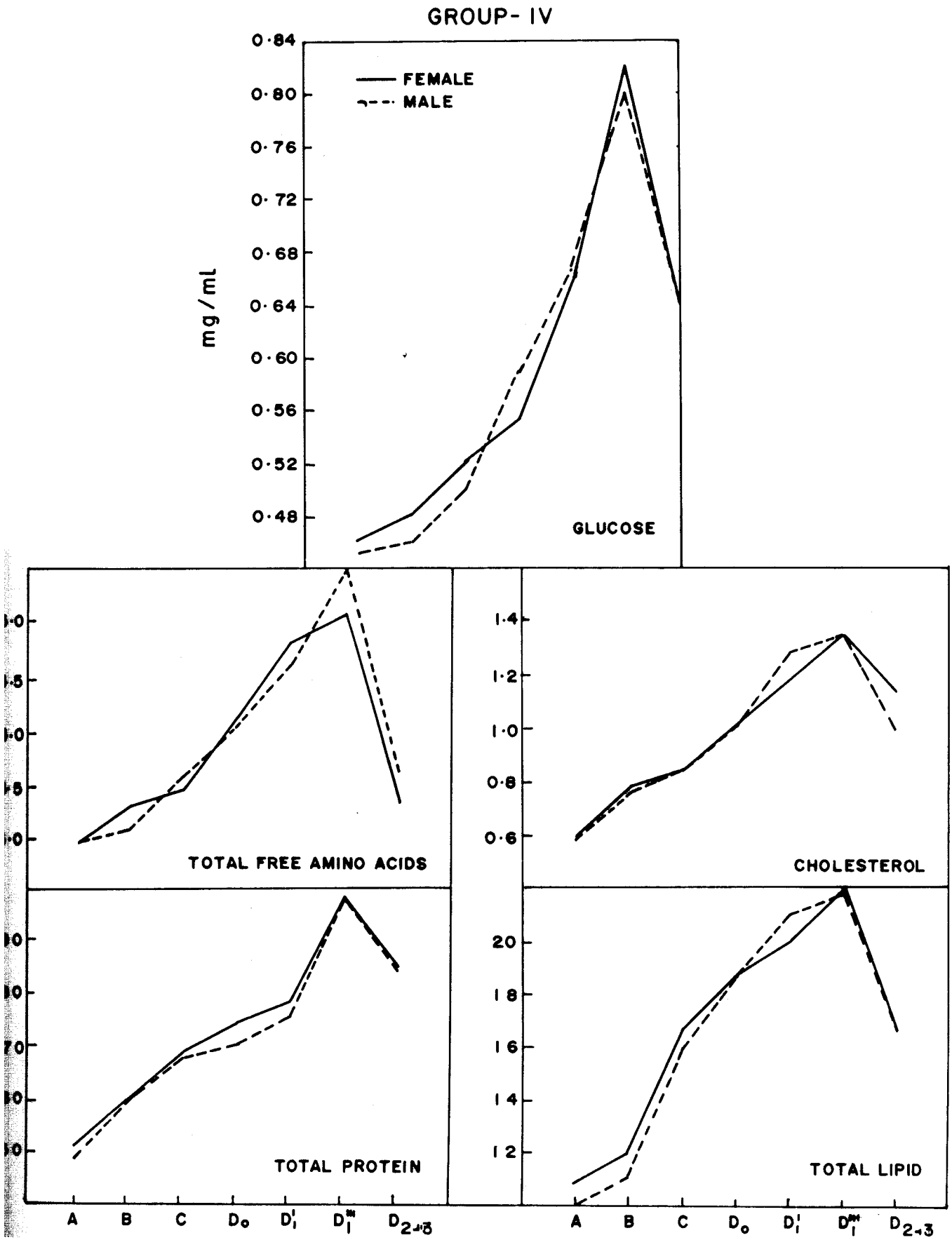
GROUP- IV
FEMALE
MALE
mg / ml
0·84
0·80
0·76
0·72
0·68
0·64
0·60
0·56
0·52
0·48
GLUCOSE
1·4
1·2
1·0
0·8
0·6
CHOLESTEROL
20
18
16
14
12
TOTAL LIPID
TOTAL PROTEIN
A
B
C
Do
D'1
D''1
D2+3

Fig. 4 a

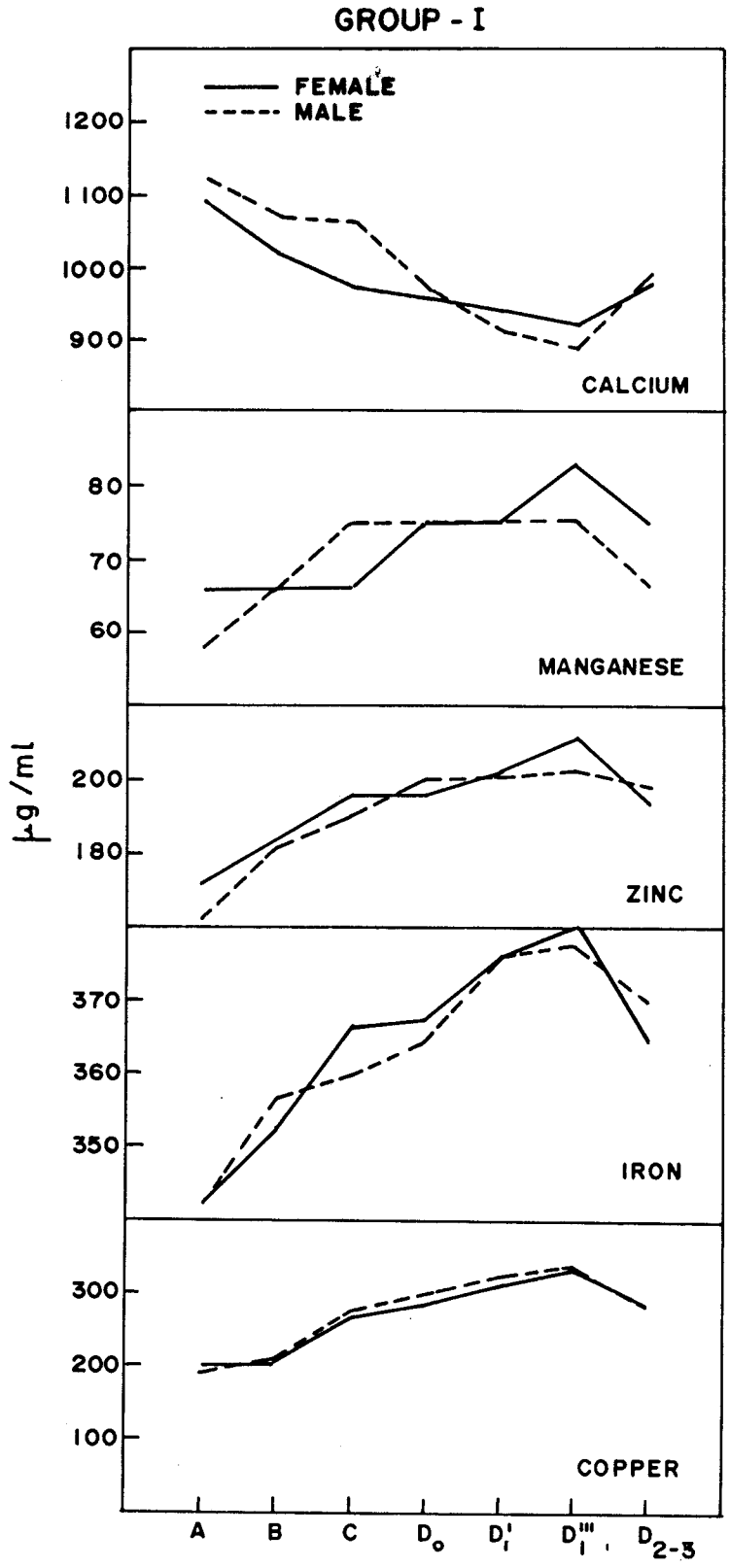
GROUP - I
FEMALE
MALE
1200
1100
1000
900
CALCIUM
80
70
60
MANGANESE
µg/ml
200
180
ZINC
370
360
350
IRON
300
200
100
COPPER
A
B
C
D_o
D_I'
D_I'''
D_{2-3}

Fig - 1 b

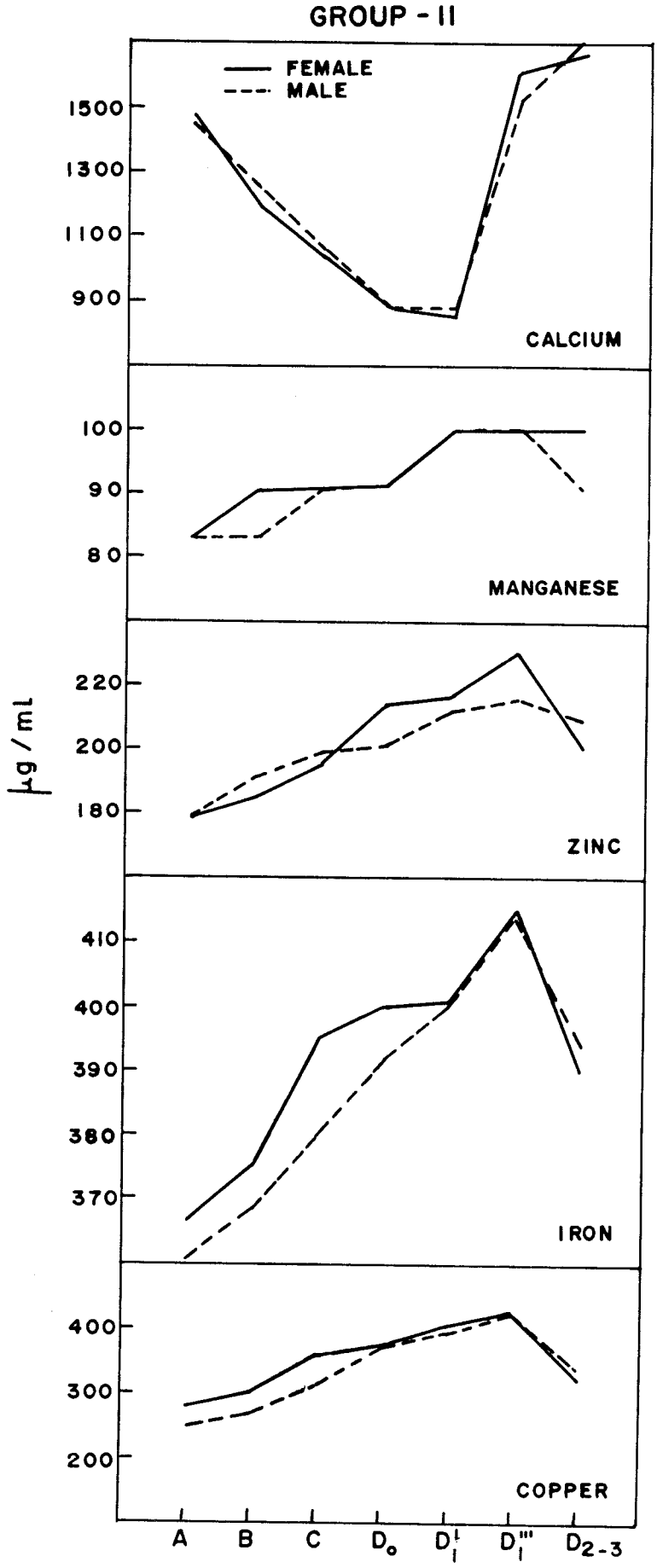
GROUP - II
FEMALE
MALE
1500
1300
1100
900
CALCIUM
100
90
80
MANGANESE
μg / ml
220
200
180
ZINC
410
400
390
380
370
IRON
400
300
200
COPPER
A
B
C
D₀
D'₁
D'''₁
D₂₋₃

Fig. 2b

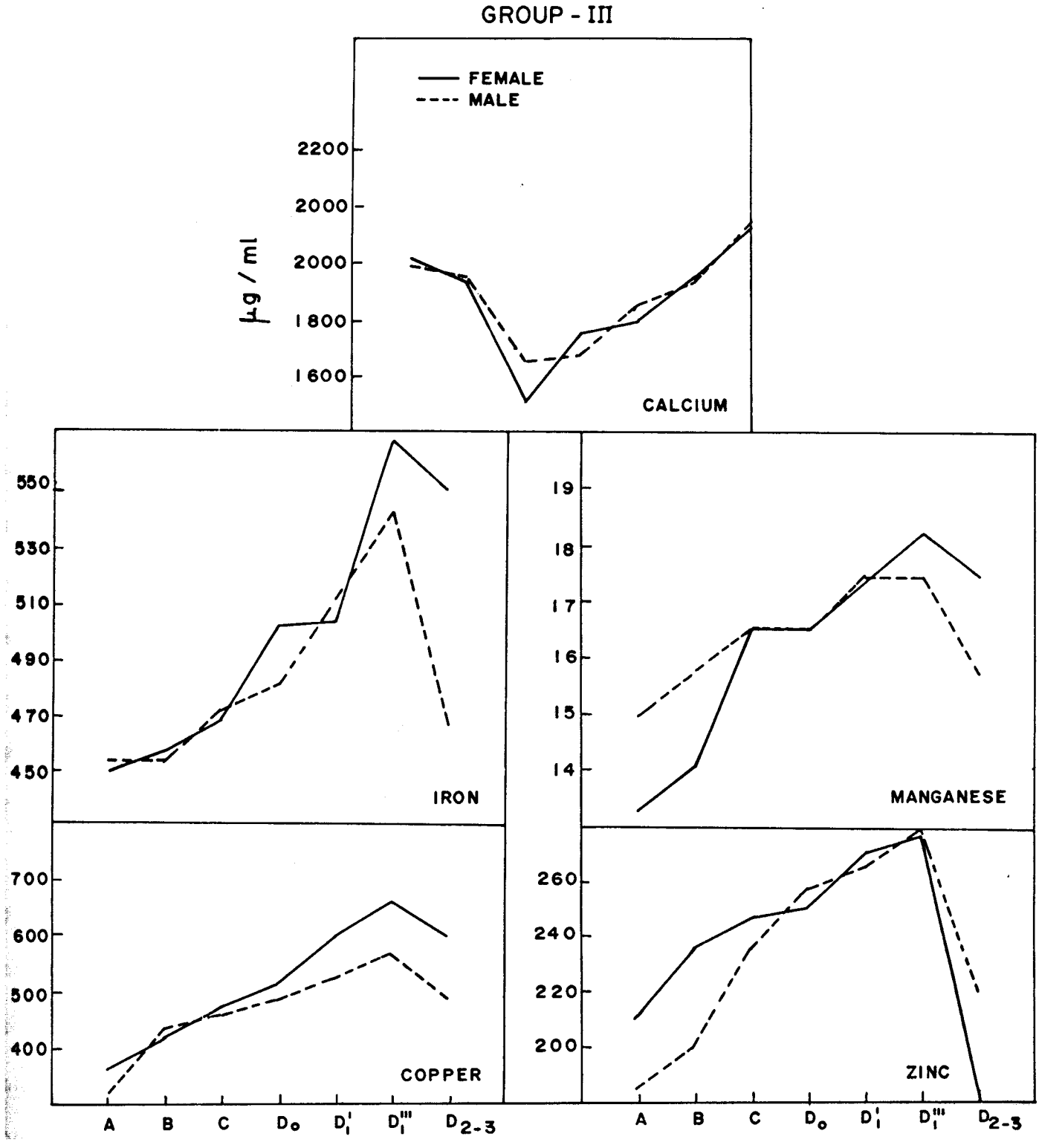
GROUP - III
FEMALE
MALE
µg / ml
2200
2000
2000
1800
1600
CALCIUM
550
530
510
490
470
450
IRON
19
18
17
16
15
14
MANGANESE
700
600
500
400
COPPER
260
240
220
200
ZINC
A
B
C
Do
D'1
D'''1
D2-3

Fig - 3 b

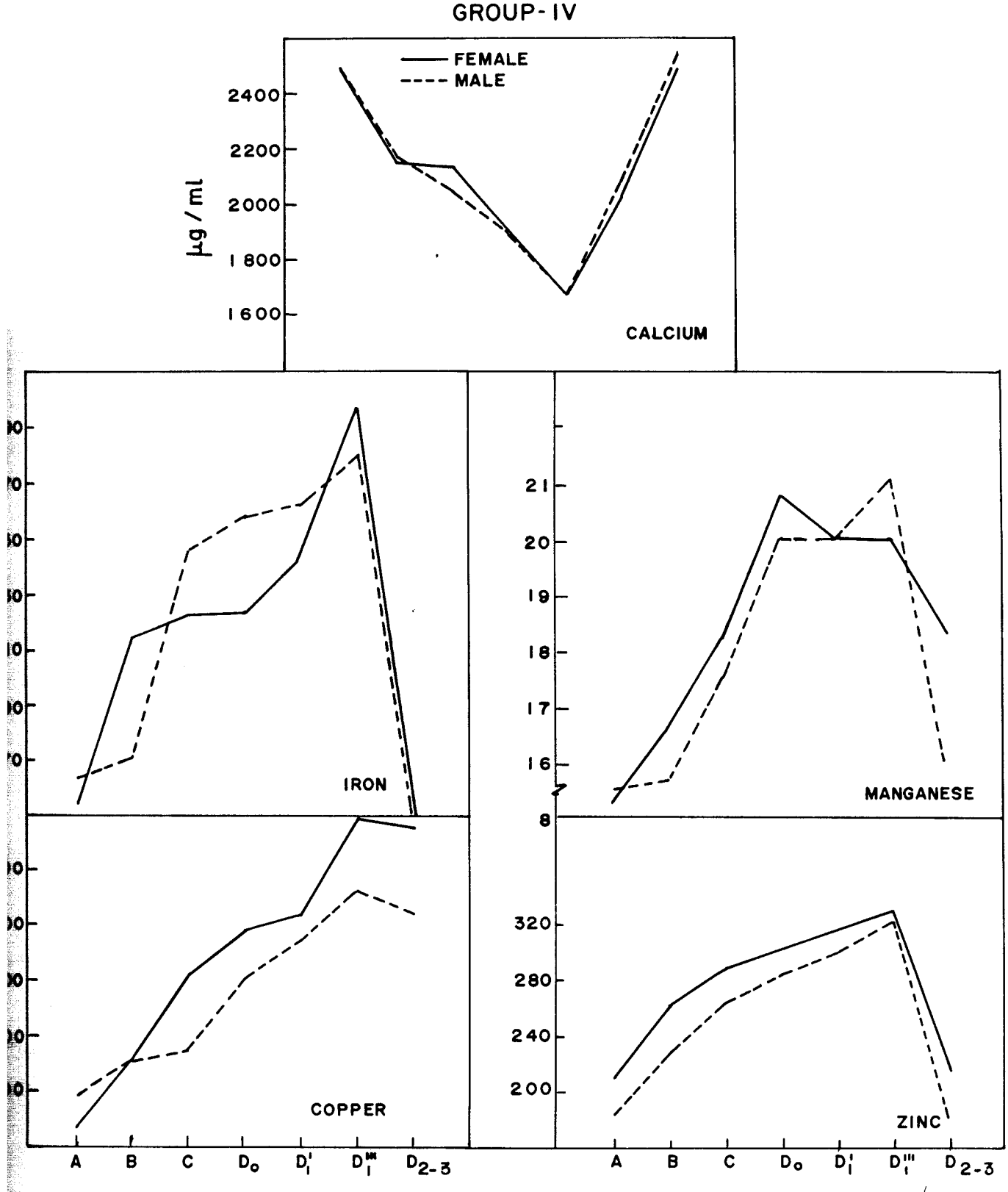
GROUP-IV
FEMALE
MALE
2400
2200
2000
1800
1600
µg / ml
CALCIUM
IRON
MANGANESE
21
20
19
18
17
16
8
COPPER
320
280
240
200
ZINC
A
B
C
Do
D1'
D1'''
D2-3

Fig. 4 b

Table 1a. TOTAL PROTEIN

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	6616.235	2205.412	12.89	HI.SIG (1%)
ERROR	52	8895.906	171.075		

Significant at $P > 0.01$

TREAT	MEAN
T 1	46.79872
T 2	49.72
T 3	66.32857
T 4	72.56357

Table 1b. TOTAL PROTEIN

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I. 60-80mm	$\bar{X}$ SD	46.62 (9.622)	46.976 (9.59)	5.135	0.0692	N.S
Gr . II 80-100 mm	$\bar{X}$ SD	48.412 (12.95)	51.027 (12.6)	6.828	0.382	N.S
Gr. III 100-120 mm	$\bar{X}$ SD	65.84 (15.513)	66.81 (14.72)	8.08	0.1208	N.S
Gr. IV 120-140 mm	$\bar{X}$ SD	71.675 (16.16)	73.45 (15.698)	8.516	0.208	N.S

Table value of T = 2.179, N.S. - Not significant

Table 1c. TOTAL PROTEIN

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	23901.750	3983.625	24.71	HI.SIG(1%)
ERROR	161	25954.410	161.207		

Significant at P > 0.01

TREAT	MEAN
T 1	31.19083
T 2	39.88542
T 3	45.275
T 4	52.14917
T 5	58.33042
T 6	70.83
T 7	46.1075

Table 2a. TOTAL FREE AMINO ACIDS

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	42.469	14.156	33.75	HI.SIG (1%)
ERROR	52	21.810	0.419		

Significant at P > 0.01

TREAT	MEAN
T 1	1.639357
T 2	2.716429
T 3	3.5585
T 4	3.893857

Table 2b. TOTAL FREE AMINO ACIDS

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	1.6317	1.647	0.193	0.079	N.S.
60-80 mm	SD	(0.403)	0.313			
Gr . II	$\bar{X}$	2.681	2.751	0.303	0.2306	N.S.
80-100 mm	SD	(0.572)	(0.563			
Gr. III	$\bar{X}$	3.528	3.589	0.407	0.149	N.S.
100-120 mm	SD	(0.718)	(0.802)			
Gr. IV	$\bar{X}$	3.925	3.862	0.472	0.1334	N.S.
120-140 mm	SD	(0.927)	(0.836)			

Table value of T = 2.179, N.S. - Not significant

Table 2c. TOTAL FREE AMINO ACIDS

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	RE
TREAT	6	44.653	7.442	7.40	HI.
ERROR	161	161.967	1.006		

Significant at $P > 0.01$

TREAT	MEAN
T 1	1.812479
T 2	2.132388
T 3	2.585092
T 4	2.968012
T 5	3.149208
T 6	3.268975
T 7	3.75916

Table 3a. TOTAL LIPID

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	5.488	1.829	15.78	HI.SIG(1%)
ERROR	52	6.027	0.116		

Significant at $P > 0.01$

TREAT	MEAN
T 1	0.8357
T 2	1.3433
T 3	1.4981
T 4	1.6757

Table 3b. TOTAL LIPID

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	0.875	0.938	0.100	0.627	N.S.
60-80 mm	SD	(0.164)	(0.207)			
Gr . II	$\bar{X}$	1.351	1.326	0.189	0.1306	N.S.
80-100 mm	SD	(0.349)	(0.358)			
Gr. III	$\bar{X}$	1.493	1.502	0.181	0.047	N.S.
100-120 mm	SD	(0.344)	(0.333)			
Gr. IV	$\bar{X}$	1.661	1.690	0.231	0.125	N.S.
120-140 mm	SD	(0.445)	(0.421)			

Table value of T = 2.179, N.S. - Not significant

Table 3c. TOTAL LIPID

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	18.078	3.013	9.57	HI.SIG(1%)
ERROR	161	50.665	0.315		

Significant at $P > 0.01$

TREAT	MEAN
T1	0.9052501
T 2	0.926125
T 3	1.113542
T 4	1.496833
T 5	1.638667
T 6	1.737208
T 7	1.60525

Table 4a. CHOLESTEROL

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	2.385	0.795	26.72	HI.SIG(1%)
ERROR	52	1.547	0.030		

Significant at $P > 0.01$

TREAT	MEAN
T1	0.4341429
T 2	0.5917143
T 3	0.6372143
T 4	0.9980714

Table 4b. CHOLESTEROL

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	0.4301	0.438	0.0279	0.286	N.S.
60-80 mm	SD	0.048	(0.056)			
Gr. II	$\bar{X}$	0.5922	0.5911	0.0805	0.014	N.S.
80-100 mm	SD	(0.151)	(0.1504)			
Gr. III	$\bar{X}$	0.6205	0.653	0.093	0.356	N.S
100-120 mm	SD	(0.1807)	(0.168)			
Gr. IV	$\bar{X}$	1.02	1.00	0.134	0.138	N.S.
120-140 mm	SD	(0.237)	(0.263)			

Table value of T = 2.179, N.S. - Not significant

Table 4c. CHOLESTEROL

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	2.893	0.482	7.45	HI.SIG(1%)
ERROR	161	10.421	0.065		

Significant at $P > 0.01$

TREAT	MEAN
T 1	0.3972583
T 2	0.452775
T 3	0.4878416
T 4	0.596925
T 5	0.7514709
T 6	0.7272541
T 7	0.6863208

Table 5a. GLUCOSE

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	0.867	0.289	47.69	HI.SIG(1%)
ERROR	52	0.315	0.006		

Significant at $P > 0.01$

TREAT	MEAN
T1	0.2672857
T 2	0.3694929
T 3	0.504
T 4	0.5924286

Table 5b. GLUCOSE

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	0.265	0.268	0.0133	0.235	N.S
60-80 mm	SD	(0.0273)				
Gr. II	$\bar{X}$	0.3645	0.3658	0.0224	0.057	N.S
80-100 mm	SD	(0.033)	(0.0494)			
Gr. III	$\bar{X}$	0.503	0.504	0.0485	0.0294	N.S
100-120 mm	SD	(0.0985)	(0.0824)			
Gr. IV	$\bar{X}$	0.5904	0.5935	0.0677	0.0463	N.S
120-140 mm	SD	(0.127)	(0.125)			

Table value of T = 2.179, N.S. - Not significant

Table 5c. GLUCOSE

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	1.174	0.196	5.71	HI.SIG(1%)
ERROR	161	5.515	0.034		

Significant at $P > 0.01$

TREAT	MEAN
T 1	0.378025
T 2	0.4101583
T 3	0.4608291
T 4	0.5179417
T 5	0.5635416
T 6	0.6140625
T 7	0.5848959

Table 6a. COPPER

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	962210.000	320736.700	22.21	HI.SIG(1%)
ERROR	52	750861.000	14439.640		

Significant at $P > 0.01$

TREAT	MEAN
T1	270.5664
T 2	347.3772
T 3	454.2715
T 4	619.9686

Table 6b. COPPER

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	272.61	268.52	29.02	0.141	N.S.
60-80 mm	SD	(53.89)	(54.69)			
Gr. II	$\bar{X}$	340.16	354.59	30.61	0.471	N.S.
80-100 mm	SD	(62.88)	(51.04)			
Gr. III	$\bar{X}$	466.85	518.83	48.75	1.065	N.S
100-120 mm	SD	(75.70)	(104.45)			
Gr. IV	$\bar{X}$	583.61	656.32	96.44	0.7539	N.S
120-140 mm	SD	(146.23)	(209.11)			

Table value of T = 2.179, N.S. - Not significant

Table 6c. COPPER

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	228773.00	38128.830	3.15	HI.SIG(1%)
ERROR	77	933157.000	12118.920		

Significant at P > 0.01

TREAT	MEAN
T 1	267.9167
T 2	290.8334
T 3	338.1667
T 4	375.4167
T 5	397.1667
T 6	425.4167
T 7	336.75

Table 7a. IRON

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	1057832.000	352610.700	241.12	HI.SIG(1%)
ERROR	52	76045.000	1462.404		

Significant at $P > 0.01$

TREAT	MEAN
T 1	364.1629
T 2	391.0093
T 3	495.1164
T 4	713.3893

Table 7b. IRON

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	363.92	364.40	6.86	0.069	N.S
60-80 mm	SD	(12.52)	(13.13)			
Gr. II	$\bar{X}$	388.44	393.57	21.39	0.534	N.S
80-100 mm	SD	(19.37)	(17.17)			
Gr. III	$\bar{X}$	481.9	499.74	21.39	0.834	N.S
100-120 mm	SD	(32.83)	(46.09)			
Gr. IV	$\bar{X}$	713.33	713.44	32.35	0.0036	N.S
120-140 mm	SD	(64.99)	(55.72)			

Table value of T = 2.179, N.S. - Not significant

Table 7c. IRON

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	23294.000	3882.333	1.17	N.S.
ERROR	77	256095.000	3325.909		

TREAT	MEAN
T 1	364.5834
T 2	372.0834
T 3	387.9167
T 4	398.75
T 5	407.0834
T 6	414.5834
T 7	392.0834

Table 8a. ZINC

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	48336.500	16112.170	15.81	HI.SIG(1%)
ERROR	52	52991.000	1019.058		

Significant at $P > 0.01$

TREAT	MEAN
T 1	193.221
T 2	201.615
T 3	237.8536
T 4	266.6043

Table 8b. ZINC

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	191.80	194.64	7.55	0.375	N.S
60-80 mm	SD	(15.02)	(13.18)			
Gr. II	$\bar{X}$	199.63	203.59	8.35	0.462	N.S
80-100 mm	SD	(13.61)	(18.06)			
Gr. III	$\bar{X}$	235.71	239.99	18.23	0.2349	N.S
100-120 mm	SD	(34.44)	(33.79)			
Gr. IV	$\bar{X}$	255.47	277.73	27.46	0.8106	N.S
120-140 mm	SD	(54.68)	(47.83)			

Table value of T = 2.179, N.S. - Not significant

Table 8c. ZINC

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	17558.250	2926.375	0.97	N.S.
ERROR	77	232358.500	3017.643		

Significant at P > 0.01

TREAT	MEAN
T 1	179.1667
T 2	188.75
T 3	197.9167
T 4	210.4167
T 5	215.8333
T 6	223.3333
T 7	196.25

Table 9a. MANGANESE

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	1033.259	344.419	66.52	HI.SIG(1%)
ERROR	52	269.258	5.178		

Significant at $P > 0.01$

TREAT	MEAN
T 1	7.137858
T 2	9.275716
T 3	16.35072
T 4	16.955

Table 9b. MANGANESE

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	7.02	7.25	0.342	0.679	N.S
60-80 mm	SD	(0.657)	(0.623)			
Gr. II	$\bar{X}$	9.154	9.397	0.357	0.680	N.S
80-100 mm	SD	(0.694)	(0.6406)			
Gr. III	$\bar{X}$	16.4	16.30	0.7825	0.1259	N.S
100-120 mm	SD	(0.929	(1.85)			
Gr. IV	$\bar{X}$	16.307	17.6	2.324	0.557	N.S
120-140 mm	SD	(4.18)	(3.983)			

Table value of T = 2.179, N.S. - Not significant

Table 9c. MANGANESE

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	27.977	4.663	0.22	N.S.
ERROR	77	1629.167	21.158		

TREAT	MEAN
T 1	8.333333
T 2	8.75
T 3	9.166667
T 4	9.166667
T 5	10
T 6	10
T 7	9.583333

Table 10a. CALCIUM

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	3	11997120.000	3999040.000	69.17	HI.SIG(1%)
ERROR	52	3006544.000	57818.150		

Significant at $P > 0.01$

TREAT	MEAN
T 1	986.1151
T 2	1258.567
T 3	1887.194
T 4	2134.318

Table 10b. CALCIUM

GROUPS		MALE	FEMALE	S.E.	T.cal.	REMARKS
Gr. I.	$\bar{X}$	987.49	1008.21	39.79	0.520	N.S
60-80 mm	SD	(58.49)	(87.53)			
Gr. II	$\bar{X}$	1251.30	1265.83	183.00	0.0793	N.S
80-100 mm	SD	(344.49)	(340.23)			
Gr. III	$\bar{X}$	1875.63	1898.44	101.14	0.225	N.S
100-120 mm	SD	(196.76)	(181.38)			
Gr. IV	$\bar{X}$	2125.06	2142.13	161.20	0.1058	N.S
120-140 mm	SD	(296.43)	(306.64)			

Table value of T = 2.179, N.S. - Not significant

Table 10c. CALCIUM

ANOVA

SOURCE	D.F.	SUM.SQR	MEAN.SQR	F-VAL	REMARKS
TREAT	6	8406792.000	1401132.000	7.99	HI.SIG(1%)
ERROR	77	13505150.000	175391.600		

Significant at P > 0.01

TREAT	MEAN
T 1	1474.167
T 2	1227.917
T 3	1050.0
T 4	893.75
T 5	855.8333
T 6	1612.083
T 7	1693.75

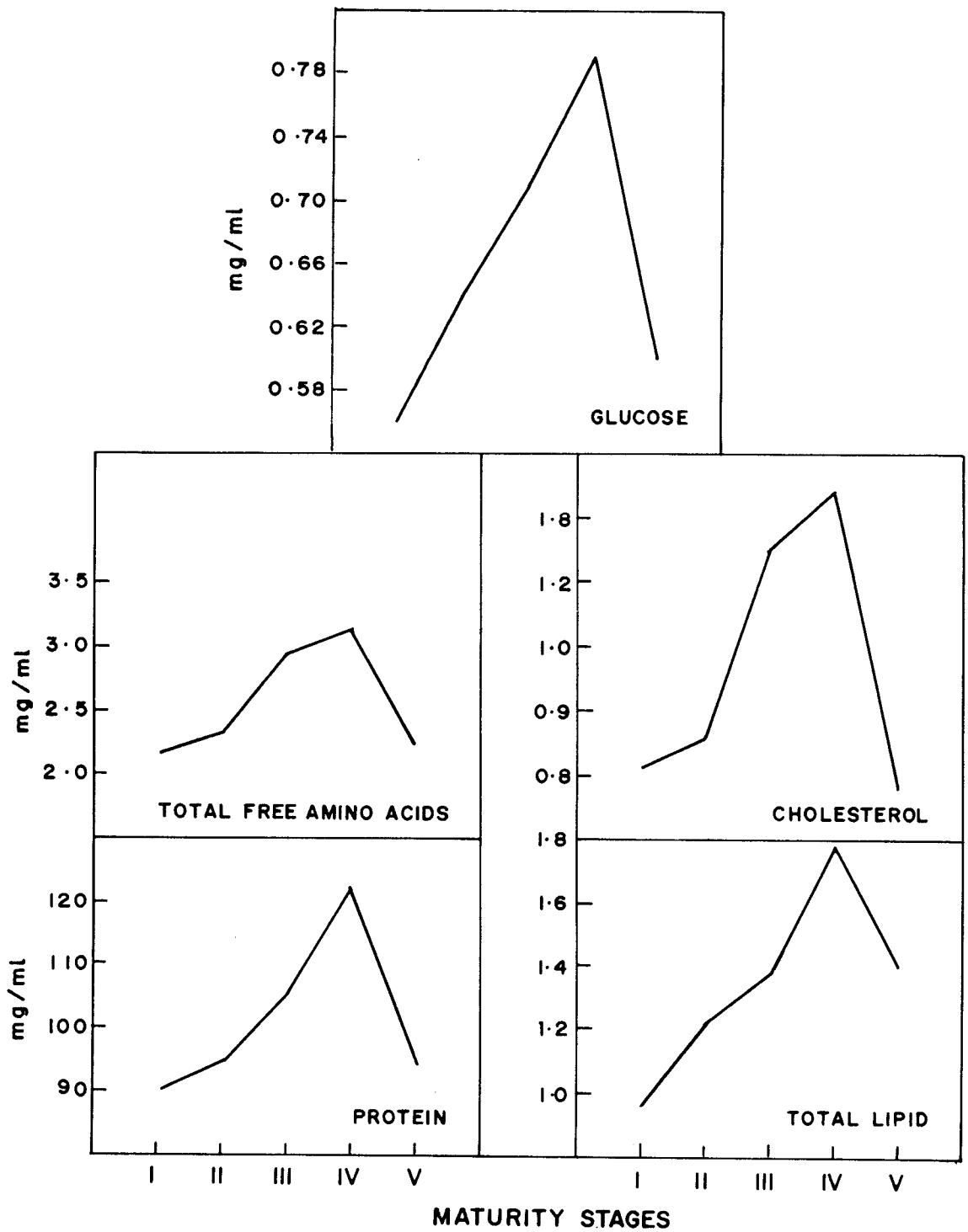
0·78
0·74
0·70
0·66
0·62
0·58
mg/ml
GLUCOSE
3·5
3·0
2·5
2·0
mg/ml
TOTAL FREE AMINO ACIDS
1·8
1·2
1·0
0·9
0·8
CHOLESTEROL
120
110
100
90
mg/ml
PROTEIN
1·8
1·6
1·4
1·2
1·0
TOTAL LIPID
I
II
III
IV
V
I
II
III
IV
V
MATURITY STAGES

Fig. 5a

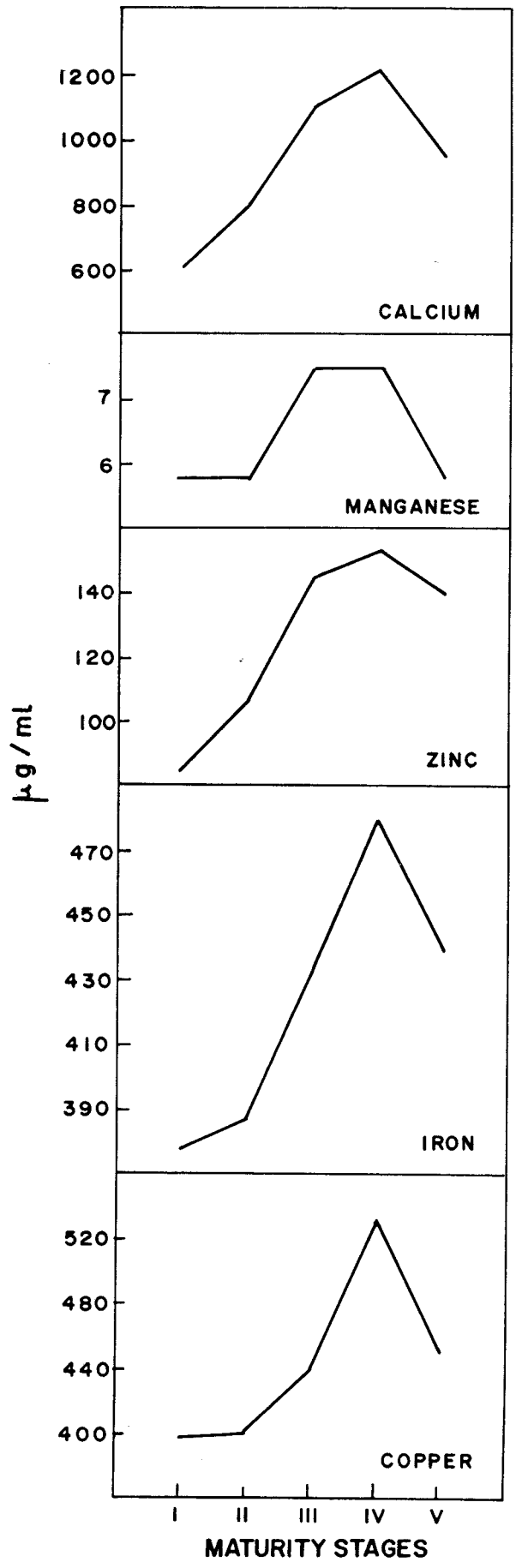
1200
1000
800
600
CALCIUM
7
6
MANGANESE
140
120
100
ZINC
470
450
430
410
390
IRON
520
480
440
400
COPPER
µg/ml
I
II
III
IV
V
MATURITY STAGES

Fig. 5b

Table 11. MATURITY STAGES

		Ist STAGE	IInd STAGE	IIIrd STAGE	MATURE	SPENT
Haemolymph Protein mg/ml	$\bar{X}$	90.55	94.99	105.55	122.21	93.88
	SD	(39.74)	(18.63)	(32.58)	(31.6)	(26.20)
Total Free amino acids mg/ml	$\bar{X}$	2.16	2.276	2.869	3.12	2.24
	SD	(0.99)	(2.04)	(1.05)	(1.52)	(1.03)
Total lipid mg/ml	$\bar{X}$	0.96	1.238	1.577	1.794	1.400
	SD	(3.14)	(0.69)	(0.843)	(0.96)	(0.779)
Cholesterol mg/ml	$\bar{X}$	0.811	0.862	1.25	1.347	0.798
	SD	(0.613	(0.843)	(0.64)	(0.72)	(0.204)
Glucose mg/ml	$\bar{X}$	0.56	0.630	0.811	0.89	0.66
	SD	(0.192)	(0.247)	(0.408	(0.444)	(0.20)
Copper /ug/ml	$\bar{X}$	399.16	404.16	439.16	534.16	456.0
	SD	(84.62)	(83.43)	(107.48)	(58.76)	(134.20)
Calcium /ug/ml	$\bar{X}$	610	808.33	1108.33	1211.6	974.16
	SD	(371.30)	(109.4)	(362.14)	(294.38)	(491.49)
Zinc /ug/ml	$\bar{X}$	85	117.5	114.16	153.33	140.0
	SD	(27.08)	(52.42)	(57.83)	(29.81)	(62.38)
Iron /ug/ml	$\bar{X}$	370.83	387.5	434.16	480.83	439.16
	SD	(44.5)	(44.5)	(71.49)	(109.19)	(51.99)
Manganese /ug/ml	$\bar{X}$	5.83	5.83	7.5	7.5	5.8
	SD	(1.86)	(1.86)	(3.8)	(3.8)	(1.86)

DISCUSSION

The chemical composition of the haemolymph in Crustacea depends more or less directly on the nature of the environment and varies during development. Despite the ontogenic and phenotypic variations, taxonomic inferences can often be based on the haemolymph composition. Since the haemolymph is the carrier of every kind of biochemical constituent from one point of the body to the other, haemolymph chemistry is concerned with the number of specific role of the substances in transit (Jeuniaux,1979).

The total protein content in the haemolymph is the most elucidated constituent in crustaceans. The wide range in the total protein content recorded in the haemolymph of *Penaeus indicus* between the four size groups (20 mg/ml to 130 mg/ml) has been recorded in other crustaceans too. Leon (1953) reported wide range in the serum total protein in Callinectus sapidus (1.83 g/100 ml to 12. g/100 ml), C. magister (1.16 to 13.75 g/100 ml), C. irroratus (1.75 to 11.45 g/100 ml), L. emarginats (0.73 to 7.25 g/100 ml), H. americanus (2.20 to 10. g/100 ml) and Limulus polyphemus (0.77 to 13.45 g/100 ml). Leone(1953) opined that wide range of values presented may be expected to occur in a natural population at any given time. In *P. indicus*, the samples were generally assembled from a single population (stock) and hence the wide variation could be expected to occur in a natural population. The haemolymph protein level also increases significantly with increase in size of the species (mean value of 46.74 mg/ml in 60-80 mm to 72.56 mg/ml in 120-140 mm size group) contributing to the wide range

recorded, unlike in the case of crabs. In C. sapidus, a ten-fold variation in the serum protein could not be attributed to the variation due to the size of the species. Significant difference in mean serum protein and copper concentrations in males and females, with lower concentration in males was also recorded in C. sapidus (Horn and Kerr, 1963). In *P. indicus* no significant difference in mean serum protein concentrations in males and females was observed. The wide range in haemolymph protein concentration is also correlated to the moult cycle; significant difference in the haemolymph protein content between the moult stages exists in *P. indicus*.

Wide intra-individual variations in the haemolymph protein concentration has been recorded in several other decapods also. In Ocypoda macrocera blood protein concentrations varied from 64.05 to 93.63 mg/ml, from the same locality (Rao et al., 1986). Comparable blood protein concentrations has been recorded in Scylla serrata (2.6 g% to 17.1 g%). Intra-individual variations as well as variations in the protein concentration during the day was also reported and attributed to the periodic sequestering of proteins from different tissues at different times of the day (Subhashini and Ravindranath, 1980). Serum protein concentrations in lobsters were similar to those observed in *P. indicus* but no correlation between the length of the species and protein concentration was found. Baztos and Bazerra (1970) recorded serum protein content in Panulirus argus (4.12 g/100 ml) and P. laevicauda (4.48 g/100 ml) and found no correlation between length and protein content in the two species unlike in the case of *P. indicus* But a positive correlation between copper and protein contents in the two species was established. In H. americanus, the total serum proteins averaged

2.5 g/100 ml in intermoult lobsters and variation among individuals was limited (Barlow and Rigway, 1969). Lower serum protein concentrations have been recorded in crayfish and Oniscoides. Kazmierczak et al. (1978) recorded protein content in the serum of three crayfish A. astacus, A. leptodactylus and Orconectes limosus and it ranged from 23.5 to 39.1 mgg/ml which is low compared to other decapods. Gondko et al. (1981) also recorded protein content in three crayfish which ranged from 2.3 to 4.4 g/100 ml and statistically significant intersex difference was noted for A. leptodactylus only. AQUACOP (1983) recorded serum protein concentrations in P. monodon ranging from 84 g/l to 104 g/l, the variation less than that in *P. indicus*. Significant difference in the haemolymph protein content between the different size groups was also reported in P. monodon, (AQUACOP, 1983) which establishes the significant metabolic role of haemolymph protein in the growth process of both P. monodon and *P. indicus*. Bouchard et al. (1985) reported a tricircadian rhythm in the serum proteein content in P. japonicus, on the other hand, variations were less in females compared to males.

Growth in crustacea is realized through periodic moulting and each stage of the moulting cycle influences the composition of the blood. During the premoult period, a resorption of the exoskeleton takes place; in the postmoult period the redeposition of chitin protein and inorganic salts in the new skeleton also affects the blood. The premoult period is characterized by an increase in the concentration of a number of blood constituents such as sugar, fat, total protein. The concentration of these constituents return again to normal values at the end of the postmoult

period (Passano, 1960; Glynn, 1968; Barlow and Ridgway, 1969; Bursey and Lane, 1971; Dall, 1974a,b; Hepper, 1978; Truchot, 1978; Cole and Morgan, 1978; Chaix et al. 1981; Castille and Lawrence, 1981a; Durliat and Vranckx, 1982; Rao et al., 1986).

In *Penaeus indicus* the blood constituents studied namely total protein, free amino acids, total lipid, cholesterol, glucose, copper, zinc, iron, calcium and manganese all registered quantitative variation during the moult cycle. Except for calcium, all the other constituents studied remained constant during intermoult (c), increased during early premoult (D_o to D_1''') and diluted at moult (D_{2-3}) and fluctuated during calcification (postmoult A & B). In the case of calcium, the haemolymph content increase during premoult and reaches a peak first prior to ecdysis and decreases after moult. This cyclic variation of haemolymph constituents has been recorded in almost all decapods, particularly the Brachyurans and Natantians. Glynn (1968) recorded the rise in serum protein concentration in the early pre-ecdysal stage (D) followed by a drop before moult in Homarus vulgaris. The fall in serum protein before moult is attributed to absorption of water, resulting in dilution of the blood protein present immediately before moulting. After ecdysis, the protein level rises within a few hours in the case of shrimps and becomes steady during intermoult. The reduced serum protein level in late premoult and early postmoult is due to the combined effects of dilution from ecdysial water intake and to the utilization of protein in the construction of the new exoskeleton. However, the quantitative variation in serum protein moult cycle could not be detected electrophoretically in *P. indicus* unlike in the case of Brachyurans. In Homarus, (Barlow & Ridgway, 1969) relative shifts in individual serum protein components

during the moult cycle was demonstrated by immunological techniques; a major protein component was present only before and shortly after moult. The quantitative variation in haemolymph protein content and association of a particular polypeptide with moulting which is present in stages D_0, D_1, D_2 and D_3 and appears in the haemolymph when the ecdysone titer increased in Acanthonyx lunulatus (Chaix et al., 1981). However, in Astacus leptodactylus no such polypeptide associated with the moult cycle could be detected although quantitative variation was observed (Durliat and Vranckx, 1982). In Homarus gammarus Hepper (1978) pointed out that the variation in blood protein level between lobsters is too great at any stage of the moulting cycle for the technique to give an indication of the moulting. He suggested that in H. gammarus the stage C_4 (inter moult) extends from 40-70% of the moulting cycle. Dall (1974a), based on starvation and feeding experiments concluded that blood protein concentration in Panulirus longipes is a conservative constituent both during starvation and moult cycle. Dall (1974) also suggested that, hemocyanin, the major blood protein component, is unlikely to be used as reserve during starvation and any synthesis of hemocyanin during obligatory starvation (moulting) would be a relatively slow process. Fall in haemolymph protein during starvation was due to haemodilution. Subhashini and Ravindranath (1982) confirmed this in Scylla serratta by starvation experiments and Rao et al. (1986) in Ocypoda macrocera. They reported wide fluctuations in the haemolymph protein concentration and recognized a direct relationship between blood protein and copper content during the moult cycle. Truchot (1978) also established in Carcinus maenas, a linear relationship between haemolymph copper and protein during the moult cycle which means that the haemolymph copper is linked to hemocyanin.

Cyclic variations of haemolymph constituents with respect to the moult cycle has been extensively studied in the Brachyurans, in which the moult cycle is an extended one unlike in Natantians with relatively short duration (lasting 15-20 days) (Subhashini and Ravindranath, 1982; Rao et al., 1986 and Truchot, 1978). While studying the osmoregulatory capacities of Penaeus monodon, Ferraris et al. (1986) concluded that the haemolymph protein content in P. monodon was not significantly different among the various moult stages and therefore compounds other than protein or calcium must have been responsible for the decrease in haemolymph osmolality after moult. They also concluded that because haemolymph protein content is also greatly affected by nutritional state as well as dietary source (Dall, 1974b; Hepper, 1978), salinity might not be an important factor in determining protein concentration in the haemolymph except in unusually high or low salinities when osmotic forces would prevail (thereby dehydrating or hydrating the haemolymph respectively). Significant drop in haemolymph protein content immediately after moulting and increase to maximum prior to moulting in Penaeus esculentus and P. plebejus was reported by Dall (1982) and these changes were mainly due to blood volume changes. Increased blood protein level during preecdysis and low at metecdysis and maximal during anecdysis was observed in Penaeus vannamei (Chan et al., 1988). While no major polypeptide involved in moulting could be detected, two minor polypeptide subunits with possible role in moulting was identified by SDS-PAGE. In P. duorarum, maximal haemolymph protein concentration at premoult and lowest values for post-moult individuals was reported by Bursey and Lane (1971). They could

detect no significant difference in the electrophoretic protein pattern as in the case of *P. indicus*. Thus it is concluded that, in general the haemolymph protein content in *P. indicus* does not vary with respect to sex and moult stages but wide individual variations observed are due to size, nutritional state and environmental factors. The statistical difference observed in the haemolymph protein content between moult stages are mainly due to haemodilution and not any apparent decrease or increase in protein concentration.

The content of free amino acids constitutes one of the biochemical parameters characterizing body fluids. The role of free amino acids in regulation of osmotic pressure has been studied in invertebrates and vertebrates (Awapara, 1962; Kitteridge et al., 1962). The content of free amino acids in haemolymph as well as muscles is dependent on the composition of food (Dall, 1974a) and physiological state of the animal. Gilbert (1959) found a dependence of the level of free amino acids in crustacean haemolymph on body size and sex of the animals. They observed higher concentrations of free amino acids in males than in females and in bigger animals than in smaller ones in both sexes. In the present study, although free amino acid content in the haemolymph varied with respect to size (1.63 mg/ml in 60-80 mm size to 3.393 mg/ml in 120-140 mm size), there was no significant difference between sexes in any of the size group. The variation with respect to size of the species contribute to the wide range in the free amino acid content (0.5 mg/ml to 9.7 mg/ml). Free amino acid content in the haemolymph of *P. indicus* recorded low values compared to other decapods although comparable to those reported in

Natantians. Dall (1974) reported values of 5.9 µm/ml in Panulirus longipes and experimentally concluded that moulting cycle did not affect the free amino acid content in the haemolymph. He also concluded that the blood amino acid concentration may be considered as a nutritional index of the species. The haemolymph of A. leptodactylus was characterized by high levels of glycine and alanine, with mean total content 62.27 µm/100 ml in males and 58.21 µm/100 ml in females. In A. astacus, the mean amino acid content was 72.05 µm/100 ml in males and 74.58 µm/100 ml in females; in Orconectes limosus, 101.20 µm/100 ml in males only was recorded. (Rogala et al, 1978). The existence of differences in the content of free amino acids in the haemolymph with respect to different seasons is also recorded (Rogala et al., 1978).

Fair and Sick (1982), determined the total amino acids (free and bound) in Macrobrachium rosenbergii as an indicator of metabolic requirements. With the exception of glycine, concentrations of all amino acids in the haemolymph decreased during starvation, but in contrast, the concentration of total amino acids (both bound and free) in the serum was not affected by a five day starvation period. Similar observations have been made during the moult cycle where obligatory starvation occurs. Species differences exist in amino acid concentrations during moulting. Proline and glutamic acid accounted for 50%. of the postmoult decrease in Gecarcinus lateralis (Duchateau et al., 1959). In the haemolymph similar changes occurred with specific amino acids being involved in energy metabolism during non-feeding premoult changes and synthesis of exoskeleton during premoult (Yamaoka and Skinner, 1976). In the present study, significant

changes in free amino acid content in the haemolymph could indicate the involvement of specific amino acids in energy metabolism during non-feeding premoult and postmoult stages, although overall differences may be due to haemodilution. This has been confirmed in the isopod, Sphaeroma serratum in which the total amino acids concentration (including taurine) of the haemolymph increases sharply during premoult (upto 267 mg/100 ml), falls after ecdysis (97 mg/ml) and then rises upto the level of the intermoult stage (120 mg/100 ml). The variations are not only due to dilution or concentration of the whole haemolymph constituents, but also due to specific modification of the concentration of some of the free amino acids with respect to others. The variations of the aminoacidemia mainly those of serine, proline, glycine, alanine, and of the amine taurine, could play a definite role in increasing the osmotic pressure of the haemolymph during premoult as preparation to ecdysis (Charmantier et al., 1979). Proline represents the highest level of total free amino acids followed by serine, glutamine, alanine, and arginine in isopods Porcellio laevis (2.18 /uM/100 ml) Armadillidium vulgare (2.66 /uM/100 ml), Armadillo officinalis (2.64 /uM/100 ml) and Ligia italica (5.11 /uM/100 ml).

The role of free intracellular amino acids in cell volume regulation has been recorded in several Brachyurans. Acclimation of euryhaline E. sinensis from one medium to another one, more concentrated, results in a slight decrease in the amount of most of the amino acids, while on acclimation from sea water to freshwater, there is a transitory increase in the blood content of various amino acids (Gilles, 1977; Gilles Schoffeneils, 1969). Volume regulation or readjustment is accomplished by increasing

the intra-cellular concentration of organic solutes, primarily free amino acids. In C. sapidus, there is no variability in the total ninhydrin positive substances (free amino acids) in the haemolymph of either sex. But positive relation between salinity and serum TNPS was observed in females but not in males, which is attributed to the migration to higher saline waters for spawning. Seasonal variations was found in C. sapidus, highest during months with lowest temperature(Lynch and Webb, 1973).

Glucose is generally considered the exclusive reducing sugar in the blood of crustacea. Wide range of values reported for reducing sugar in the haemolymph of crustacea suggest that physiological condition of the animal influences the blood sugar levels (Florkin, 1960). Great individual variation is seen in the blood glucose level of *P. indicus* wide range of 0.1 mg/ml to 1.1 mg/ ml glucose is observed which is related to the size and moult stage. The glucose content in the blood increased with the size of the species (0.267 mg/ml in 60-80 mm to 0.592 mg/ml in 120-140 mm size) but no significant difference between either sex was noticed. Similar results are reported for haemolymph glucose in Paratelphusa hydrodromus, C. sapidus, Chasmagnathus granulata, Paranephrops zealandicus (Ramamurthi and Veerabhadrachari, 1975; Lynch and Webb, 1973; Santos and Colares, 1986; Balazs et al., 1974; Quilter, 1977). However, in Crangon crangon, mean glucose concentrations tend to decrease with increasing size and were highest in males, lowest in non-ovigerous females and intermediate in ovigerous females. The glucose content also tends to increase with salinity, lowest at extreme salinities and reduced at lower temperature concurrent with reduced metabolic activity and implication in the osmo-

regulatory process (Spaargaren and Haefner, Jr., 1987). In Penaeus marginatus the females had higher glucose levels compared to males (Balazs et al., 1974). Dean and Vernberg (1965) verified the blood glucose determinations in Callinectes, Hepatus, Libinia and Panopeus and found that the blood glucose was 20-25% of the total reducing sugars. Significant changes in the glucose level occurred in the females as eggs develop. They also observed a diurnal cycle in blood glucose level in Uca pugilator as well as 79% reduction in haemolymph glucose even after 10 days starvation.

High individual variation in glucose concentration might be related to its integral role in metabolic activity. Crustaceans are known to be capable of rapidly changing their internal glucose concentrations in response to varying demands. Thus, the effect of handling during sampling may evoke large changes in the mobilization of glucose (Lynch and Webb, 1973; Spaargaren and Haefner Jr., 1987) but individual variations does not completely mask environmentally induced changes (Spaargaren 1972; Williams & Lutz, 1975).

The nature and role of blood sugars in the moulting process has evidently generated much studies in decapods. The large amount of sugar expended for the synthesis of new chitin at the time of moulting and the resorption of the constituents from the old cuticle before it is shed, alters the normal sugar levels of the haemolymph. This has been reported in Ligia exotica (Parvathy, 1971), Emerita asiatica (Parvathy, 1970), Penaeus vannamei (Chan et al., 1988) and C. maenas (Spindler-Barth, 1976). In *P. indicus*, the haemolymph glucose level showed a marked increase in

premoult phase, subsequent fall during moult and postmoult phase. Since glucose levels are lowest just before and after ecdysis, it is unlikely that, in *P. indicus* glucose was essential for chitin synthesis of the new cuticle or as a source of energy during moulting and does not reflect concurrent changes in metabolism. Instead, the haemolymph glucose variation may be correlated to the feeding pattern. Maximal levels of haemolymph glucose in *P. indicus* during intermoult and early premoult, probably resulted from an accumulation of food reserves during period of active feeding. Likewise, the gradual decline in glucose level during the preecdysis and postecdysis corresponds with reduced feeding and rapid uptake of water resulting in haemodilution. This also indicates the role of haemolymph glucose in the nutritive physiology of the species.

The control of blood sugar levels by the production of hyperglycaemic hormone (HGH) in crustaceans has been well established (Scheer and Scheer, 1951; Ranganekar and Madyastha, 1971; Parvathy, 1972). Eyestalk removal causes hypoglycaemia after initial period of variable glucose concentrations in Paranepthrops zealandicus (Quilter, 1977), Chasgmagnathus granulata (Dos Santos and Colares, 1986), Astacus leptodactylus (Kallen et al., 1988) O. limosus (Hamann, 1974) and Metapenaeus (Dall, 1970). This has also been established in *P. indicus* by earlier workers (Rajesh, 1985; .Vijayan, 1988). In starvation, all the energy stores are diminished and on feeding, the rapid rise in blood glucose, followed by increase in glycogen. In the normal state, the haemolymph accounts for 84% of the total glucose and 85% of the glycogen in Metapenaeus (Dall, 1964). It appears that haemolymph is the major organ involved in carbohydrate metabolism (William and Lutz, 1975).

The level of lipids in the haemolymph of crustaceans has been hardly documented. Recent studies indicate that total lipid and cholesterol content in the haemolymph is higher in Brachyurans compared to other groups. In *P. indicus*, wide range in the total lipid as well as cholesterol content is observed due to the increase in content with respect to size (0.56 mg/ml to 3.8 mg/ml - Total lipid and 0.1 to 2.3 mg/ml Cholesterol). The total lipid content in the haemolymph increases from 0.836 mg/ml in 60-80 mm size to 1.67 mg/ml in 120-140 mm size group which was found to be statistically significant. The cholesterol content in the haemolymph also increased from 0.434 mg/ml in 60-80 mm size to 0.99 mg/ml in 120-140 mm size group. No significant difference in the total lipid and cholesterol content in either sex was noticed. Similar variation in lipid content was reported in Carcinus maenas (Spaargaren and Mors, 1985). The lipid concentrations registered, significant increase in ovigerous females as in *P. indicus*. In Macrobrachium rosenbergii, the mean cholesterol concentration in the haemolymph was 35 mg/100 ml; the males had higher cholesterol concentration (47 mg/100 ml) than females (214 mg/100 ml) (Balazs et al., 1974). Most of the lipids in the haemolymph of spiny lobster Panulirus interruptus were associated with high density lipoproteins. The lipid of this protein was composed of 88% phospholipids, 4% sterol and 3% triglyceride (Lee and Puppione, 1978). In isopods, the major lipid component in the haemolymph appears to be cholesterol (Serrazanetti et al., 1982). The mean total lipid content in haemolymph of isopods Ligia italica (2.2 mg"ml), Porcellio laevis (1.58 mg/ml), Armadillidium vulgare (1.31 mg/ml) and Armadillo officinalis (1.85 mg/ml) reflects the possible difference in lipid profile with respect to adaptation

to terrestrialness (Sevilla and Lagarrigue, 1975).

The cyclic variation in total lipid and cholesterol content in the haemolymph with respect to the moult cycle was registered in *P. indicus*. The total and cholesterol content in the haemolymph increased from intermoult to mid premoult, decreased at premoult and remained low at postmoult and then again increased gradually. Most crustaceans probably accumulate lipids at the beginning of premoult and may be utilized as energy source for subsequent ecdysis. Similar results are recorded in C. maenas (Spaargaren and Mors, 1985; O'connor and Gilbert, 1968),Palaemon paucidens (Teshima and Kanazawa, 1976). P. japonicus, (Kanazawa et al. 1976), Palaemon serratus (Teshima et al., 1975). In Palaemon paucidens, more polar lipids than neutral lipids accumulate throughout the moulting cycle (Teshima and Kanazawa, 1976). In Palaemon serratus, it has been shown that the shrimp is capable of converting desmosterol to cholesterol at all stages of the moulting cycle, although the activity varied during the stages, being highest at stage B (postmoult) (Teshima et al., 1975). Variations in the haemolymph total lipid content with respect to moult cycle is recorded in female Pachygraspus marmoratus (Lautier and Lagarrigue 1976), with maximum (17.99%) recorded at premoult and minimum in intermoult (6.7%). Phospholipids account most in the total haemolymph lipid content. Circulating cholesterol appears to be the important metabolic precursor for ecdysone biosynthesis in crustaceans. Large increase in uptake of cholesterol by Y-organs occurs during premoult, coinciding with rise in circulating ecdysteroid titers in Cancer antennarius (Spaziani and Kater, 1973; Watson and Spaziani 1982a, Vensel et al., 1984).

The copper content of blood, containing hemocyanin has been generally used as a measure of the respiratory pigment in crustacea (Djangmah 1970; Djangmah and Grove 1970; Horn & Kerr 1963; Bryan 1964; 1968; Colvocoresses and Lynch, 1975; Boone and Schoffeniels, 1979; Hagerman 1983). Most of these studies have shown that there exists a wide variation in copper concentrations in the haemolymph of crustacea. In *P. indicus* the copper containing respiratory pigment, hemocyanin, constitutes over 60% of the total copper content. This was established electrophoretically also. Two hemocyanin molecules appear to exist in the native state. Quantitatively, the haemolymph copper reflects wide variation ranging from 135 /ug/ml to 1225 /ug/ml. The variation is correlated with size of the species but not sex, 270.57 /ug/ml in 60-80 mm size to 619.97 /ug/ml in 120-140 mm size. Wide range of values for blood copper concentrations have been reported in the literature for decapod crustaceans. In Maia squinado, the range of blood copper concentration was found to be 0.5 to 82 /ug/ml (Zuckerkandl, 1960), in Carcinus maenas 20-120 /ug/ml (Kerkutt, et al., 1961); Callinectes sapidus - 8 to 173 /ug/ml, 16-176 /ug/ml in egg bearing females and 43-165 /ug/ml (Horn & Kerr, 1963) Homarus vulgaris - 34 to 124 /ug/ml (Bryan, 1964), Austropotamobius pallipes 6 to 59 /ug/ml (Bryan, 1967); Crangon vulgaris from Rhosneigr area - 58 to 200 /ug/ml and C. vulgaris from Red Wharf area 15 to 160 /ug/ml (Djangmah and Grove 1970); Ocypoda macrocera 27.75 to 63.32 /ug/ml in the intermoult stage and 54.55 to 101.70 /ug/ml in premoult stage (Rao et al., 1986).

Hemocyanin values in Carcinus maenas recorded was 40-50 mg/ml (Uglow, 1969a), Panulirus argus - 3.92 mg/100 ml and P. laevicauda

- 4.47 mg/100 ml (Bastos & Bezerra, 1970), 75 mg/ml in Callianassa californiensis (Miller et al. 1976), 50-60 mg/ml in Crangon crangon (Djangmah, 1970), 45-70 mg/ml in Callinectes sapidus (Mangum and Weiland, 1975); 100 mg/ml in Palaemon adspersus (Hagerman and Weber, 1981); Homarus americanus 50 mg/ml (Senkbeil and Wristen, 1981). Assuming that 90% of the copper is bound to hemocyanin (Horn and Kerr, 1963), the blood copper concentration indicate that the Brachyurans have the least copper concentration, compared to higher values in crayfish and lobsters The haemolymph copper concentrations in *Penaeus indicus* are much higher than those recorded so far (135 /ug/ml to 1225 /ug/ml). This may be of respiratory significance to the marine habitat of the species unlike in most other forms studied so far.

In *Penaeus indicus* significant correlation was found between blood copper concentrations and moult stages : highest values was recorded in premoult (C to $D_1^{'''}$) with lowest during late premoult (D_{2-3}) and postmoult (A-B) stages. These variations are significantly linked to the total protein content in the haemolymph. Such variations have been recorded in all other crustaceans studied. Zuckerkandl (1960) showed that a great variation in the blood copper concentration was related to moult cycle in Maia squindo. He showed that in Maia, the blood copper concentration falls during moult and continues to fall after the moult to a minimum at the early intermoult stage (C_1 and C_2), when hemocyanin is withdrawn from the blood. Reciprocal relationship between hepatopancreas copper and blood copper was recorded, but this could not be confirmed in Carcinus maenas by Kerkut et al. (1961). Low postmoult blood copper concent-

trations have been recorded in Palaemon serratus and Palaemon squilla.

The low preecdysial blood copper concentrations, recorded in *Penaeus indicus*, like the blood proteins can be explained in terms of the dilution of the blood with water at the moult. Unlike in Maia and perhaps other Brachyurans, a breakdown of hemocyanin does not occur after the moult. The steady rise in the blood copper concentration after moult may be due to steady dimunition in blood volume due to withdrawal of water into the growing tissues (Drach, 1939; Zuckerkandl, 1960; Dhangmah, 1970, Djangman and Grove, 1970). This has been reported in Crangon vulgaris (Djangmah and Grove, 1970), Homarus gammarus (Hagerman, 1983), Ocypoda macrocera (Rao et al.,1986) and Crangon crangon (Hagerman, 1986). In all these studies, inverse relationship between hepatic copper and blood copper concentrations was established. The increase in haemolymph copper during the late postmoult stages evidently shows serum copper re-entering blood in the postmoult stage as it is found that blood copper level increased in early C stage even before feeding begins (Kerkut et al., 1961; Djangmah and Grove, 1970; Rao et al., 1986). Truchot (1978) found similar variations in C. maenas with low haemolymph copper level in postmoult, their values increase during intermoult and early premoult and steep decrease at ecdysis due to concomitant seawater uptake. Hemocyanin concentrations were also found to vary with respect to starvation as well as moult, in H. gammarus and C. crangon. Two consequences of low hemocyanin concentration due to starvation was reduced oxygen supply to muscles during very active periods and prolonged clotting time of haemolymph. This is of particular significance in culture practices, where prolonged holding of lobsters

results in loss of hemocyanin with time, probably due to deficiency of some constituent in the feed (Uglow, 1969a, Hagerman, 1983, 1986).

Hemocyanin synthesis during hyposmotic stress has been reported in Carcinus maenas and C. crangon (Boone and Schoffeniels, 1979; Hagerman 1986). Copper content in the haemolymph of crabs adapted to low salinity was twice the concentration found in seawater adapted animals and total body copper remained constant under low salinity and seawater conditions. In C. crangon, hemocyanin synthesized under hypoxia in all moult stages at about 5-6% of the normoxic mean value per day. The synthesis of new hemocyanin may be natural response to hypoxia, however since many factors influence hemocyanin concentration, important among them being starvation and type of feed, the animals living under 'low' food conditions must be considered to be more sensitive to hypoxia than well fed ones. During hypoxia, however it is important that sufficient hemocyanin is available for oxygen uptake and transport (Hagerman, 1983, 1986). In *Penaeus indicus* the high values of haemolymph copper recorded may be in accordance with the highly diverse euryhaline habitat of the species. Low and fluctuating salinities of brackishwater, lower the hemocyanin oxygen affinity and it is possible that the high hemocyanin content plays a compensatory role in osmoregulation.

Zinc is one of the most important essential trace elements and more than 90 zinc containing enzymes and proteins have been discovered. Further more zinc increases the activity of many other enzymes (Vallec, 1978). In decapod crustacea, zinc is a known cofactor of carbonic anhydrase, the principal enzyme involved in calcification (Florkin, 1960). Zinc

concentrations have been found to be many times higher than in normal seawater and is well regulated and relatively independent of environmental conditions under normal circumstances (Bryan, 1966, 1967). In *P. indicus* fairly high level of haemolymph zinc is recorded with range of 60-455 µg/ml. The zinc concentration does not vary with sex, but with size and moult stages, significant differences exist. In C. sapidus, the serum zinc concentrations was 6-22 ppm (Lynch and Webb, 1975). According to them (1973) the relation between serum protein level and sex and year class should apply to the serum zinc level as well. Bryan's extensive work (1964, 1966, 1967, 1968) on zinc regulation in decapods has indicated that there are species specific ratios of zinc to blood solid content. This depends on the binding properties of the blood proteins and efficiency of the mechanisms of zinc removal from the blood. Bryan (1967) found that zinc was chiefly associated with the non-hemocyanin proteins. He also found that zinc and carbonic anhydrase levels to be loosely related and found that a 10 - fold carbonic anhydrase activity registered only 2-3 fold increase in zinc concentration in H. vulgaris. Therefore, it is probable that common binding with other serum proteins may be more important. This could also be the case in *P. indicus* where wide variation in haemolymph zinc concentration due to size and moult stages may be due to its binding activity with haemolymph proteins other than hemocyanin. More detailed specific studies on the zinc metabolism is called for in decapod crustaceans.

Manganese is likely to be present in all marine organisms because of its role as an activator of enzyme systems (Lehninger, 1950). The role or influence of trace elements like iron and manganese in the proper

functioning of living systems as they move through the ecosystem has been recognized, but absolutely no data or record of the nature and amount of these elements in the haemolymph of crustaceans is available. In *P. indicus* iron content is fairly high, almost equal to that of copper content Wide range of 290 to 1740 µg/ml is observed. The manganese content in the haemolymph varies from 5 to 25 µg/ml. It seems likely that the iron and manganese are linked to non-hemocyanin proteins in the haemolymph as in the case of zinc since variations with respect to size and moult stages is similar to those exhibited by protein in the haemolymph. The manganese concentration in the blood of Homarus vulgaris was found to be 1.4 to 3.4 µg/g (Bryan and Ward, 1965). They found that manganese is lost from blood during starvation and is absorbed so slowly from solution in seawater that normally an appreciable amount of the body manganese must be absorbed from food. About 98% of the manganese in the body lies in the calcified skeleton and it is possible that some absorption and release of manganese by the shell can take place which may assist in controlling the blood and tissue manganese concentrations. Similar phenomenon may be involved in *P. indicus* judging by the appreciable amount of iron and manganese in the haemolymph.

Calcium, from which most skeletal salts that strengthen the exoskeleton is made, is the most important mineral resource for successful moult in crustaceans. Calcium is withdrawn from the exoskeleton during the premoult period to weaken it so that exuviation can occur. Some of this calcium is stored internally in discrete tissues for use in postmoult calcification. In marine decapods, the concentration of calcium in the haemolymph

is above that in seawater during the intermoult stage (Robertson, 1960; Greenaway, 1983).

In *Penaeus indicus* fairly high amount of calcium is present in the haemolymph. Being euryhaline and also subject to periodic moulting (15-20 days) the calcium transport pattern through the haemolymph from the tissues as well as the medium is very much dynamic. The total calcium content in the haemolymph exhibits wide range (625 to 3770 µg/ml) from 60 mm to 140 mm size. The calcium content increases with increase in size but is not affected by sex. The wide range exhibited in *P. indicus* is accounted for by the individual differences within the population affected by the salinity as well as the stage of moult. Such lack of intersex difference is reported in three species of crayfish A. astacus, A. leptodactylus and Orconectes linosus by Gondko et al. (1981), and in Oniscoides, Oniscus asellus, Porcellio scaber and Cylisticus convexus (Gondko et al., 1984).

The changes in the internal distribution of calcium has been of particular interest because calcium is the major inorganic component of the carapace involved in its degradation and the rapid postmoult hardening of new exoskeleton as well as the concomitant changes in permeability associated with both these processes. Calcium cycling throughout the moulting cycle has beeen reported in several species - Carcinus maenas (Robertson, 1960; Roer, 1980) Procambarus centus (Adegboye et al., 1975) Austropotamobius pallipes (Adams et al., 1982) and Macrobrachium rosenbergii (Fieber and Lutz, 1982). These studies have indicated the dynamic interplay between haemolymph and exoskeleton and the other organs capable of storing

calcium during moulting.

In *Penaeus indicus* (Bursey and Lane, 1971) the calcium level of the haemolymph falls immediately after moulting, when a large amount of calcium is required for mineralization of the new exoskeleton and this is taken directly from the haemolymph. Haemolymph concentrations are reestablished by mobilization of calcium from the hepatopancreas, decreased excretion and increased uptake. The increase in calcium in the premoult haemolymph is accounted for by resorption from the old exoskeleton. Similar changes have been reported for Sesarma catenata (Hecht, 1975). Carcinus maenas (Greenaway, 1976; Robertson, 1960, 1977), Armadillidium nasatum and Orconectes limosus (Bohn and Eibisch, 1976) Macrobrachium rosenbergii (Fieber and Lutz, 1982, 1984), Callinectes sapidus and C. maenas (Greenaway 1983), P. monodon (Ferraris et al., 1986 and Baticados et al., 1987) C. sapidus (Cameron, 1989).

In *Penaeus indicus* as in the case of other species investigated so far, calcium concentration of the haemolymph increase significantly over the intermoult (C) and remains elevated until postmoult calcification. This increase in haemolymph concentration occurring at the same time as the calcium peak in the hepatopancreas as reported in M. rosenbergii (Fieber and Lutz, 1982) indicates that the blood is acting as the organ of transport for calcium between the exoskeleton and the hepatopancreas. The haemolymph also serves as a premoult sink for calcium. The calcium peak in the hepatopancreas during intermoult in *P. indicus* has been reported by Vijayan (1988). Consolidation of calcium postmoult calcification results

in part from these internal sources (haemolymph, hepatopancreas, gastroliths (Mantel and Farmer, 1983) and in part from calcium ions taken up from seawater (Fieber and Lutz, 1982; Rao et al., 1982; Ferraris et al., 1986 and Vijayan, 1988).

The enhanced level of calcium in the haemolymph may be an advantage to the moulting *P. indicus* due to enhanced oxygen transport, particularly because the moulting period is associated with increased oxygen consumption at a time when ventilation may be poor due to weakness of the cuticle. The role of calcium as an important effector in this process has been demonstrated in Callinectes sapidus (Mason et al., 1983). The role of calcium ions in the control of the state of aggregation, the magnitude of the Bohr effect and the degree of cooperativity between the oxygen binding states in hemocyanins of Carcinus mediterraneus and Potamon edulis has been effectively proved by Chantler et al. (1973). In *P. indicus* it has been found (in this study) that hemocyanin fractions separated electrophoretically are linked to or bound to calcium ions (ie. the hemocyanins are also calcium binding proteins) and therefore contribute significantly in enhancing the oxygen affinity of *P. indicus* hemocyanin and might therefore facilitate oxygen loading at the gills (Miller and Van Holde, 1974).

Vitellogenesis is a crucial period in the female reproductive cycle of crustacea, characterized by rapid yolk deposition. Biochemical studies on vitellogenesis have largely dealt with components of yolk protein and their autoheterosynthesis and been confined to evaluation of their level in the ovary and hepatopancreas. Vitellogenin or female specific protein,

the seric precursor of lipovitellin is lipoglycocaroteno-protein in nature of high molecular weight and the quantitative and qualitative variation as vitellogenesis progresses is reflected in the haemolymph.

In *Penaeus indicus* the biochemical components of the haemolymph register an increase with vitellogenesis. Total protein, total free amino acids, total lipid, cholesterol and glucose rise during the reproductive cycle. Calcium, copper, iron and zinc also exhibit a similar pattern, but is absent in the case of manganese. The content in haemolymph in each case increases from stage I (immature) to stage IV (fully mature stage) rapidly and declines after spawning (spent). The rapid increase in total protein, lipid cholesterol and carotenoid content in the haemolymph between each stage has been found to be statistically significant by Mohammed (1989).

The rise in protein content during the reproductive cycle has been noted in several other species viz. in cirripedes (Barnes et al., 1965), Portunus pelagicus, Metapenaeus affinis and Uca annulipes (Pillay and Nair, 1973), P. pelagicus (Rahaman, 1967), Ocypoda platytarsis and O. macrocera (Nadarajalingam and Subramoniam, 1987) Squilla mantis (Ferrero et al., 1973). Quantification of other haemolymph constituents such as copper iron, zinc and manganese during vitellogenesis in other species is entirely lacking.

Quantification of vitellogenin in Pandalus kessleri in the haemolymph in several species revealed an increase in the vitellogenin level, as vitellogenesis advanced and reached a peak when the oocytes were observed to be yolky histologically (Quinitio et al., 1989). Similar condition was

reported in Homarus americanus by Nelson et al. (1989).

Vitellogenin and lipovitellin are high molecular weight proteins associated with lipids, carbohydrates and carotenoids. Thus, the increase in total protein, total lipid, free amino acids, cholesterol, glucose and other components explains the accumulation of vitellogenin in the haemolymph as vitellogenesis progresses and the sudden decline after spawning since hemocyanin (Copper protein) is the major component of haemolymph, the specific role of the proteins in vitellogenesis is not understood, although this study revealed peculiar pattern exhibited by the copper protein fractions electrophoretically during various stages of ovarian maturation and corresponding high levels of copper in the haemolymph. It is probable that the copper protein enhances the availability of oxygen during the energy demanding process of vitellogenesis.

Various studies have dealt with the biosynthesis of vitellogenin and none of other constituents of the haemolymph (Lin and O'Connor, 1977; Nakagawa et al., 1982, Yano and Chinzei, 1987, Nelson et al., 1988 Rankin et al., 1989, Quinitio et al., 1989). These studies demonstrated that ovary is responsible for synthesis of major ovarian polypeptides that appear during vitellogenesis and hepatopancreas is not involved in this process since it does not produce appreciable amounts of these components. This implies that the haemolymph serves as the conduit for the ovarian polypeptides during ovarian maturation which is reflected in the increase in the biochemical constituents from stage I to stage IV (Fully mature stage) and a sharp drop after spawning when the eggs have been released,

which is the case in *P. indicus* also. Calcium serves as transporting agent through the haemolymph to the developing oocytes, by binding with proteins during vitellogenesis as apparent by the high levels of calcium in the haemolymph during ovarian maturation.

CHAPTER II

CHARACTERIZATION OF PROTEIN CLASSES IN THE HAEMOLYMPH

INTRODUCTION

Hemocyanins are respiratory proteins occurring in the haemolymph of a wide variety of invertebrate species belonging to the Arthropoda and Mollusca. Arthropodan hemocyanins are found in the Chelicerates (horse shoe crabs), Arachnids and Araeinids (scorpions and spiders), Chilopodes (centipedes) and the Malacostracans which includes the Isopods, Amphipods Stomatopods (mantis shrimps), Euphausiids (krill) and Decapods (crabs, cray-fishes, lobsters and shrimps).

Hemocyanins are copper-containing respiratory proteins which occur extracellularly in the haemolymph. The copper content in arthropods is 0.17% (w/v). The molecular weight of hemocyanin ranges from 400,000 to 9 million daltons. Typically, they consist of 75 kdal polypeptide chains, which are associated into hexamers. Each polypeptide or subunit contains two copper atoms which can bind one molecular oxygen (Van Holde and Van Bruggen, 1971). In the oxygenated form these copper ions are bivalent, which causes the typical blue colour of hemocyanin. Aggregation states with sedimentation coefficients of 5S, 7S, 16S, 24S, 34S and 60S are observed for crustacean hemocyanins. The striking feature of crustacean hemocyanins is their subunit heterogeneity; three to eight subunits have been distinguished in them.

Hemocyanins show very interesting homotropic and heterotropic interactions. Divalent cations, especially calcium (Ca^{2+}) and magnesium (Mg^{2+}) have been shown to play a role, both,in controlling subunit interactions

and oxygen affinity of the copper sites. Bohr effects of hemocyanin are sometimes positive and sometimes negative. Oxygen affinities, pH dependent characteristics and degree of cooperativity resemble those of hemoglobins in many instances. These characteristics of the copper-protein in the haemolymph of crustacea has generated considerable interest and have been subjected to electrophoretic, immunochemical, circular dichroic spectra, and electron microscopic investigations.

Apart from hemocyanin, various specific protein fractions are also reflected in the haemolymph of crustaceans.

i) Female specific proteins (FSP) associated with the vitellogenic process, which serve as useful indicators of developmental changes in the species. These are of low molecular weight and are glyco-lipo-proteins.

ii) Proteins associated with the moulting cycle, also of low molecular weight (Busselen, 1971).

iii) Heteroagglutinins - agglutinating factors with molecular weights larger than 150 kdal.

iv) Fibrinogen - although these factors are yet to be properly identified they are definitely of high molecular weight.

Electrophoretic and immunochemical techniques have been employed to investigate the pattern and nature of proteins in the haemolymph of several crustaceans. Immuno-diffusion, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, (SDS-PAGE), Sephadex Gel Chromatography, and electron microscopy techniques have been employed to determine the molecular

weights of hemocyanin subunits. Apart from providing information on the variation in blood constituents with respect to sex, reproductive and moult cycle, these techniques have aided in establishing species specificity as well as phylogenetic relationship among crustaceans based on similarities among hemocyanins.

Among Arthropods, haemolymph characterization studies have largely been directed towards the larger decapods and to a limited extent, the isopods, amphipods and chelicerates. Chelicerate and Mandibulate arthropod hemocyanins have been much investigated, among which, the living fossil, the horse shoe crab Limulus polyphemus has been extensively studied. Pezalla and Herman (1977) quantified and analysed the haemolymph lipids, while Shishikura and Sekiguchi (1978, 1983) conducted comparative studies on horse shoe crab coagulogens (L. polyphemus, Tachypleus tridentatus, T. gigas and Carcinoscopius rotundicauda). Bijholt et al. (1982) conducted extensive electron microscopy studies on Limulus hemocyanin while Brenowitz et al. (1982) investigated the subunit composition by various biochemical methods. Armstrong et al. (1985) investigated the α_2-macroglobulin like activity in the blood of Limulus polyphemus.

The earliest studies on crustacean haemolymph proteins and particularly hemocyanin, was initiated by Redfield (1934), Klotz et al. (1948) and Redmond (1955). Isopods, amphipods and stomatopods have been investigated by several workers. Weiser (1965) separated hemocyanin and other proteins in the blood of fifteen species of marine and terrestrial amphipods and isopods. The electrophoretic analysis of haemolymph of Ligia italica, Porcellio laevis, Armadillidium vulgare, Armadillo officinalis (Sevilla

and Lagarrigue 1973, 1976; Sevilla, 1975), Orconectes propinquus (Decapod) P. laevis and A. vulgare (Alikhan and Lysenko 1973, 1975; Alikhan and Akhtar, 1980) Gammarus fosarum, G. lacustris, G. roesli, A. aquaticus, O. asellus (Jazdzewski et al. 1975); Idotea balthica (Kaim-Malka et al., 1983) has revealed interesting features. Picaud (1971, 1976) and Picaud et al. (1974) demonstrated variations due to inter sexuality and presence of FSP and protein variations during moult cycle in the haemolymph of Ligia oceanica and A. vulgare. Picaud (1978a,b) also demonstrated and determined by immuno-diffusion the vitellogenin (FSP) and vitellin and their molecular weights in Porecellio dilatatus. Gilbert (1971, 1972) analysed haemolymph proteins of Niphargus virei according to physiological, ecological and systematic factors. Various aspects of haemolymph proteins of Orchestia gammarella has been elucidated by Croiselle et al. (1974); Junera et al. (1974); Saiag et al. (1979) and Blanchet et al. (1979). Sevilla (1977) studied the influence of pH on the dissociation of hemocyanin of L. italica, Tylos latreillei, P. laevis, A. granulatum, A. vulgare and A. officinalis. Sevilla and Lagarrigue (1979a,b, 1978) employed various techniques such as agar immuno-diffusion, quantitative immuno-precipitation, amino acid sequencing and absorption spectra to study the structure and characteristics of the hemocyanins of the above mentioned isopods and amphipods. Jokumsen et al. (1981) studied the hemocyanin of giant Antartic isopod Glyptonotus antarticus while Terwilliger et al. (1982) studied the hemocyanin subunit structure of Ligia pallasii. Protein variability of haemolymph of stomatopod Squilla mantis was investigated by Ferrero et al. (1983).

The Brachyurans have been subjected to extensive haemolymph characterization studies. Several species have been investigated. Horn & Kerr (1969), Cole & Morgan (1978) and Ceccaldi (1971) characterized electrophoretically, the haemolymph proteins of the blue crab Callinectes sapidus and Scyllarus arctus to identify hemocyanin and other major constituents. Uglow (1969a,b) characterized haemolymph protein concentrations as well as effect of imposed fasting on protein in Carcinus maenas. Godbillon and Frentz (1972 a,b,c) conducted immuno-electrophoresis to separate the haemolymph proteins of C. maenas and identified hemocyanin fractions as well as those proteins associated with moulting and vitellogenesis. Further investigations on C. maenas hemocyanin was conducted by Gabriel et al. (1972) by normal and magnetic circular dichroisim while Rizzoti (1974) studied the quaternary structure of C. maenas hemocyanin. Baron (1975) separated the serum proteins of Portunus validus. Martin et al. (1977) studied the zinc-hemocyanin binding in the haemolymph of C. maenas. Zatta and Salvato (1981) investigated the reconstitution of C. maenas hemocyanin in the presence of non-ionic detergent. Electrophoretic and immunochemical analysis to study the variation in proteins of haemolymph during moult cycle of C. mediterraneus was carried out by Herberts et al. (1978). Ghidalia (1972) and Ghidalia et al. (1973, 1975, 1976) have conducted extensive electrophoretic and immunochemical studies on Macropipus puber male serum to detect a haemoagglutinin as well as other proteins. Kannupandi and Paulpandian (1975) separated the general proteins, glyco and lipoproteins and hemocyanin fractions from the blood of Ocypoda platytarsus, O. macrocera, Uca annulipes, U. triangularis, Thalamita crenata, Scylla serrata and Cardisoma carnifex.

Attempts to identify proteins associated with moulting, in the haemolymph has been limited. Zuckerkandl (1956) attempted to study the variations in protein fractions in the haemolymph of Maia squinado during the moult cycle. Effects of moulting and nutritional conditions on the haemolymph proteins was investigated by Busselen (1970) in C. maenas while Adiyodi (1968) investigated the mode of utilization of soluble proteins during moulting and reproduction in Paratelphusa hydrodomous. Vacca and Fingerman (1975a,b) traced the carrier proteins in the blood of Uca pugilator while Lautier and Lagarrigue (1976) investigated them in Pachygraspus marmoratus and Chaix et al. (1981) in spider crab Acanthonyx lunulatus.

Lipoproteins associated with vitellogenesis has hardly been investigated in crabs. Feilder et al (1971) revealed by disc electrophoresis, a female limited lipoprotein and diversity of hemocyanin component in the dimorphic variant of fiddler crab Uca pugilator. Vendrely et al. (1979) and Madaras et al. (1981) attempted to purify and characterize the coagulogen from the haemolymph of Cancer pagurus and Ovalipes bipustulatus respectively. Zatta (1983) investigated the protein-lipid interactions in C. maenas hemocyanin. Spaziani et al. (1986) and Spaziani (1988) studied the pattern of serum high density lipoproteins in Cancer antennarius. The influence of serum lipids on oxygen binding of Callinectes sapidus hemocyanin was investigated by Mangum et al. (1987) while Lee and Puppione (1988) isolated Lipoprotein I and II from the haemolymph of blue crab C. sapidus associated with vitellogenesis.

Interest on the structure and function of hemocyanin of crabs has led to concerted efforts to decipher more information about them. Subunit and antigenic structure of hemocyanin of several brachurans has been brought to light through biochemical studies on hemocyanins of Maia squinado, Callinectes gladiator, Neptunus validus, Macropipus puber Carcinus maenas, Cancer pagurus; (Fine et al. (1975), Lambin et al. (1976); Hamlin and Fish (1977), Rochu and Fine (1977, 1978, 1980, 1984a,b), Ghidalia et al. (1985)). Amino acid composition, amino terminal analysis, subunit characterization of C. magister hemocyanin was performed by Carpenter and Van Holde (1973) and dimorphism in C. magister hemocyanin subunits by Loer and Mason (1973). Bonaventura et al. (1979) examined the properties of hemocyanins isolated from the little red crab Dilocarcinus pagei cristacus, the little brown crab Sylviocarcinus pardalinus and Macrobrachium amazonicum from Amazon river. The function of hemocyanin in respiration of C. sapidus, the structural changes during development in C. productus and immunological comparison of hemocyanins from different populations has been investigated by Mangum and Weiland (1975), Wache et al. (1988) and Jaccarini and Harris (1975). Neuteboom et al. (1989a,b) investigated the relationship between N-terminal sequences and immunological sequences in C. pagurus, C. maenas, Cherax destructor and Palinurus vulgaris. Sullivan and Tentarin (1981) studied the genetics and evolution of hemocyanin multigene in Uca sp.

Aspects of physiology and ecology of hemocyanin in crabs has been worked out in several species. Oxygenation and aggregation properties of hemocyanin from Carcinus mediterraneous and Potamon edulis and

C. maenas was investigated by Chantler et al. (1973) and Truchot (1975) respectively. Young (1972a,b,1973) studied the physiology of hemocyanins in West Indian mangrove crabs, Weiland and Mangum (1975) in C. sapidus, Morris et al. (1988) in robber crab, Birus latro. Maguire and Fielder(1975a,b) examined by disc electrophoresis the haemolymph proteins of 12 species of portunid crabs with respect to effects of storage and physiological and taxonomic aspects. Heavy metal ion interactions with C. sapidus hemocyanin and effect of organic acids on C. magister hemcyanin was investigated by Brouwer et al . (1982), Graham et al. (1983) and Graham(1985) and intracellular buffering at high and low temperatures by dipeptides in the blue crab C. sapidus by Cameron (1989).

Murray and Jeffrey (1974) studied the subunit heterogeneity of crayfish Cherax destructor hemocyanin. Brodski et al. (1976) conducted electrophoretic investigation of the haemolymph serum of Astacus leptodactylus to analyse the population structure.

Durliat and Vranckx (1976,1978,1982,1983) investigated the changes in water soluble proteeins from integument and haemolymph during the moult cycle of A. leptodactylus. Durliat and Vranckx (1976a,b,1982b) also analysed and identified immunoelectrophoretically the fibrinogen like coagulogen protein and hemocyanin in the haemolymph of A. leptodactylus and attempted to isolate the coagulogen. Jeffrey and Treacy (1982)determined the molecular weight of C. destructor hemocyanin. Patak and Baldwin (1984) investigated structural similarities among hemocyanin of six genera of Australian Parastacid crayfish viz. Cherax sp, Euastacus sp. Astacopsis sp. Engacus sp. Geocharax sp and Gramastacus sp. by electrophoretic and immunoelectrophoretic techniques.

Sevilla and Attard (1986) analysed the amino acid composition of the hemocyanins from six species of crayfish to study the homology among them - A. astacus, A. leptodactylus, Austropotamobius pallipes, Pacifastacus leniusculus, Orconectes limosus and Procambarus clarkii. Morris et al. (1986, 1987) investigated the regulation of hemocyanin oxygen affinity during emersion, of the crayfish Austropotamobius pallipes.

Studies on lobster haemolymph has been restricted to three species. Lauffer et al. (1955) conducted extensive physical and biochemical studies on the hemocyanin molecule of lobster, and Stewart et al. (1966) conducted various extensive studies on the constituents of the haemolymph of H. americanus. Barlow and Ridgway (1969) studied the changes in the serum protein during moult and reproductive cycles of H. americanus. Senkbeil and Wriston Jr.(1981) investigated hemocyanin synthesis in the lobster Homarus americanus while Goldenberg and Greenberg (1983) studied the functional heterogeneity of carbohydrate binding haemolymph proteins, providing evidence of a non-agglutinating opsonin. Hagerman (1983) measured the hemocyanin concentration of juvenile lobsters H. gammarus in relation to moulting and feeding while Spoek (1974) studied the relationship between hemocyanin level, oxygen uptake and heart beat in the same species. Beinhard et al.(1983) conducted small angle x-ray scattering study on the quaternary structure of the 24S component of the hemocyanin of H. vulgaris and C. pagurus. Gaykema et al.(1984) elucidated the 3.2A° structure of Panulirus interruptus hemocyanin

Haemolymph protein characterization in Natantia has been restricted to few species. Schwab (1974) identified sex-specific protein in Artemia

while Freel (1978) worked out the oxygen affinity of haemolymph of the mesopelagic mysidacean Gnathophausia ingens. Herberts (1981) has described the occurrence of hemocyanin in the Rhizocephalan, Sacculina carcini. Munuswamy and Subramoniam (1987) characterized the sexual and developmental differences in protein pattern of fairy shrimp Streptocephalus dichotomous. Arcier (1973) and Arcier and Tournamille (1974) conducted immunochemical studies on the haemolymph of Palaemon adsperus in relation to vitellogenesis. Hagerman (1981) studied the respiratory rate, haemolymph oxygen tension and hemocyanin level in P. adspersus. Nakagawa et al.(1982) characterized the Female specific lipoprotein level in the haemolymph of freshwater shrimp P. paucidens during vitellogenesis. Respiratory properties and electrophoretic characterization of the haemolymph of the intertidal prawn P. elegans was worked out by Morris et al.(1985) and Van Wormhoudt (1988). Quinitio et al. (1989) characterized vitellin in the hermaphrodite shrimp Pandalus kessleri. Information on protein characterization of haemolymph in penaeids is scanty. Haemolymph composition of Penaeus kerathurus was evidenced by Cuzon and Ceccaldi (1971). Lee & Kim (1973) and Kulkarni et al. (1980) separated proteins from different tissues of some penaeid prawns in relation to sex. Extensive studies on the structure and function, subunit association and dissociation and effect of environmental variables on the hemocyanin of ghost shrimp Callianassa californiensis was carried out by Roxby et al. (1974), Miller et al (1977). Miller and Van Holde (1981 a,b). Subunit structure of the hemocyanin from the penaeid prawn Penaeus monodon was examined by Ellerton and Anderson (1981). Evidence for the presence and specificity of an unidentified plasma cofactor increasing the oxygen affinity in Palaemon elegans and P. serratus was

provided by Morris et al. (1985). Yano and Chinzei (1987) separated vitellogenin from the kuruma shrimp, Penaeus japonicus and Rankin et al. (1989) from South American white shrimp, P. vannamei.

In the present study, proteins in the haemolymph of *Penaeus indicus* has beeen characterized. The haemolymph proteins have been separated by sodium dodecyl sulphate - Polyacrylamide gel disc electrophoresis (SDS-PAGE). General proteins, lipoproteins, and copper proteins (Hemocyanin) in the haemolymph has been identified by specific staining methods with respect to sex, size groups, moulting stages and reproductive cycle. An attempt has also been made to determine the molecular weight of hemocyanin in the haemolymph of *Penaeus indicus*.

MATERIALS AND METHODS

Haemolymph of *Penaeus indicus* was analysed electrophoretically to identify the species-specific haemolymph proteins. The haemolymph protein pattern of both male and female, of different size groups and moulting stages of size group 100 - 200 mm and maturity stages was traced and compared with normal human serum as standard. Specific staining techniques were used to identify the copper protein (hemocyanin) fractions, calcium binding proteins, glycoproteins and lipoproteins. Determination of molecular weight of the copper-protein fraction - hemocyanin was also done electrophoretically using standard molecular weight markers.

The polyacrylamide gel disc electrophoresis (PAGE) method of Davis (1964) as given by Subhashini and Ravindranath (1981) and Gordon (1974) was followed with necessary modifications.

2.1. **Apparatus:**

The cylindrical perspex tanks manufactured by Dalal & Co. with facilities to run 12 tubes at a time was used for the electrophoretic study. A steady current was maintained with an electrophoretic power pack manufactured by Biochem, Madurai. Glass tubes of uniform length of 85 mm and an inner diameter of 5 mm was used for setting the separating gel. Each tube has three markings engraved on it at a distance of 10 mm, 15 mm and 20 mm respectively from the upper edge (Plate 1).

2.2. Separating gel concentrations:

Separating gels of concentrations ranging from 5% to 10% were prepared by varing the amount of acrylamide and bisacrylamide. Stock solutions were prepared and stored in the refrigerator in amber-coloured bottles and brought to room temperature before use. They were diluted and mixed following the method of Bo Gahne et al.(1977), to get various concentrations of running or separating gel.

In the gels of higher concentrations (10%), migration of protein samples was negligible while in 7% acrylamide and 2% bisacrylamide concentration, the separated fractions were confined to the upper zone only and poor resolution was obtained. Gel concentration of 5% acrylamide and 2% bisacrylamide gave best separation and resolution and was therefore chosen for further study. The addition of sodium-dodecyl sulphate (SDS) promoted better separation and hence SDS - PAGE was adopted. The presence of 2-mercaptoethanol in the sample buffer did not give any significant result in the protein separation and hence SDS-PAGE without 2-mercaptoethanol was employed for general protein separation. Spacer gel in the concentration of 1:2:1:4 (Large pore monomer, Large pore buffer, Riboflavin and 40% Sucrose) was used to obtain clear separation of fractions by concentrating the sample.

2.3. Stock solutions:

1. 40% Acrylamide stock solution - 40 g Acrylamide made up in 100 ml double distilled water.

2) 2.1% Bis-acrylamide stock solution - 2.1 g, Bis-acrylamide made upto 100 ml double distilled water.

3) Tank Buffer

Tris	-	60 g	Made upto 100 ml. in double distilled water, pH adjusted upwards with 10 N NaoH.
Boric acid	-	30 g	
Na_2 EDTA	-	6 g	
SDS	-	1 g.	

4) Small pore buffer - 60 ml of the tank buffer was diluted to 100 ml and 0.23 ml. TEMED (N_1, N_1, N_1', N_1' methylethylene diamine) was added.

5) 0.14% Ammonium per sulphate solution:- 0.14 g ammonium per sulphate in 100 ml double distilled water. This solution was prepared afresh daily.

6) Large pore buffer (Spacer gel) - 60 ml of the tank buffer was diluted to 100 ml with double distilled water and 0.46 ml TEMED was added.

7) Large pore monomer - 12.5 g Acrylamide & 2.5 g Bisacrylamide made upto 100 ml DDW.

8) 0.04% Riboflavin - 4 mg Riboflavin dissolved in 100 ml DDW.

9) 40% sucrose - 40 g sucrose dissolved in 100 ml DDW.

2.4. Buffer:

Various buffers viz. Tris glycine (pH 8.9),Trisboric Na_2EDTA (pH 9.0) Tris-Citric-EDTA (pH-8.0), Phosphate buffer (pH-7.0) and Tris Boric-Na_2 EDTA (pH 8.0) were used in the separating gel as well as tank buffer.

Of these, Tris-boric acid - Na_2EDTA (pH-8.0) gave best resolution and separation and was therefore adopted for further study.

2.5. **Sample:**

Different solvents were used before loading the haemolymph sample for electrophoresis.

i) 40% sucrose.

ii) Tris-boric Na_2EDTA Buffer (TBE)

iii) TBE with SDS and 2-Mercapto-ethanol.

iv) Double distilled water (100 μl sample + 1.0 ml DDW)

v) Haemolymph sample as such (10 μl).

Of these, while the haemolymph sample when used as such gave best results, the diluted sample (ie. with DDW) also gave comparable results. The others resulted in smudged separations. For larger specimens, of *P. indicus* where the protein concentration is very high, haemolymph samples diluted with water was used. In all other cases, haemolymph as such was used. 40 μl/tube of the diluted sample gave best separation and 10 μl/tube in the case of undiluted sample.

2.6. **Procedure:**

Clean dry gel tubes were fixed firmly in the gel setting stand. The separating gel mixture (5% acrylamide and 2% bisacrylamide)was prepared by thoroughly mixing the solutions of acrylamide, bisacrylamide, small pore buffer, distilled water and ammonium per sulphate according to Bo Gahne

et al (1977). The mixture was carefully poured into the fixed gel tubes along its sides using a 50 ml syringe fitted with a long No.19 needle. This mixture was poured upto the lowest marking on the gel tube. Care was taken to avoid trapping of air-bubble in the gel. Few drops of double distilled water was layered carefully over the gel to avoid meniscus formation The tubes were left undisturbed for a 15-20 minutes to allow polymerization of the gel.

After completion of polymerization, the overlying water was carefully removed by inserting bits of blotting paper into the gel tubes. The spacer gel solution was prepared by mixing large pore buffer, large pore monomer, riboflavin and 40% sucrose solution in the ratio 1:2:1:4. This mixture was carefully poured over the set separating gel upto the second marking on the gel tube (10mm middle mark). Few drops of double distilled water was overlaid to avoid meniscus formation. The tubes were then placed near a fluorescent lamp for 10-15 mins. for complete photopolymerization.

The haemolymph sample (10 /ul/gel tube) was applied to each tube after blotting out the excess water from the tubes. Few drops of the marker dye (0.1% bromophenol blue) was added over the sample in each tube using a finnipipette. The tubes were overlaid with dilute tank buffer upto the brim using a syringe. Tank buffer is prepared by diluting 60 ml of stock tank buffer to 600 ml with double distilled water.

Each gel tube was carefully removed from the stand and inserted into the grommets of the upper tank. Drops of dilute buffer was suspended

from the lower end of the gel tubes to avoid trapping of air bubbles during the run. About 300 ml of the diluted buffer was added to the lower tank and the upper tank with the tubes was placed over this. Buffer was added to the upper tank, carefully through the sides using a clean glass rod, so as not to disturb the sample in the tubes. The upper tank was then covered with the lid. The whole unit was placed in the refrigerator and connected to the power pack. A steady current of 1 mA/tube was supplied till the marker dye crossed the spacer gel after which the current was increased to 3 mA/tube. The correct supply was stopped when the marker dye reached the lower end of the tubes.

On completion of the run, the tubes were removed from the grommets. The gels were carefully removed from the gel tubes by forcing a jet of water between the gel and inner tube using a syringe fitted with a thin hypodermic needle. The gels were then treated with appropriate stains.

2.7. **Staining procedure:**

Detection of protein fractions:- The gels were incubated for 30 mins in 10% Trichloroacetic acid solution. They were then transferred to stain (0.25% Coomassie brilliant blue prepared in 100 ml mixture of methanol; acetic acid and water in the ratio 5:5:1) and kept in dark for 15-20 mins; destained in mixture of methanol : acetic acid : water in the ratio 5:5:1 for 30 mins. The gels were stored in labelled test tubes containing 7% acetic acid. The protein fractions retained the blue colour of the stain used.

Detection of copper-protein fractions (Hemocyanin):- To detect the presence of copper containing protein fractions, the gels were treated for 48 hrs at room temperature in a saturated solution of rubeanic acid in methanol, acetic acid and water in the ratio of 5:2:5 (v/v/v). The appearance of greenish brown colour indicated the presence of copper (Horn and Kerr, 1969).

Detection of polysaccharide moiety:- The method of Smith (1968) and Gordon (1980) was followed. Polysaccharides associated with proteins were localised, with periodic acid - Schiff (PAS) test. The gels were fixed for 1 hr in 1% periodic acid in 3% acetic acid and leached for 1 hour in double distilled water and then treated with Schiff's reagent (Mc Manus, 1948) for 1 hr in the dark under refrigeration. The gels were destained in 1% sodium metabisulphite solution and by repeated washing in 7% acetic acid. The polysaccharide moiety developed a majenta colour. These gels were stored in 7% acetic acid in labelled test tubes.

Detection of lipoprotein moiety:- The gels were incubated in a saturated solution of Oil red 'O' in 50% methanol containing 10% TCA for 2 hrs at 60°C. The neutral lipids developed a reddish orange colour. The gels were destained and stored in 7% acetic acid solution in labelled test tubes.

Localization of calcium - binding protein fractions:- The presence of calcium was detected by following the method of Dahl (1952) as given by Pearse (1968). The gels were treated with Alizarin red S staining solution (pH-6.5) for 20 mins. Fixation in 10% TCA was avoided as the staining method was pH specific. The gels were washed with double distilled water and

rinsed with acid ethanol (10^{-3}M Hcl in 95% ethanol) till the background was destained completely. The gels were then stored in 7% acetic acid. Calcium containing protein fractions stained deep reddish orange colour.

2.8. Relative mobility (R_f) values and electropherograms:

The total length of the separating gel, distance travelled by the marker dye in the separating gel and the various distances migrated by the different protein fractions were measured. The relative mobility/relative fraction (Rf) values of each band was calculated as follows:-

$$\text{Relative mobility (Rf)} = \frac{\text{Distance travelled by the protein fraction}}{\text{Distance travelled by the marker dye.}}$$

Separate electropherograms were drawn based on these results. Each protein fraction was numbered serially with the fastest migrating protein fraction as the first and the slowest fraction, the last number.

2.9. Determination of molecular weight of hemocyanin:

Molecular weight of hemocyanin, the major copper containing protein of the haemolymph of *P. indicus* was determined by sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE) using standard molecular weight markers. The method adopted was a modification of the methods of Weber and Osborn (1969) and Davis and Stark (1970). MW SDS-280 Kit of Sigma Co. was used for this procedure.

Electrophoresis of the sample as well as the standard markers was done by employing the same procedure as followed for general protein separation. The gel concentration was 5% acrylamide and 2% bisacrylamide

and sample applied was 10 µl. The gels were stained with 0.75% coomassie blue. The relative mobility of each fraction was measured. The check proteins used in this experiment are Limulus hemocyanin and cross-linked bovine serum albumin. The best linear fit was computed by the equation $Y = a+bx$ where Y = molecular weight and x the refractive mobility (Rf value) and the molecular weight of the hemocyanin protein was obtained.

RESULTS

COMPONENTS OF THE HAEMOLYMPH OF *PENAEUS INDICUS*

Quantitative and qualitative variability of the components of individual samples of haemolymph of *Penaeus indicus* makes it difficult to describe a characteristic or 'normal' pattern for the species. Fig. 1 represents the composite pattern specific to the species after sodium-dodecyl-sulphate-polyacrylamide gel discontinuous electrophoresis (SDS-PAGE) of hundred individual samples of haemolymph from males and females of size range 60-140 mm. Beginning with the components of highest mobility (anodic), the fractions are serially numbered in the ascending order.

Generally, 10-16 protein fractions are observed in the haemolymph, the major fractions being the copper bearing proteins - the fast and slow hemocyanins (Plate 2).

a) Fast hemocyanin (Fraction 7) - The broad, intensely staining somewhat diffuse, major component,trailing approximately midway through the separating gel, represents hemocyanin. This component constitutes nearly 80% of the total protein in the haemolymph along with the slow hemocyanin fraction. This fraction stains for copper, calcium and is also glycolipoproteic in nature. It varies quantitatively with size of the species. The relative mobility (Rf value) ranges between 0.285 to 0.30.

b) Slow hemocyanin (Fraction 10) - This copper bearing protein, a narrow zone of high intensity of stain is termed as the slow hemocyanin. This slow hemocyanin fraction was invariably present in all samples of haemolymph and stained positively for copper and calcium and is also a glyco-lipo-protein complex like the fast hemocyanin. The relative mobility (Rf) was invariably 0.14.

c) Heteroagglutinin (Fraction 9) - A streaky band closely associated with the slow hemocyanin fraction is considered to be the heteroagglutinin, a component associated with clotting of the haemolymph (Terminology adopted from Ghidalia et al. (1970) and Baron, (1975). This fraction is non-copper bearing but stains for calcium and rarely for PAS and Oil Red O. The exact nature of this protein fraction is still unknown. The relative mobility in all cases was found to be 0.142.

b) Clotting protein (Fraction 16) - this is a streaky band, with almost no mobility, observed near the cathodic end, intensely stained. This protein fraction is believed to represent the "Fibrinogen" (Manwell and Baker 1963b). Amylase activity is associated with this fraction (the present study did not include this test). This fraction is also a complex protein, staining for PAS and Oil Red O but not for copper and occasionally for calcium. The relative mobility (Rf) in all cases was found to be 0.014.

The slow and fast hemocyanin fractions present in all samples of haemolymph and no polymorphism is noticed in the population. Apart from the above mentioned specific major protein fractions, several other

protein fractions of varying intensity is observed in the haemolymph. These vary greatly with respect to size, sex, moult stage and reproductive cycle and nutritional state. The fast moving fractions 1, 2, 3, 4 and 5 are apparently simple proteins since they do not stain for PAS, Oil Red 0 or Alizarin Red S. The slow moving fractions apart from those mentioned above 6, 8, 11, 12, 13, 14 and 15 are complex proteins staining for either calcium, glycogen or lipid although they vary a great deal. The qualitative differences in the proteins in the haemolymph reflects their functional significance in the metabolism.

A peculiar feature noticed during the electrophoretic separation of haemolymph proteins, is the occurrence of half fractions stained quite intensely so as to be considered as protein fractions. Since the occurrence of such half fractions has not been recorded earlier, these are probably artifacts. Such fractions have been noticed occasionally in several individual samples and they also stain for PAS and Calcium.

VARIATIONS WITH RESPECT TO SIZE AND SEX

Haemolymph protein pattern of four size groups (60-80 mm, 80-100 mm, 100-120 mm and 120-140 mm) are represented in Fig. 2, 3, 4, 5 and 6. In size group I (60-80 mm), the number of protein fractions varies between 12-14. While the basic protein pattern does not vary, the quantitative difference in the protein content is represented by the reduced thickness of the copper staining fraction - the fast hemocyanin.

In the male haemolymph, 13 fractions are separated (Fig. 2). While fractions 5 and 8 are copper bearing proteins staining positive with Rubeanic acid, fractions 1, 5, 7, 8 and 13 stain for calcium, lipid and glycogen thus indicating a complex protein nature. Fractions 7, 10, 12 also stain for PAS thus indicating that most of the fractions separated are glycoproteins The copper bearing proteins (fractions 5 and 8) stain for Oil Red 0, PAS and Alizarin Red S thus reflecting the complex glyco lipo-protein nature of the hemocyanin molecule. The role of calcium in binding with hemocyanin molecule is reflected by Alizarin Red positively.

In the female haemolymph, 14 fractions are observed (Fig. 2). The relative mobility of the various fractions appears to differ slightly from that of the male haemolymph fractions. Fractions 7 and 10 stain positive for copper, PAS, Oil Red O and Alizarin Red S, fractions 2, 4, 5, 7, 9, 10, 14 for PAS, fractions 2, 4, 5, 7, 10 and 14 for Oil Red O and fractions 2,7,10 for calcium. The apparent difference in the fractions separated from male and female haemolymph is attributed to intra-individual variation within the size groups. No female-specific protein fractions is observed in this group. The basic components in the haemolymph, namely the hemocyanin fractions (slow and fast fractions), the fibrinogen and hetero-agglutinin remain unaltered in all cases, while variations occur in the remaining fractions within individuals of the same size group.

In size group II (80-100 mm), the number of proteins, fractions varies between 11-12. In this size group also, the quantitative variation in the protein content in the haemolymph is represented by the copper

bearing protein - the fast hemocyanin (Fig. 3). In this size group, while fractions 8, 9 and 10 in the males stain for PAS fraction, fractions 4 and 7 only stain for PAS in females. Fractions 2, 4, 5, 6, 8, 9 and 10 stain for Oil Red O in the case of males while, in females only fractions 4 and 7 stain for lipid. Fractions 2, 6, 8, 9 and 10 stain for calcium in males and 2, 4, 7 and 8 in the case of females. Again qualitative difference in the fractions of males and females does not indicate sex specificity but intraindividual variation within the size group.

In size group III (100-120 mm), 11-12 fractions occur in the haemolymph of males and females (Fig. 4, Plate 3). The overall relative mobility of fractions appears to be reduced compared to the above mentioned size groups. In this case, the increased protein content in the haemolymph is represented by the thicker fast hemocyanin fraction (fraction 3 in female and 4 in male). The glycoproteins are more in females than in the males, fractions 2, 3, 5, 6, 8 and 9 stain for PAS while fractions 2, 3, 8, 9 are glycolipoproteins. Calcium binding fractions are 3, 5, 6 and 9 in females and 4 and 8 in males. Slow and fast hemocyanin fractions are also present. There is an obvious increase in the glycoprotein in females from this size group onwards. No sex-specific proteins are present.

In size group IV (120-140 mm), 13-16 protein fractions are separated from the haemolymph (Fig. 5, Plate 4). The hemocyanin fractions are more thick and densely stained reflecting the increase in the protein content in the haemolymph. In the males, 13 fractions are observed. Fractions 4, 5, 6, 7, 8, 9, 10 and 13 stain for PAS while fractions 4, 5, 6, 7, 8,

9, 10, 12 and 13 are calcium binding fractions and fractions 5 and 8 hemocyanins.

In the female haemolymph, 16 protein fractions are observed among which mos of them are glycoproteins (fractions, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 16). Fractions 5, 6, 7, 9, 10, 11, 12 are glycolipoproteins and fractions 7, 10, 11 are calcium binding proteins. There is obvious increase in the glycoproteins in the haemolymph in the females. Fractions 7 and 10 are hemocyanin fractions. In this size group, the obvious difference between the male and female reflects the individual variations within the size group as well as sex.

Comparison of the haemolymph protein pattern of the four size groups shows that the basic species specific pattern prevails among all the size groups. Fig. 6, (Plate 5) represents the comparative electropherograms of haemolymph protein pattern of females in intermoult stage, from the four size groups, compared with normal human serum protein pattern. In Group I, the overall relative mobility of all the fractions is greater than in other groups. This is probably due to the presence of more simple protein fractions. The presence of more protein fractions may be due to the proteins occurring as simple proteins. In group II and III, the relative mobility is reduced and fractions are fewer. This may be due to altered nature of the fractions and formation of complex polypeptides thereby increasing the molecular weight of the protein molecules. This is also more obvious in group IV. The protein fractions are more in number, also their qualitative nature changes viz., most of the fractions are glycolipo-

proteins. This reflects the progressive alteration of the nature of the proteins in the haemolymph to suit the functional requirement of the species as size increases.

HAEMOLYMPH PROTEIN VARIATIONS WITH RESPECT TO MOULT CYCLE

Electropheregrams of haemolymph protein pattern in different moult stages in size group II (80-100 mm) and Group III (100-120 mm) is represented in Figs. 7 and 8 (Plate 6). The haemolymph protein pattern in different moult stages is identical in each stage except for minor differences. These minor differences cannot be attributed to any particular moult stage but due to intra-individual variation. In both size groups, it is obvious that, the species specific polypeptides or protein fractions are present in all stages of the moult cycle and minor variations such as the occurrence of half fractions cannot be denoted to the physiological phenomenon of moulting. The quantitative increase in the total protein content in the haemolymph from the post moult stages (A and B) through inter moult (C) to late premoult (D_1''') and sudden decline just prior to ecdysis (D_{2-3}) in not reflected in the qualitative analysis of proteins, indicating that no particular major polypeptide is involved in the moulting process.

HAEMOLYMPH PROTEIN PATTERN DURING OVARIAN DEVELOPMENT

Haemolymph protein fractions separated from individuals in different stages of ovarian maturity compared with male haemolymph protein pattern is represented in Fig. 9. In the male haemolymph, 13 fractions are separated

with high protein content represented by the very densely stained fast hemocyanin fraction. In the females, the number of fractions varies from 12 to 15 from stage I to spent stage. In stage I, 14 fractions are separated of which fractions 4, 5, 6, 7, 8 and 9 are very intensely stained. These are obvious in stage II also. Fractions 4, 6, 7, 8, & 9 seem to be highly specific in these stages. These fractions which become intense during the development of ovary are the yolk proteins (Vitellogenin) and are termed as female specific proteins (FSP) as they develop during the development of the ovary only. They stain positive with PAS and are glycoproteins. The fractions become less obvious in stage III and are highly disoriented in the mature and spent stages. These specific protein fractions are noticed only during ovarian development and not in any other size groups (Plate 7, 8, 9, 10).

The specific nature of the Female specific proteins (Vitellogenin) is indicated in Figs. 10 and 11. In stage I, fractions 4, 5, 6, 7, 8, 9, 10, 13 & 14 are glycoproteins of which fractions 4, 5, 7 & 8 are lipoproteins also. Calcium binding fractions are 5, 6, 8, 10 & 11. The peculiar feature of copper binding fractions is that only fraction 5 stains positive with Rubeanic acid, indicating the altered role and structure of the hemocyanin molecule during vitellogenesis (Plate, 8, 9, 10).

In stage II, again the glycoproteins are predominant, fractions 4, 5, 6, 7, 9, 11, 12 & 15 staining for PAS while lipoproteins are similar to stage I, fractions 4, 5, 6 & 7 stain for Oil Red O. The calcium binding proteins are same as the glycoproteins stressing that FSP are complex

proteins with calcium also modulating the process of vitellogenesis. In stage II also, the copper bearing fraction is represented by a single fraction - the fast moving fraction (Plate 8, 9, 10).

In stage III, the intensity and number of glycoproteins increases, represented by fractions 3, 4, 5, 6, 8, 9, 11, 12 & 14. The lipoproteins also increase in this stage, represented by fractions, 3, 4, 5, 6, 8, 9, 11, 12. The calcium binding proteins are similar to the glycoproteins, 3, 4, 5, 6, 8, 9, 11, 12 & 14 stressing the role of calcium in vitellogenesis. In the stage III, the slow moving hemocyanin fraction which is absent in the earlier stages reappears.

In the fully mature stage, there is a drastic change in the haemolymph protein pattern the female specific proteins are reduced and seem to have disappeared. The increased protein content compared to the other stages is represented by the very broad and intensely stained fast hemocyanin fraction. In this stage again a peculiar re-orientation of the copper bearing fraction occurs. The slow moving fraction is replaced by a fast moving, more thickened fraction just below the normally occurring fast hemocyanin. The glycoproteins and calcium binding fractions are 1, 2, 3, 4, 6 and 12 while lipoproteins are 1, 2 and 3.

In the spent stage, again there is an altered haemolymph protein pattern. The female specific proteins are present but reduced in intensity compared to stages I, II and III. The glycoproteins are 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 15. In this calcium fractions are reduced, represented

by 4, 5, 8, 9, 10 and 11. The lipoproteins are represented by fractions 4, 5, 8, 9, 10, 11 and 12. In spent stage again, only copper binding fraction noticed is fraction 5. The slow moving fraction is absent (Plate 7).

MOLECULAR WEIGHTS OF HEMOCYANIN FRACTIONS

When *Penaeus indicus* haemolymph is subjected to SDS-gel electrophoresis - a method which separates proteins on the basis of their molecular weight, more than 80% of the protein is found in two bands of slightly different mobility. The two bands stain for copper with Rubeanic acid and hence are identified as hemocyanin protein molecules. The molecular weight of these two hemocyanin subunits was determined by calibration with proteins of known molecular weight namely Limulus hemocyanin and Bovine serum albumin. This calibration method yielded values of 280,000 daltons for the slow fractions and 1,37, 183.9 daltons for the fast fraction as molecular weights of the two hemocyanin units (Fig. 12, Table 1).

The two hemocyanin subunits although present in unequal amounts are not affected by sample treatment. Similar results are obtained whether or not the sample is heated in the presence or absence of mercapto-ethanol, though heating does decrease the amount of the residual high molecular weight (MW 280,000) component. Reduced mobility is observed with increase in gel concentration proving their high molecular weights. The subunit structure is however altered greatly with increase in pH conditions. The mobility and number of fractions increased with increase in pH.

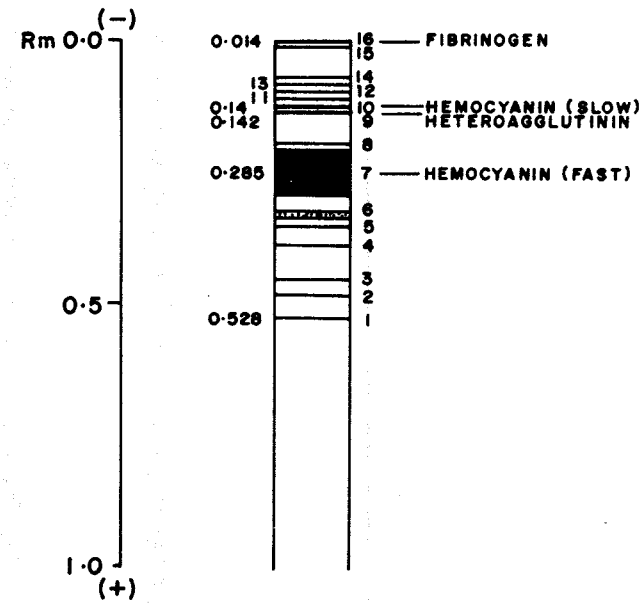

Fig. 1 COMPOSITE PATTERN OF HAEMOLYMPH PROTEIN FRACTIONS OF PENAEUS INDICUS

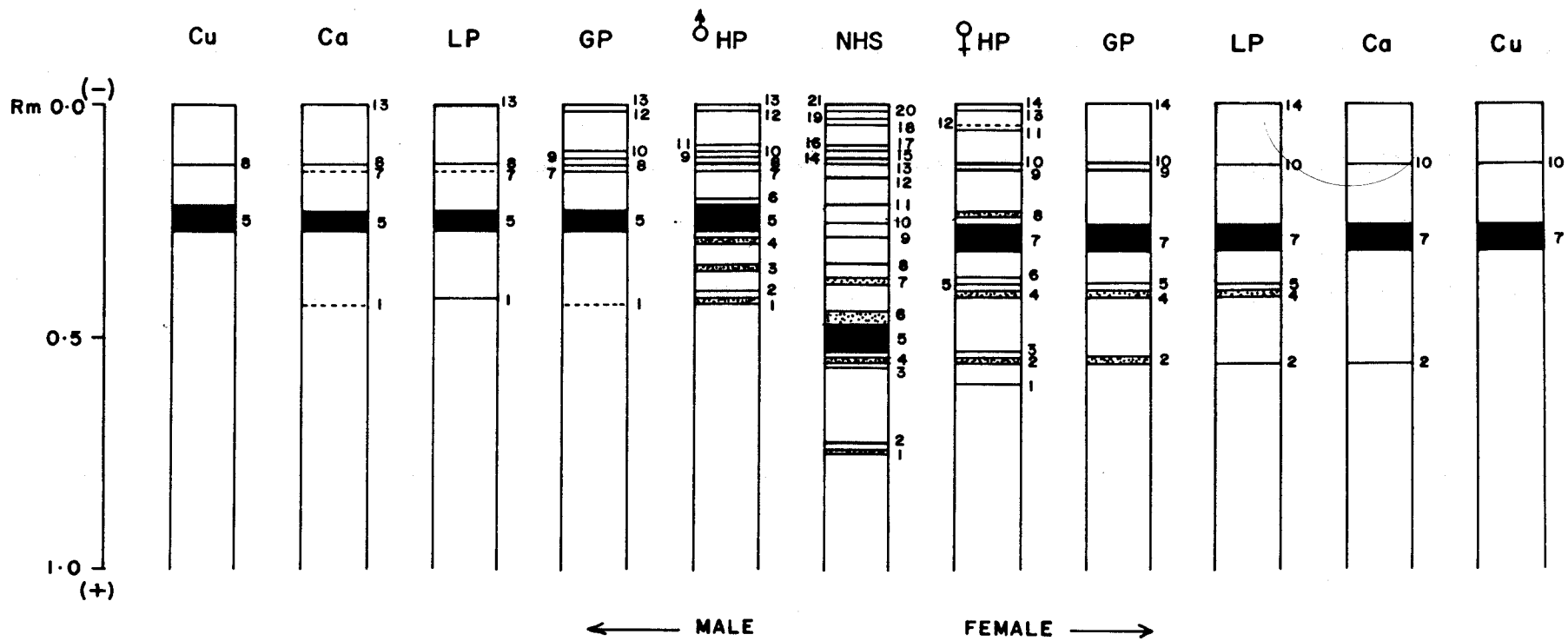

Fig. 2. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH PROTEINS OF PENAEUS INDICUS, MALE AND FEMALE, SIZE GROUP I (60-80mm), INTERMOULT STAGE, ALONG WITH NORMAL HUMAN SERUM PROTEINS.

HP - HAEMOLYMPH PROTEINS, NHS - NORMAL HUMAN SERUM, GP - GLYCOPROTEINS, LP - LIPOPROTEINS, Ca - CALCIUM BINDING PROTEINS, Cu - COPPER BINDING PROTEINS.

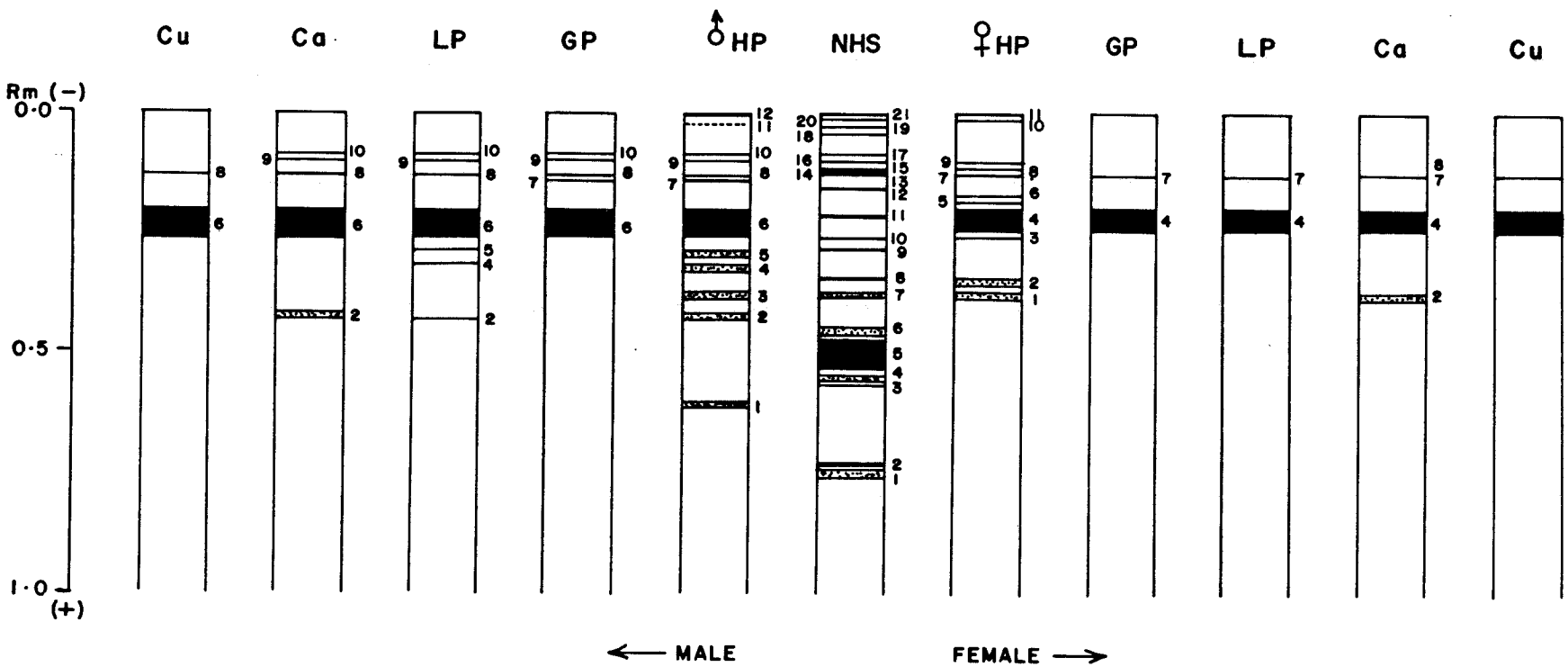

Fig. 3. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH PROTEINS OF PENAEUS INDICUS, MALE AND FEMALE, SIZE GROUP II (80–100), INTERMOULT STAGE, ALONG WITH NORMAL HUMAN SERUM PROTEINS.

HP – HAEMOLYMPH PROTEINS, NHS – NORMAL HUMAN SERUM, GL – GLYCOPROTEINS, LP – LIPOPROTEINS, Ca – CALCIUM BINDING PROTEINS, Cu – COPPER BINDING PROTEINS.

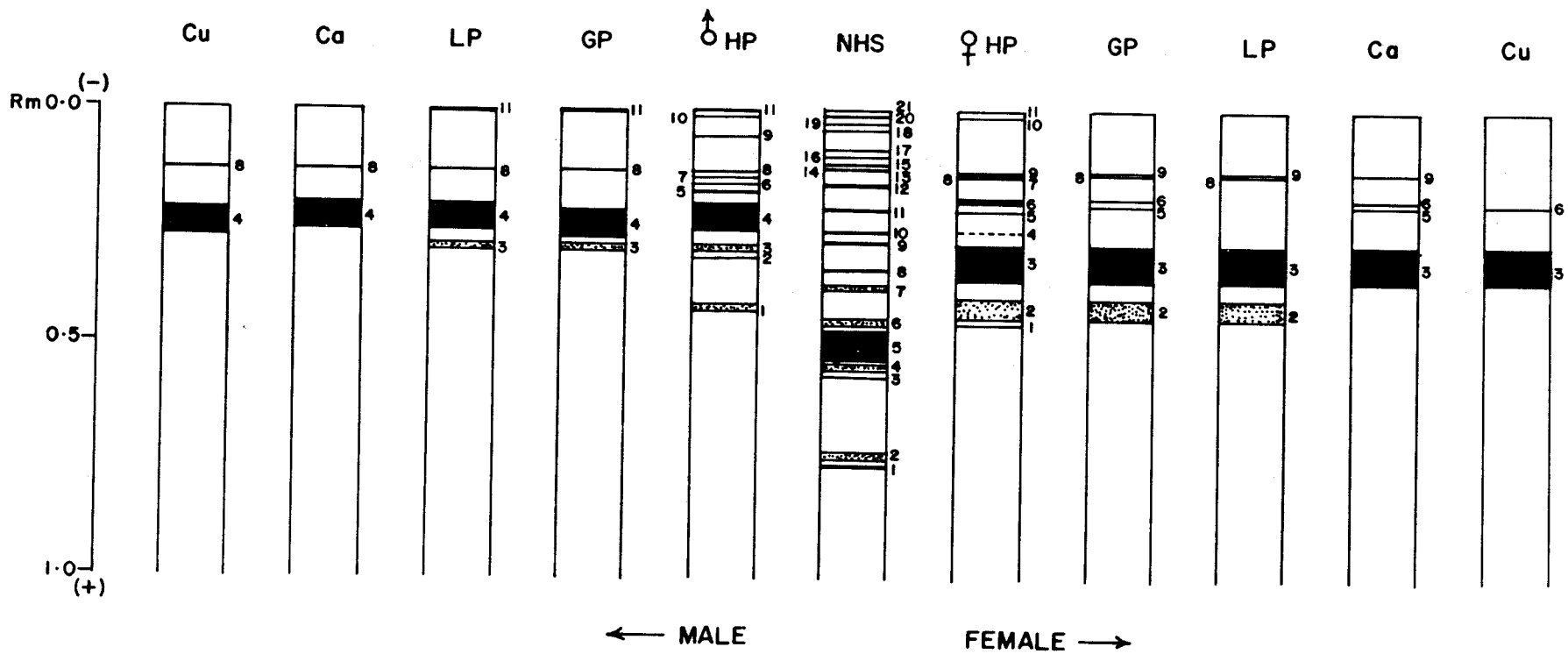

Fig. 4. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH PROTEINS OF PENAEUS INDICUS, MALE AND FEMALE, OF SIZE GROUP III (100-120mm), INTERMOULT STAGE, ALONG WITH NORMAL HUMAN SERUM PROTEINS.

HP - HAEMOLYMPH PROTEINS, NHS - NORMAL HUMAN SERUM, GL - GLYCOPROTEINS
LP - LIPOPROTEINS, Ca - CALCIUM BINDING PROTEINS, Cu - COPPER BINDING PROTEINS.

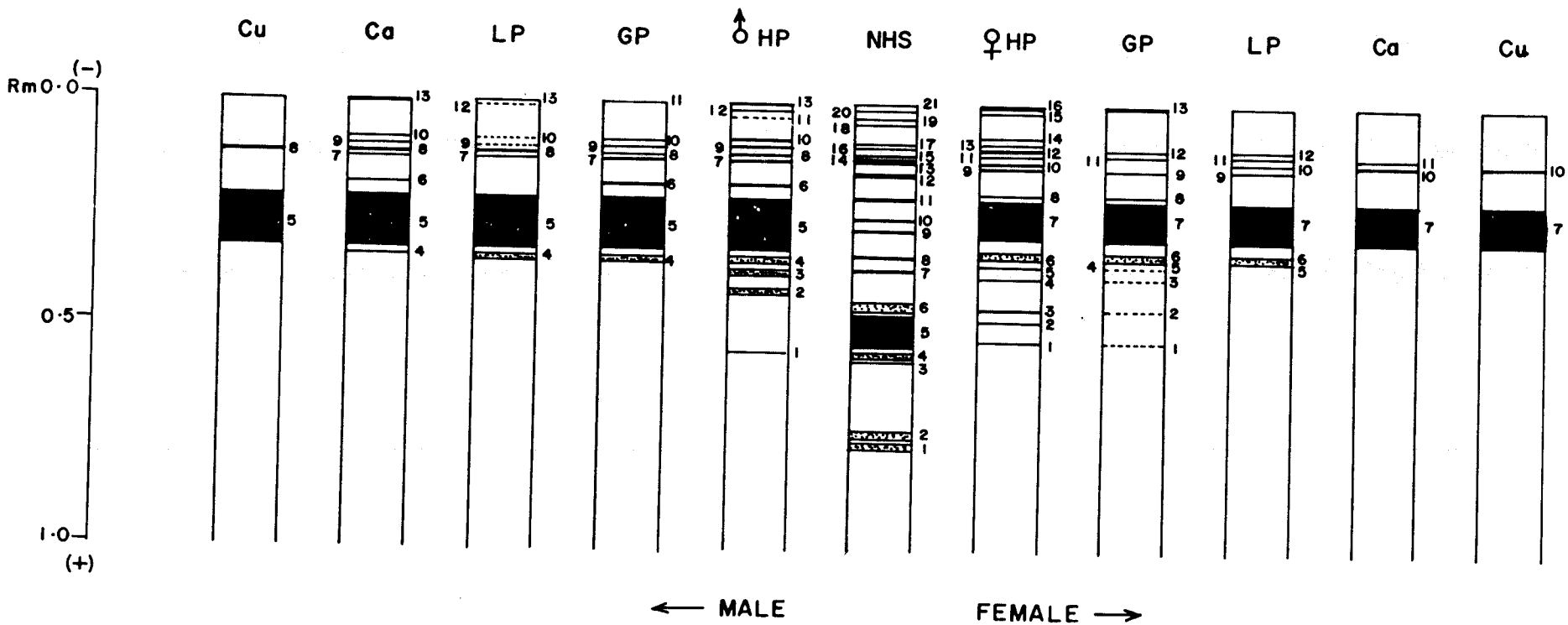

Fig. 5. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH PROTEINS OF PENAEUS INDICUS, MALE AND FEMALE, OF SIZE GROUP IV (120-140mm), INTERMOULT STAGE, ALONG WITH NORMAL HUMAN SERUM PROTEINS.

HP- HAEMOLYMPH PROTEINS, NHS - NORMAL HUMAN SERUM, GL - GLYCOPROTEINS, LP- LIPOPROTEINS, Ca - CALCIUM BINDING PROTEINS, Cu - COPPER BINDING PROTEINS.

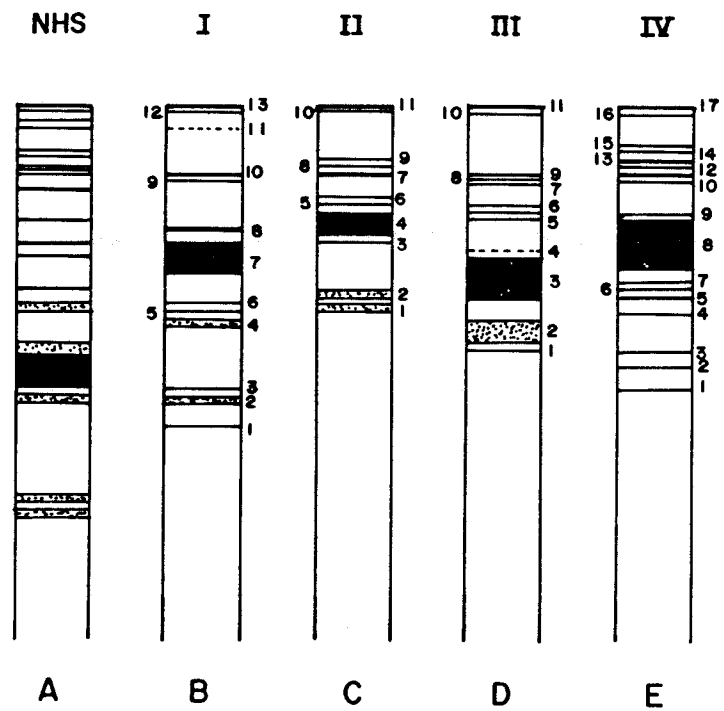

Fig. 6. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH PROTEINS FROM FOUR SIZE GROUPS, ALONG WITH NORMAL HUMAN SERUM PROTEINS.

A – NORMAL HUMAN SERUM PROTEINS, B – GROUP I 60-80mm.
C – GROUP II 80-100mm, D – GROUP III 100-120mm, E – GROUP IV 120-140mm.

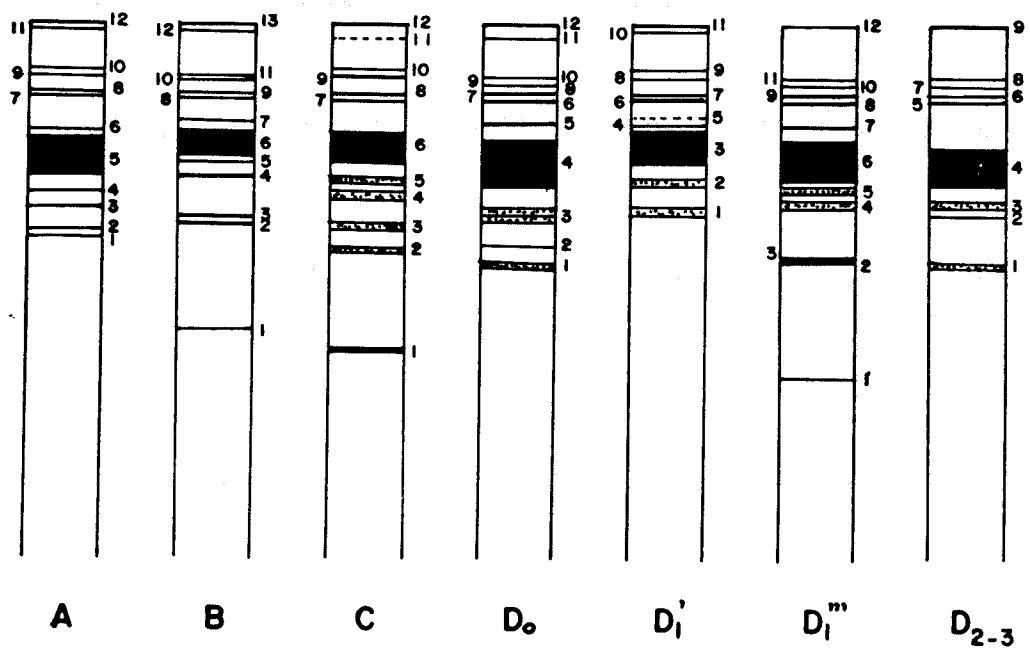

Fig. 7. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH PROTEINS OF PENAEUS INDICUS (MALES) IN DIFFERENT MOULT STAGES, SIZE GROUP II 80 - 100mm.

A - EARLY POSTMOULT B - LATE POSTMOULT C - INTERMOULT D_o - EARLY PREMOULT
D_1' - MID PREMOULT D_1'' - LATE PREMOULT D_{2-3} PREMOULT

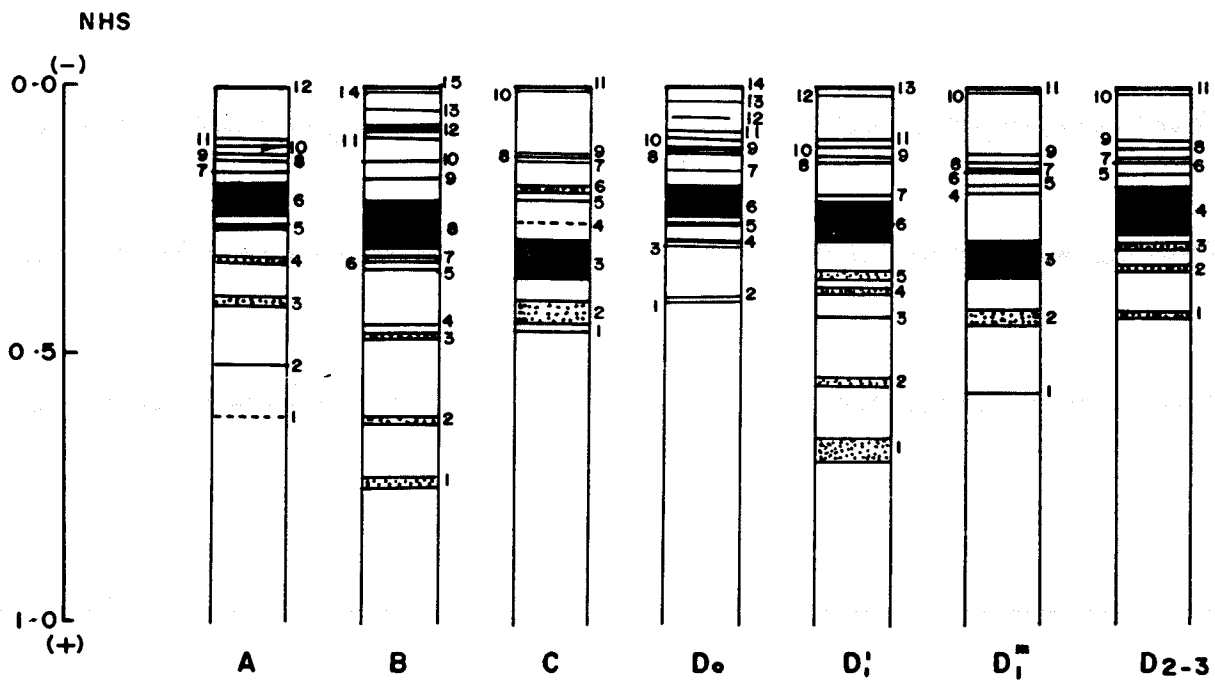

Fig. 8. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH PROTEINS OF PENAEUS INDICUS (FEMALE) IN DIFFERENT MOULT STAGES, SIZE GROUP 100-120 mm.

A - EARLY POST MOULT
B - LATE POST MOULT
C - INTERMOULT
D_o - EARLY PREMOULT
D'_1 - MID PREMOULT
D''_1 - LATE PRE MOULT
D_{2-3} PREMOULT

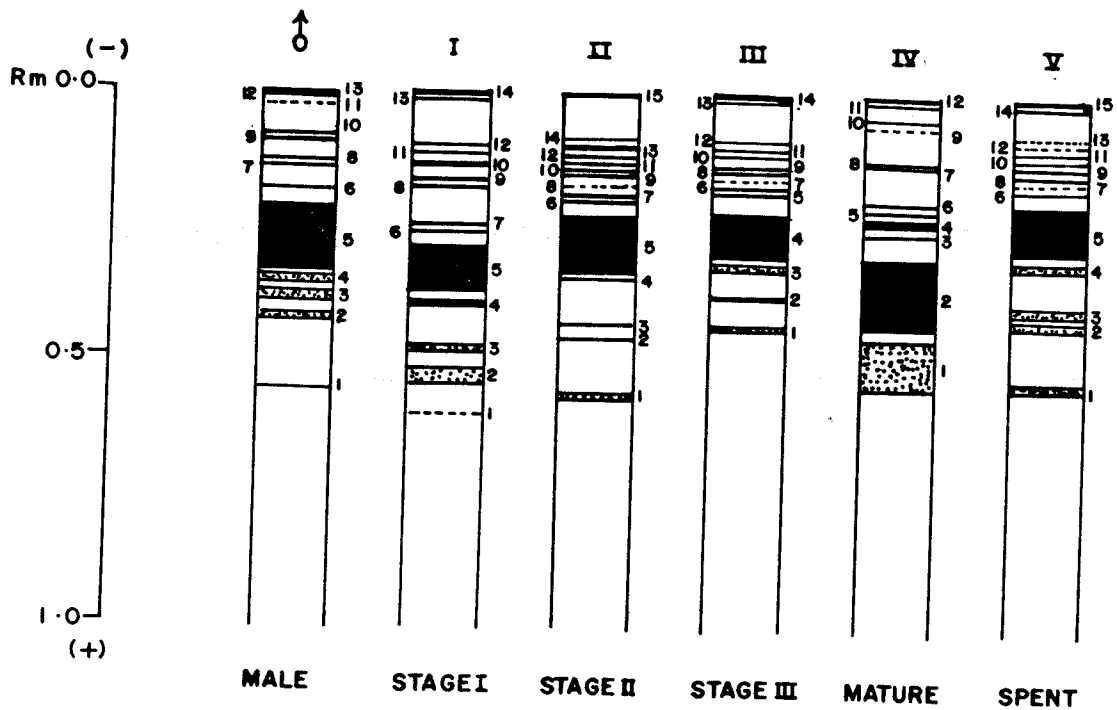

Fig. 9. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH PROTEINS OF PENAEUS INDICUS IN DIFFERENT STAGES OF OVARIAN DEVELOPMENT, ALONG WITH MALE HAEMOLYMPH PROTEINS.

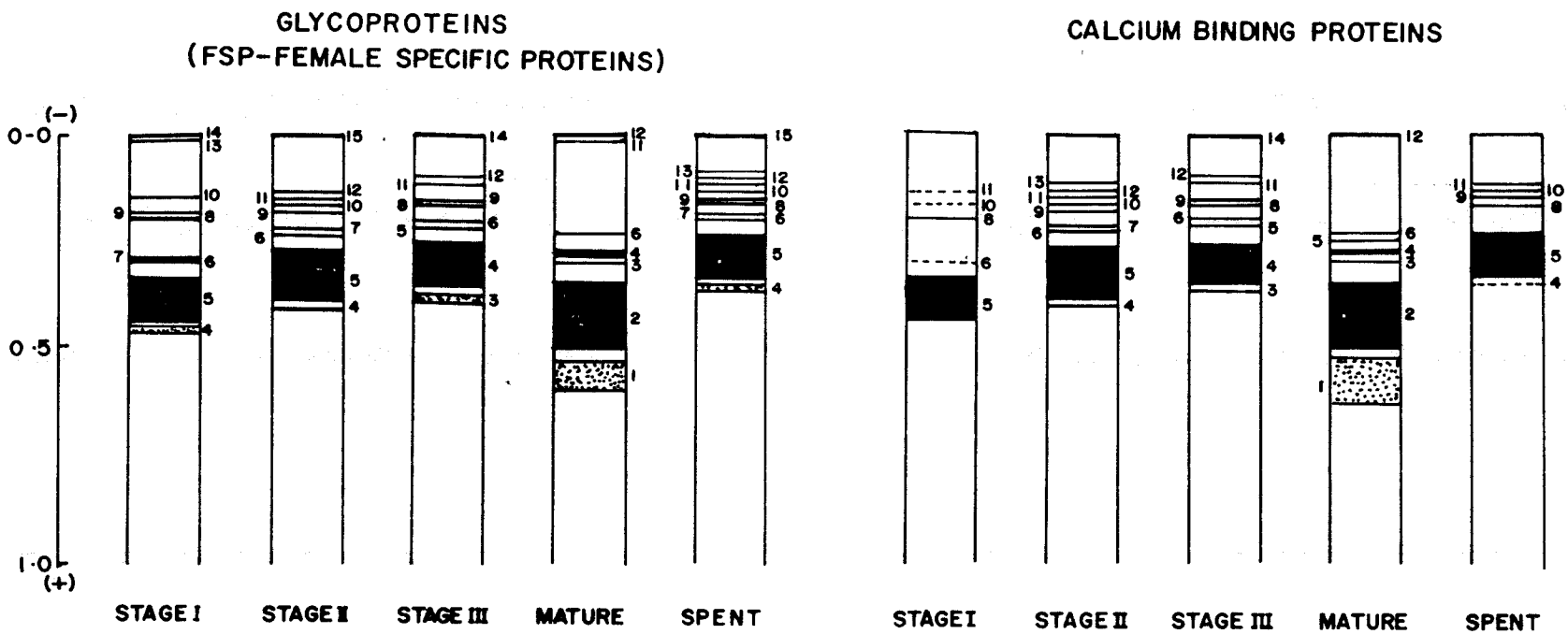

Fig. 10. COMPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH GLYCOPROTEINS AND CALCIUM BINDING PROTEINS OF PENAEUS INDICUS, IN DIFFERENT STAGES OF OVARIAN DEVELOPMENT.

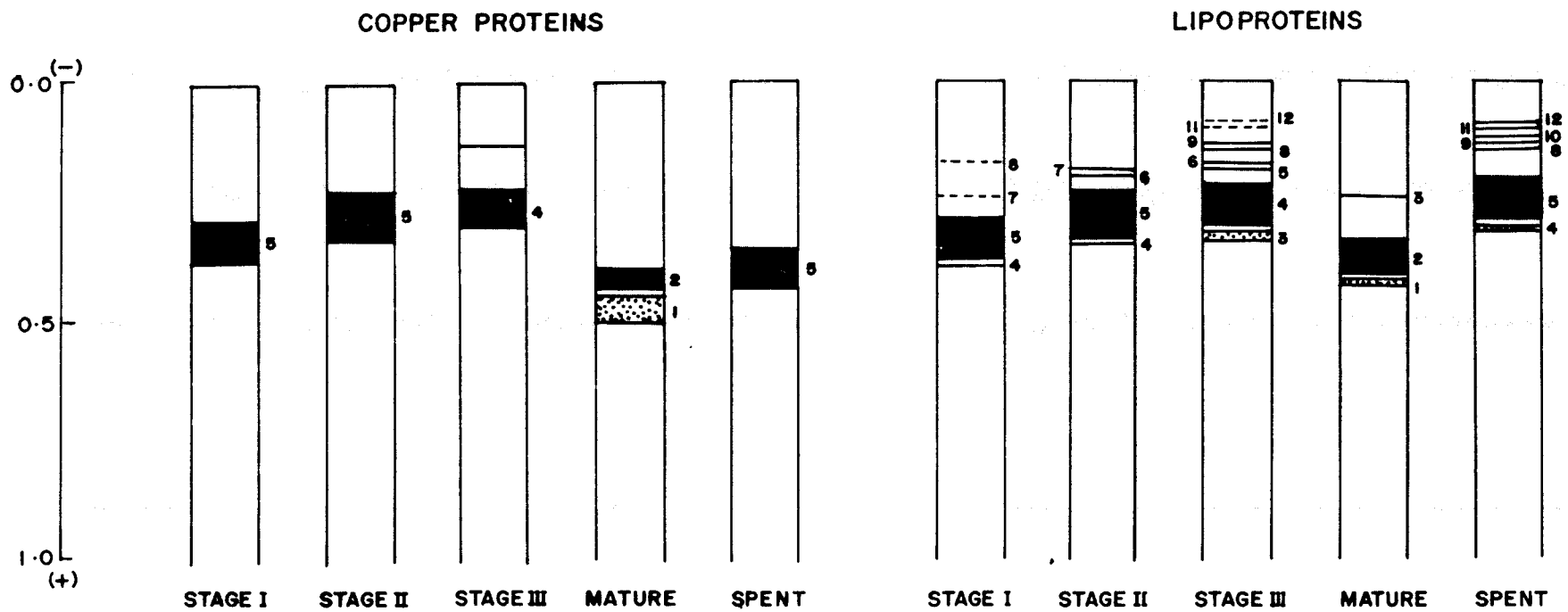

Fig. II. COPARATIVE ELECTROPHEROGRAMS OF HAEMOLYMPH COPPER BINDING PROTEINS AND LIPO-PROTEINS OF PENAEUS INDICUS, IN DIFFERENT STAGES OF OVARIAN DEVELOPMENT.

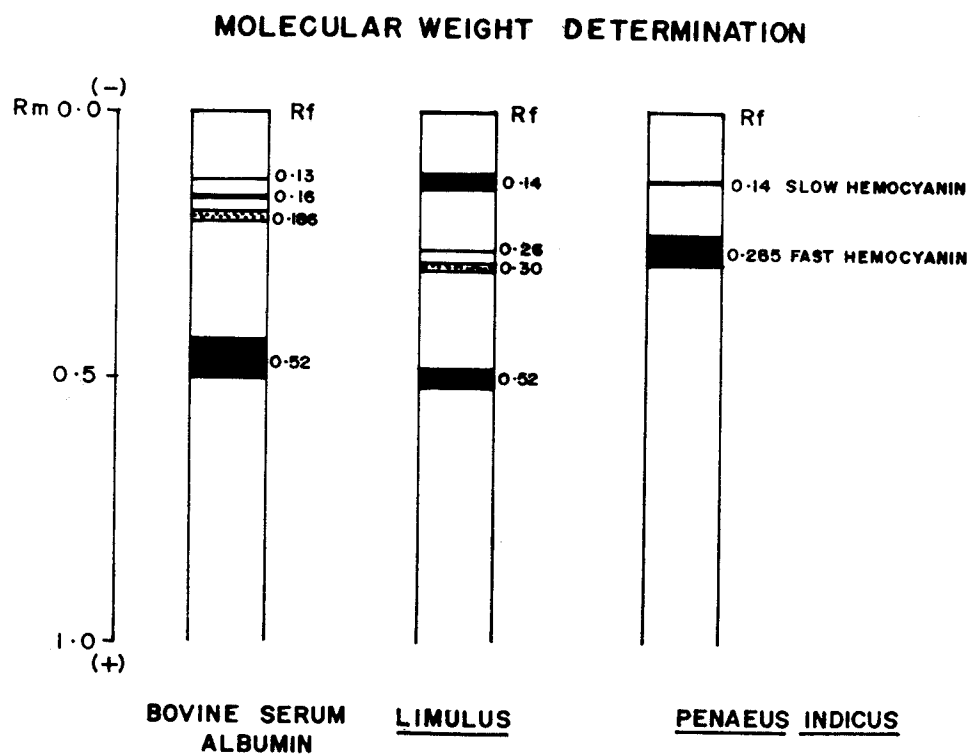

Fig. 12. ELECTROPHEROGRAMS OF BOVINE SERUM ALBUMIN, LIMULUS HEMOCYANIN AND COPPER BINDING PROTEINS (HEMOCYANIN) OF PENAEUS INDICUS, FOR DETERMINATION OF MOLECULAR WEIGHT OF P. INDICUS HEMOCYANIN

Table 1.

Relative mobility (Rf)	Approximate molecular weight in Daltons
Bovine Serum Albumin	
0.13	2,64,000
0.16	1,98,000
0.186	1,32,000
0.52	66,000
Limulus Hemocyanin	
0.14	2,80,000
0.26	2,10,000
0.30	1,40,000
0.52	70,000
Penaeus indicus (Hemocyanin fractions)	
0.14 Slow hemocyanin	2,80,000
0.285 Fast hemocyanin	1,37,183.9

DISCUSSION

Electrophoretic separation of specific proteins has been one of the most useful techniques used to delineate specific biochemical characters. Early studies used paper (Sibley, 1960) or starch gel (Manwell and Baker 1963a) but better separations of proteins have been achieved using polyacrylamide gel disc electrophoresis (Davis, 1964, Ornstein, 1964). This method has been used to describe specific proteins from a variety of tissues including vertebrate eye lens (Calhoun and Koening, 1970), the foot muscles and crystalline style of molluscs (Davis and Lindsay, 1970; Bedford & Reid, 1969) respectively. However, blood has been the most commonly used tissue and comparisons of blood proteins using a wide variety of electrophoretic techniques have been useful in investigating population structure (Lush 1969; Davis and Lindsay, 1970), dimorphic variants (Fielder et al., 1971), hybridization (Manwell et al., 1963a) sibling species (Manwell and Baker, 1963b) and species groups (Guttman, 1970).

In recent years, electrophoresis in stabilizing media, has been widely used for accurate and rapid characterization of crustacean haemolymph proteins. Unfortunately, these results, obtained by different electrophoretic techniques are not easily comparable and often lead to conflicting results. Although data concerning the blood protein composition lack uniformity, they clearly demonstrate the occurrence of an important intraspecific variation of the haemolymph constituents - this may be partly attributed to differences in physiological factors such as sex, season (Uglow, 1969a),

diet (Zuckerkandl, 1957; Uglow, 1969b), Moult cycle (Frentz, 1954; Zuckerkandl, 1956; Chaisemartin et al., 1968; Barlow and Ridgway, 1969; Nusselen, 1970; Chaix et al., 1981) and reproductive cycle (Fielder et al., 1971; Picaud 1971, 1976; Adiyodi and Adiyodi, 1972; Maguire and Fielder 1975a,b; Durliat and Vanckx, 1976; Junera et al., 1979; Nagakawa et al., 1982; Munusamy and Subramoniam 1987; Rankin et al., 1989). In view of this specificity, at least at the generic level, Woods et al. (1958) suggested that the electrophoretic analysis of serum proteins may be useful in certain racial studies, taxonomic problems, considerations of biochemical individuality. This was supported by Manwell and Baker (1963) who reported that although the multiplicity and quantity of the haemolymph components varied from individual to individual, the variations was not so great as to obscure the species specific pattern.

In *Penaeus indicus* by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), of the haemolymph, a species specific pattern was observed, in spite of variations attributable to physiological factors such as size, moult cycle, season, nutritional state etc. Thus, it is in agreement with Woods et al. (1958) and Manwell and Baker (1963a). In *P. indicus* four main components - the slow and fast hemocyanin, heteroagglutinin and fibrinogen are invariably present, in both males and females, irrespective of size, along with other simple protein fractions. the species specificity is indicated by the characteristic relative mobility of the specific slow and fast hemocyanin fractions. In other words, these fractions are observed in other species with relative mobilities characteristic to the particular species. The maximal number of protein fractions recorded

is 16 while the minimum observed is 10. The basic pattern remains the same in all size groups studied. The quantitative variation in the haemolymph protein is indicated by the thickness and intensity of the fast hemocyanin fraction. In general, the least number of protein fractions were observed in the haemolymph of younger prawns (60-80 mm) which increased with size. This change correlated with factors such as size, weight etc., probably corresponds with the change in size and properties of the various molecules. The increase in protein content with increase in size of the species is reflected by the broader and dense fast hemocyanin fraction and progressive occurrence of other simple protein fractions. Absolutely no change occurs to the slow hemocyanin, heteroagglutinin and fibrinogen in either of the sexes. Apparently, the respiratory role of the hemocyanin and role of heteroagglutinin and fibrinogen is significant in *P. indicus* since these remain steady throughout the life cycle of the species. The occurrence or development of vitellogenin, which is considered as the female specific protein in other related species, is observed during vitellogenesis.

In haemolymph protein characterization in crustaceans, it has been observed that, undoubtedly the respiratory pigment - the hemocyanin, is the major component in the haemolymph and constitutes over 50-80% of the total protein content. But a wide variety of electrophoretic technique have been used, resulting in varied and complex patterns such that the true nature and role of the hemocyanin molecule is still not clearly understood.

Qualitative changes in haemolymph protein pattern associated with sex and age of the animal has been recorded by few authors. Among isopods and amphipods, Porcellio laevis, Amadillidium vulgare, Ligia italica, L. oceanica, Armadillo officinalis, Gammarus sp. and Niphargus virei have been subjected to extensive study. Wieser (1965) investigated 15 species of marine and terrestrial amphipods and isopods by cellulose acetate electrophoresis of whole blood samples. In all species, a fast moving hemocyanin fraction with distinct peroxidase activity was observed. Several species specific slow proteins was also observed. A third major fraction represented by the narrow band with no electrophoretic mobility - the fibrinogen, also reported by Manwell and Baker (1963) was observed. In Ligia italica, P. laevis, A. vulgare and Armadillo officinalis, 13, 14, 10 and 10 fractions were identified respectively by PAGE (Sevilla and Lagarrigue, 1973). Only one major fraction is reported in all these species. The almost identical relative mobility (Rf value) and protein content in the four species reflected their systematic correlation as well the functional significance of hemocyanin (Sevilla and Lagarrigue, 1973). Picaud (1971, 1976) by PAGE described in L. italica, 14 fractions and reported that the Proteinogrammes were identical in both sexes. The hemocyanin fraction was not identified and probably represented by the intense staining 9th and 10th fractions. In Ligia oceanica, 15 fractions with one hemocyanin fraction, and 20 fractions in A. vulgare was separated. Picaud (1976) also noted characteristic fractions in the intersexual forms of both species.

Alikhan and Lysenko(1973) by means of PAGE separated the haemolymph proteins of Porcellio laevis, into seventeen stainable fractions,

identifying three hemocyanin fractions, a lipoprotein, a glycoprotein and an esterase. The haemolymph protein pattern of P. laevis belonging to different growth instars clearly indicated a direct relationship between blood protein concentration and age/size/weight of the isopod. The haemolymph of seventh instar nymphs revealed seven fractions, eihth instar showed eight fractions, ninth instar eleven fractions and tenth instar (adult) seventeen fractions. The hemocyanin content also varied correspondingly. Starvation seemed to have a profound effect on the hemocyanin and other fractions. A 15 day starvation period tended to lower hemocyanin content by 10%, while the glycoprotein, slow and fast fractions also disappeared. Alikhan and Akhtar (1980) compared the haemolymph protein pattern by PAGE in P. laevis, A. vulgare and decapod, Orconectes propinquus. Typical patterns of adults revealed three bands for P. laevis, five in A. vulgare and eight in Orconectes. In P. laevis, a fast moving hemocyanin, a glycoprotein and a lipoprotein; in A. vulgare, a fast and slow glycoproteins, fast and slow hemocyanins and a slow moving lipoprotein; in Orconectes, two fast moving lipoproteins, one fast and one slow hemocyanins, one fast and two slow glycoproteins and a slow moving lipoprotein was identified. No major difference was found in male and female patterns except for the concentration of the glycoprotein which was somewhat lesser in the male haemolymph than that in females. The other protein fractions were not identified. Jazdzewski et al. (1975) determined by acetyl cellulose electrophoresis, the protein composition of Gammarus fossarum, G. lacustris, G. roesli (amphipods) Asellus aquaticus and Oniscus asellus (Isopods) and described five major protein fractions - apohemocyanin, hemocyanin,

heteroagglutinin, fibrinogen and glycoproteins. The haemolymph protein pattern of G. lacustris was similar to that of G. roesli but not with that of G. fossarum which seems due to ecological affinity but not of systematic position. In Niphargus virei (amphipod), Gibert (1971), revealed 15-16 fractions by PAGE and reported wide intra-individual variations but the species specific pattern revealed three hemocyanin fractions exhibiting peroxidase activity. Glycoproteins are majority while lipoproteins are few. Kaim-Malka et al. (1983), using PAGE separated 25 fractions in males and 22 fractions in females of Idotea balthica. They recorded characteristic variations for each sex during the moult cycle. Two hemocyanin fractions were identified.

Haemolymph of crayfish and lobsters has been subjected to much immunoelectrophoretic study compared to shrimps. Durliat and Vranckx (1976, 1982) separated the haemolymph proteins of Astacus leptodactylus by various preparative methods and revealed the presence of clottable protein in plasma, which is cathodic in position. By immunological studies it was found to be heterogenous and of large molecular weight, analogous to the fibrinogen like factor. While they demonstrated that the monomer has its origin in the haemocytes, it is also present as an integral part in the plasma. The fibrinogen - like factor identified in *P. indicus* by SDS-PAGE, may be similar in properties and further immunological methods of identification will provide the clue to the true nature of this protein factor. As in crayfish, the fibrinogen in *P. indicus* has its origin in the haemocytes since it initiates clotting within seconds of contact with the syringe, during extraction in the absence of anticoagulant. It is also

presence of two hemocyanins, fast and slow, interconvertible; clotting protein in the serum and less in plasma, and apohemocyanin which had different electrophoretic mobilities to hemocyanin when dialysed. The slow hemocyanin was found almost exclusively in females. Species specificity of the electrophoretic pattern was confined to the characteristic mobility and dissociation of the fast hemocyanin, the only protein found in all samples of the blue crab haemolymph. In *P. indicus* the four fractions namely the fast and slow hemocyanin, the heteroagglutinin and the fibrinogen apparently form the characteristic pattern of the species, all these present in all samples of hemolymph irrespective of size, sex and moult. Modifications of the fast and slow hemocyanin are noticed only during the development of the ovary stressing the significant role of haemolymph in transporting the specific vitellogenin to the developing oocytes. Ceccaldi (1971) revealed by PAGE, in Scyllarus arctus, 14 fractions, of which fractions, 7, 9 and 10 were important quantitatively and fraction 7 was identified as hemocyanin. Ghidalia (1972) analysed electrophoretically, the male serum of Macropipus puber using two selective media followed by ultra-violet absorption of the protein fractions. Two cuproprotein fractions exhibited two absorption bands at 280 nm and 345 nm. At 280 nm, three unequal but distinct peaks are visible and at 345 nm also three peaks are present. Both these have high electrophoretic mobility and are cuproproteins in nature. Certain heterogeneity between the cuproproteins is indicated by the absorption peaks. Such heterogenecity probably exists in *P. indicus* hemocyanin also, which can be proved only by further detailed studies, which was beyond the scope of this study.

Fielder et al. (1971) reported the electrophoretic pattern of the components of hemocyanin from the light patch variant of the crab, Uca pugilator, in which there are 4 components compared to the 3 hemocyanin components in the dark patch variant of the same species in both males and females. The functional significance of this variation is not known. Maguire and Fielder (1975a,b) by PAGE of haemolymph of 12 species of portunid crabs revealed species specific pattern. In each species,the fractions segregated into three distinct regions; neither sex, length of gel or storage had any effects on the mobility or pattern of protein fractions. All the fractions in the lower region was identified as hemocyanin and also stained for PAS. The hemocyanin fractions varied between 3 and 5, thus species specific. Species groups suggested by the pattern bore little similarity to the accepted genera. Fractions in the upper and middle regions reflected high individual variation. Hemocyanin was not affected by the physiological state of the crabs. In *P. indicus*, while sex, and storage of haemolymph did not affect the basic pattern of protein fractions, preliminary standardisations revealed reduced mobility of the protein fractions with decrease in length of gel. The relative mobility of the two hemocyanin fractions in *P. indicus* is high and hence found confined to the upper region. The physiological changes occurring during ovarian development is reflected in the haemolymph protein pattern unlike in Uca pugilator. Baron (1975) by starch gel electrophoresis, separated 12 fractions in Portunus validus, of which 4 main fractions was hemocyanin, fractions 6 and 9 were sex-specific, found only in females. The presence of heteroagglutinin and a fraction similar to prealbumin in human serum was also identified. In *P. indicus* also, the heteroagglutinin and fibrinogen have been identified

and their specific role requires further detailed examination.

Among shrimps, haemolymph characterisation has been very limited. Cuzon and Ceccaldi (1972) by PAGE revealed the presence of 17 protein fractions in Penaeus kerathurus; only one hemocyanin fraction is identified and its role as reserve during starvation seems to be significant. As in *Penaeus indicus* wide individual variation is recorded. In P. monodon also 17 protein fractions were identified in the adult; the number of fractions increased with increasing size and no marked difference in the male and female haemolymph protein was observed. The hemocyanin fractions were not identified (Prathibha, 1984). In *P. indicus* 16 fractions are revealed by SDS-PAGE, in the adult; also the number of fractions increased with size as in the case of P. monodon. In Squilla mantis, Ferrero et al. (1983) by SDS-PAGE, revealed 16 major proteins and quantitative variation of individual fractions. The hemocyanin molecule is reported to be homogenous represented by one fraction. In *P. indicus* 2 hemocyanin fractions exist in the nature state; in P. monodon, possibly 3 exist (Ellerton and Anderson, 1981) and P. kerathurus only one fraction is revealed (Cuzon and Ceccaldi, 1972). These point to the possible polymeric existence of the hemocyanin fraction. The pH dependence of the hemocyanin molecule is also to be taken into consideration. At pH higher than 8.0, the molecule yields different subunits, which are electrophoretically different from native hemocyanin. As most electrophoretic studies cited above were done at pH above 8.5, by varied electrophoretic techniques, the variability and complexity of the reported patterns are probably due to the dissociation of the hemocyanin molecule as well as other proteins.

Quantitative variations in the serum protein content, during the moult cycle has been recorded in several crustaceans. In almost all crustaceans, the total serum protein content usually builds up before the moult, is diluted at the moult, fluctuates during calcification and remains constant throughout the intermoult period. Qualitative variations such as development and disappearance of specific moult related fraction in the haemolymph proteins during the moult cycle has been documented in few crustaceans (Zuckerkandl, 1956; Barlow and Ridgway, 1969; Busselen, 1970; Djangmah, 1970; Picaud et al., 1974; Vranckx and Durliat, 1976; Herberts et al., 1978; Chaix et al., 1981). In *P. indicus*, haemolymph protein characterization by SDS-PAGE during moult cycle did not reflect any significant qualitative variation in the protein pattern. No specific or characteristic moult-related fraction could be traced throughout the moult cycle.

Barlow and Ridgway (1969) by starch gel electrophoresis and immuno-diffusion techniques, observed a relative shift in the concentration of certain antigens in lobster, Homarus americanus sera with changes in the moult cycle. Both sexes possessed an antigen that disappeared about one month after moult and appeared again in about four weeks. Busselen (1970) by means of cellulose acetate electrophoresis of haemolymph proteins of C. maenas traced the protein pattern during moult cycle. Moulting causes a four to five fold decrease in the concentration of hemocyanin, premoult levels are progressively restored during intermoult. At ecdysis, the glyco-protein fraction I, disappeared completely and reappeared after 10 days. The concentration of fraction I is highly dependent on the nutritional status.

Starvation causes complete disappearance of the glycoprotein. During a period of excessive feeding, fraction I accumulates in the blood. This is reflected during the obligatory starvation that takes places during the moult cycle. In Maia squinado, the hemocyanin fraction disappeared during premoult and postmoult stages and reappeared during intermoult (Zuckerkandl, 1956). In C. mediterraneus immunochemical analysis revealed two groups of fractions at the end of intermoult which increased at beginning of D_0 and became magnified as ecdysis approached. An addition fraction was found in the haemolymph during intermoult and D_0 only;which disappeared by the time of the appearance of two new hypodermal fractions (Herberts et al., 1978). Vranckx and Durliat (1976) by two dimensional electrophoresis of Astacus leptodactylus haemolymph recorded variations during moult cycle. Of the 8 fractions observed, which included hemocyanin and fibrinogen in serum and plasma, fraction II showed a characteristic pattern. It was stable during C_4, increased steadily from D_0 to D_1'' and then decreased upto D_3 and postmoult A. In spider crab, Acanthonyx lunulatus, two dimensional electrophoresis revealed a new polypeptide different from other fractions present in D_0, D_1, D_2, and D_3 stages. This fraction appears in the haemolymph when the ecdysone titer increases (Chaix et al. 1981). In *Penaeus indicus* by SDS-PAGE, no polypeptide corresponding to those observed in other species could be detected during the moult cycle. Quantitative variations registered during the moult cycle did not reflect a corresponding qualitative change in the haemolymph protein pattern. Intra-individual variations, combined with variations due to factors such as size, season, moult stage etc. resulted in quantitative variation of the fast moving

hemocyanin fraction only. Specific moult-related proteins could not be observed. It is suggested that in lobsters, crayfishes and crabs, the moult cycle is an extended one (almost one year) and leads to synthesis of specific moult related proteins. Also, the appearance and disappearance of particular polypeptides could be attributed to the obligatory starvation prior to ecdysis. In shrimps, particularly penaeids, the moult cycle is comparatively shorter (15-20 days) and quantitative variations observed are due to dilution of blood proteins by water uptake prior to ecdysis, rather than an increase or decrease in protein content in the haemolymph and hence does not reflect qualitatively. It is also probable that in *P. indicus* more refined electrophoretic techniques such as two dimensional immunoelectrophoresis may aid in detection of particular polypeptides, if any, involved in the moulting process, which might have escaped detection in SDS-PAGE.

Vitellogenin (VG) a blood serum precursor of the egg yolk protein, is secreted into the haemolymph during the process of vitellogenesis. This circulating ovarian precursor is otherwise known as Female Specific Protein (FSP). When vitellogenin enters the oocytes, it is known as vitellin or lipovitellin (LV). In Crustacea, several authors have determined vitellogenin in the haemolymph which is known to vary during the reproductive cycle (Ceccaldi and Martin, 1969; Horn and Herr, 1969; Fielder et al., 1971; Fyffe & Connor, 1974; Derell et al., 1986; Suzuki, 1987; Nelson et al., 1988). In *Penaeus indicus* the presence of vitellogenin in the haemolymph could be detected during the different stages of ovarian maturity. The female haemolymph revealed the presence of vitellogenin in the early

developing stages (I & II) and increased in concentration in stage III, indicated by increased intensity and disappeared partially during the fully mature stage and reappeared in the spent stage although with reduced intensity. These fractions stain for PAS and Oil Red O confirming their glyco-protein nature. The pattern strongly reflects the mode of utilization of lipovitellin which is believed to be synthesized by the ovary and rarely by extraovarian tissues and through the haemolymph in the form of vitellogenin, is transferred to the developing oocytes. Thus, in the fully mature stage, the vitellogenin is absorbed by the developing oocytes as lipovitellin, thereby resulting in the altered pattern of haemolymph FSP in the fully mature stage. The reappearance of FSP in the spent stage (although decreased level) indicates the resorption of vitellogenin by the extraovarian tissues.

Studies on other crustaceans, including decapods, have revealed one or more ovarian lipovitellins with immunologically corresponding haemolymph vitellogenins (Wolin et al., 1973; Fyffe and Connor, 1974; Durliat, 1984; Nelson et al., 1988). The electrophoretic pattern of haemolymph and ovarian extract of Pandalus kessleri on SDS-PAGE revealed two strong bands in both cases. The molecular weight of the strong bands was estimated to be 68-80 KD for female haemolymph and 78-110 KD for ovarian extract (Quinitio et al., 1989). In Parapenaeopsis longirostris, Tom et al. (1987) reported one protein band in the purified vitellin. However, in Armadillidium vulgare four forms of vitellin and vitellogenin was identified (Suzuki, 1987). In Porcellio dilatatus, a reaction of complete identity was demonstrated by immunodiffusion between vitellin and vitellogenin. The molecular weights of the two FSP was determined to be 315,000

and 3,77,000 daltons respectively (Picaud, 1978a,b). The presence of FSP in the haemolymph of females with mature ovary was demonstrated by PAGE in L. oceanica and O. gammarella (Picaud 1971; Junera et al., 1974). In Penaeus kerathurus three proteins seem implicated in the vitellogenesis process (Ceccaldi, 1970). In Macrobrachium rosenbergii, purified vitellin gave a positive reaction by ELISA titration with vitellogenin also. The molecular weight of vitellogenin and lipovitellin was found to be 92,200 and 84,000 daltons respectively (Derelle et al., 1988).

Nakagawa et al. (1982), revealed by PAGE, in the freshwater prawn Palaemon paucidons, the presence of three lipoprotein fractions in the haemolymph during egg formation, but its electrophoretic mobility did not correspond to that of vitellin in the ovary. The molecular weight was found to be approximately 1000 x 10^3. Munuswamy and Subramoniam (1987) by PAGE revealed the appearance of FSP (glycoproteins) in stage III of maturity and mature stage in the fairy shrimp Streptocephalus dichotomous In Squilla mantis, SDS-PAGE revealed three female specific protein fractions in the haemolymph and was considered vitellogenins since their amount increased with ovarian maturity. The molecular weights was around 12 Kdal (Ferrero et al., 1983). In Homarus vulgaris, cellulose acetate electrophoresis and immunodiffusion revealed a female specific component in the haemolymph during ovarian development (Barlow & Ridgway, 1969). In Homarus americanus, the presence of two immunochemically related VGS, were detected, corresponding to an ovarian lipovitellin. As vitellogenesis progresses, VG_1 appears to change slowly to VG_2 (Nelson et al., 1988).

Female specific proteins associated with ovarian development have been detected in the haemolymph of crabs too. Horn and Kerr (1969) detected a lipoprotein in female sera of Callinectes sapidus. In Uca pugilator Fielder et al. (1971) found the FSP in haemolymph of females with developing oocytes and the lipoprotein was detected in both, the light and dark patch variants. Maguire and Fielder (1975) identified a glycoprotein which increased in the haemolymph as ovarian development progressed in portunid crab, Portunus pelagicus, but it occurred in few males also and hence cannot be considered as FSP. In the hermit crab, Clibanarius clibanarius, Varadarajan and Subramoniam (1982) by PAGE revealed the development of FSP in the haemolymph during vitellogenesis. In the haemolymph, three fractions (3,4,5) separate discretely in ovarian stages I and II of which fraction 5 is the only PAS staining fraction in stage II and disappearing in stage III, when its full intensity is displayed in the ovary. During resorption the pattern is the same as in stage III and resembles the male haemolymph pattern. This is observed in *P. indicus* also, wherein the FSP (fractions 4, 7, 8 and 9) disappears in the fully mature stage (IV) although partially and reappears in the spent stage (V) probably for the next cycle. Further immunological studies may reveal its immunological identity with lipovitellin in the ovary.

In *Penaeus indicus*,the copper-bearing proteins-haemocyanins behave differently during the ovarian developing stages. In this case, the two copper-binding components, which are present in all individuals examined, irrespective of sex, size and moult stage, exhibit a peculiar pattern during the vitellogenic process. Only one component, the fast moving component

is present in stage I, II and III of ovarian maturity. In fully mature individual, the pattern is slightly different, with two broad, diffuse components, one with same mobility as seen in normal cases, and the other with greater mobility than the first component. In the spent stage, the two components as seen in normal case is observed. This phenomenon is not recorded in any other species. Wache et al. (1988) studied the structural changes of hemocyanin during early development in the crab, Cancer productus and found two hexamer hemocyanin in the oocytes which have structure exactly same as the adult hemocyanin. She suggested that the oocyte probably takes up the maternal hemocyanin through endocytosis. Probably this is the case in *P. indicus* too. The developing oocytes may be absorbing the maternal hemocyanin by endocytosis. It will be of interest to know which fraction, the slow or fast hemocyanin is involved in this function. Perhaps the hemocyanin functions to facilitate oxygen diffusion to the metabolizing oocytes. Similar occurrence of hemocyanin in early development stages has been reported in Eurypelma californicum (Kempter, 1986); Astacus leptodactylus (Durliat, 1984); C. maenas (Busselen, 1971) and Tachypleus tridentatus (Sugita and Sekiguchi, 1979). The presence of hemocyanin in the oocytes of *P. indicus* has not been elucidated, and if present, it will be of interest to compare the functional properties of the embryo hemocyanin with those of adult hemocyanin, as has been reported in C. magister. Megalopa and juvenile hemocyanins of C. magister, differ from adult hemocyanins in that they lack one of the adult polypeptide chains and relative concentrations of the other chains are not the same as in adult protein. These structural changes result in striking changes in functional properties - purified hemocyanins from megalopa and juveniles

of C. magister have about 50% lower oxygen affinity than adult hemocyanins (Terwilliger and Terwilliger, 1982; Terwilliger et al., 1986).

The female specific protein (vitellogenin) is a complex protein staining positive for calcium. The increase in calcium binding proteins during vitellogenesis stresses the role of calcium in transporting vitellogenin to the developing oocytes through the haemolymph. There is no record of qualitative estimation of calcium binding proteins during vitellogenesis, in the haemolymph, in other species, although the role of calcium in transporting vitellogenin to the developing oocytes is well recorded. In *P. indicus* almost all of the haemolymph proteins are of high molecular weight including the lipoprotein fractions, as apparent by their low relative mobility.

MOLECULAR WEIGHT DETERMINATION

Hemocyanin is undoubtedly the major component in the haemolymph, although not the only one, in almost all crustaceans studied to date. It is a complex protein composed of several subunits or polypeptides. The molecular weight of hemocyanin in crustacean ranges from 400,000 to 9 million daltons. The subunit molecular weight is between 66,000 to 75,000 daltons, each subunit containing two copper atoms which can bind one molecular oxygen (Van Holde and Van Bruggen, 1971). The subunit or polypeptide is the minimal functional unit and the oligomeric forms are made up of 6, 12, 24 and 48 subunits in decapod crustaceans (Rochu et al., 1978). In nature, the aggregation level is species specific (Ghiretti, 1968; Van Holde and Van Bruggen, 1971; Van Holde and Miller, 1982; Van Bruggen et al., 1982; Ellerton et al., 1983).

Arthropod hemocyanin particles dissociate at alkaline pH into their subunits, which are still capable of reversibly binding oxygen. Among crustacea, detailed investigations on hemocyanin have been almost exclusively limited to the larger decapods (Redmond, 1971). Extremely flexible in function, arthropod hemocyanins compensate for environmental and physiological changes during the life cycle of the organism (Mangum 1980, 1983) and it has demonstrated flexibility in the evolution of crabs, crayfishes shrimps, isopods, scorpions, spiders, each adapted to a special aquatic or terrestrial situation (Markl, 1986).

Native hemocyanin has been shown to be heterogenous in its susceptibility to dissociation by salt and pH. Differences have also be observed in the electrophoretic behaviour of undissociated as well as dissociated hemocyanin molecules (Horn and Kerr, 1969; Decleir and Richard, 1970; Jeffrey and Treacy, 1982). Whether this heterogeneity is due to differences in primary structure of hemocyanin subunits or to variability in conformation of subunits or aggregates has not been conclusively established at the level of the smallest oxygen binding unit. In *P. indicus* two hemocyanin components of slightly different mobilities, which differ in molecular weight by almost 50%, has been detected by polyacrylamide gel (5%) electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). The two components stain for rubeanic acid. SDS-PAGE is a method which separates proteins on the basis of their molecular weight. Calibration of gels with proteins of known molecular weight yielded values of approximately 2,80,000 daltons and 1,37,184 daltons for molecular weights of the slow and fast hemocyanin components respectively. The two components are not present in equal

amounts. Similar results are obtained, whether or not, the sample is heated in the presence or absence of mercaptoethanol. But preliminary standardisations revealed the effect of increased pH on the hemocyanin components resulting in dissociation productions and altered mobility. Varying gel concentration from 6 to 10% reduced the mobility reflecting the high molecular weights of the components. Thus, the native stage of *Penaeus indicus*, hemocyanin occurs in the form of two components of differing mobilities in SDS-PAGE at pH 8.0 when almost all hemocyanin molecules remain in the native aggregation state. The two components were present in all individuals irrespectively of sex, size (60-140 mm) or physiological condition except during the period of vitellogenesis wherein the slow hemocyanin component disappeared and reappeared after spawning. The functional significance of this phenomenon is inexplicable at the moment.

Molecular weights of hemocyanin molecules and subunits, in crustacea has been determined by several authors by several methods - ultracentrifugation, SDS-PAGE, immunoelectrophoresis, isoelectric focussing and immunochemical methods. These methods (although there exists diversity of opinion) have established the existence of subunit heterogeneity, the specific antigenic determinants as well as common antigenic determinants in the hemocyanin molecules of decapods. The heterogeneity of hemocyanin subunits could be due to variable carbohydrate content, single amino acid substitution, incomplete denaturation and differing degrees of detergent binding, all of which may influence the apparent molecular weight (Brenowitz et al.1981) The molecular weight of the aggregate form of hemocyanin has been determined in several species, 9,50,000 daltons in Cancer magister (Ellerton

et al., 1970); 7,50,000 dalton in Eriphia spinifrons (Di Giambernardino, 1967) 6,10,000 daltons for Cancer pagurus (Boone et al., 1983); 8,25,000 daltons in Homarus vulgaris (Lauffer and Swaby, 1955) 4,71,000 daltons by ultacentrifugation in Penaeus monodon (Ellerton and Anderson, 1981). By PAGE, they determined three monomers of 82,000; 91,000 and 1,07,000 daltons and the main monomer 1,76,000 daltons in P. monodon. Fielder et al. (1971), in Uca pugilator, have shown the existence of many forms of rapid hemocyanin, the number of which changes according to the morphological variants. In *Penaeus indicus* the molecular weight of the two hemocyanin components is comparatively less, being 2,80,000 daltons and 1,37,814 daltons respectively.

The subunits of hemocyanin in Decapods have molecular weights of 70,000 - 80,000 daltons; 75,000 - 80,000 in Cancer magister (Ellerton et al., 1970) 75,000 - 84,000 for two other estimates for the same species (Carpenter and Van Holde, 1973; Loer and Mason, 1973); 80,000 for Eriphia spinifrons (Di Giambernardino, 1967); 76,000 - 78,000 for Homarus americanus (Waseman, 1975); 78,000 for Neptunus validus, 80,000 for Macropipus puber, 82,000 for Panulirus regius (Lambin et al., 1976); 60,000 in Idotea balthica (Kaim-Malka et al., 1983); 76,000 for Panulirus regius (Rochu et al., 1978), 75,000 in P. interruptus (Gaykema et al., 1984), 70,000, 77,000 and 85,000 in P. monodon (Ellerton and Anderson, 1981). The subunits of hemocyanin of Limulus polyphemus also have same characteristics. (Sullivan et al., 1976; Bremowitz et al., 1981). It is highly probable that the subunits of *P. indicus* hemocyanin are also of similar composition and magnitude as in other decapods. The subunits may be heterogeneous

as reported in P. monodon, a closely related species. Further immunological analysis is worth being conducted to elucidate the subunit structure and molecular weight.

The mechanisms of cooperative oxygen binding, Bohr effects and the anionic regulation of the allosteric constants, must all result from specific subunit interactions (Brenowitz et al., 1981). Attempts to study the subunit structure and molecular weights of these subunits has been confined to the larger decapods, Limulus polyphenus and few oniscoides. (H. americanus - Lauffer and Swaby, 1955); (C. magister - Loer and Mason, 1973); (Cherax destructor - Murray and Jeffrey, 1974); (Porcellio laevis - Alikhan and Lysenko,1975) (Callianassa californiensis - Roxby et al., 1974); (C. magister - Carpentor and Van Holde, 1973); (Oniscoides - Sevilla 1978; Sevilla and Lagarrigue, 1979); (Limulus - Brenowitz et al., 1981) Cancer pagurus - Rochu & Fine 1980, 1984a,b) (Macropipus puber - Ghidalia et al., 1985). The studies on the oxygen binding properties of hemocyanin in crustacea have established the influence of several factors such as pH, presence of divalent cations (Ca^{2+} and Mg^{2+}) and temperature. Chantler et al. 1973; Miller and Van Holde, 1974, 1981ab, b) Roxby et al., 1974; Bonaventura et al., 1975; Mangum et al. 1976; Sevilla, 1977; 1978; Ellerton et al., 1977; Klarman and Daniel, 1977; Wacjman et al., 1977; Freel, 1978; Sevilla and Lagarrigue, 1979; Mason et al., 1983; Durliat, 1983). Such studies on the hemocyanin of *P. indicus* may throw light on the functional, adaptive and systematic significance on the two hemocyanin components present. Analysis of the hemocyanin subunit structure by

ultracentrifugation, immunochemical, amino-acid analysis, immunodiffusion and electron microscopy techniques will further explain the functional significance of the hemocyanin molecule.

CHAPTER III

HAEMOCYTE IDENTIFICATION AND CLASSIFICATION

INTRODUCTION

Crustaceans, like other Arthropods, have an open circulatory system. For this reason, the blood has been termed 'haemolymph'. The haemolymph of crustaceans carries numerous non-pigmented cells which was termed 'Haemocytes' for the first time by Carus in 1824, instead of lymphocytes or leucocytes, to prevent any confusion with analogous blood cells of vertebrates. Since then, studies on crustacean haemocytes have been directed along two main lines of investigation viz - characterization of different morphological types of cells and the determination of their particular role in haemolymph clotting.

Early attempts to establish a convenient classification of haemocytes were largely unsuccessful due to lack of classical guidelines in classification schemes as well as due to lack of proper sampling procedures. While some authors have adopted morphological criteria others have resorted to functional properties of the haemocytes resulting in conflicting accounts. Haemocytes are very reactive cells which undergo considerable transformations when removed from the hemocoel such that lack of sufficient caution in sampling leads to ambiguous results and discrepancies in classification. More recent classifications have tried to overcome these shortcomings by attempting to define morphological types, both, at the light and electron microscopic levels and subsequent biochemical and physiological characterization.

Since an open circulatory system is more susceptible to repeated intrusions from the surrounding environment, interest in haemocytes has been revived, and enlarged because of their implication in wound-repair and defense mechanism against parasites like fungi, viruses, rickettsiae, bacteria protozoa, helminth and even other crustaceans. Considerable progress is now also being made in crustacean pathology and immunology.

Crustacean haemocytes, as mentioned earlier, are known under different names, but at least three broad classes of haemocytes have been recognized. Hyaline cells are the smallest of the three with large central nucleus surrounded generally by basophilic cytoplasmic fringe. Cytoplasmic inclusions are scarce or few. Granulocytes are the largest cells with small occentric kidney-shaped nuclei. A Golgi is present most of the time. Dominant smooth and rough endoplasmic reticulum are present. Free ribosomes are scattered in the cytoplasm literally filled with large membrane-bound granules Semi-granular cells are an intermediate type between the hyaline and large granulocyte. They are intermediate in size, have a central or eccentric or spherical or lobed nucleus. They also contain free ribosomes, smooth and rough ER, more developed than in other two types and two or more Golgi producing a fairly large number of eosinophilic granules sparsely distributed in the cytoplasm. These three main types apparently form a continuous differentiation series with many intermediate forms. Besides these, lipoprotein cells and cyanocytes have been identified in some crustacea by few carcinologists (Sewell, 1955 and Fahrenbach 1970).

The haemocytes of crustaceans are reported to participate in numerous physiological activities such as the production of hemocyanin

(Stang-Voss, 1971), Storage of glycogen (Johnston et al. 1973), ecdysis (Bauchau and Plaquet, 1973; Vacca and Fingerman 1975, 1983)& hemostasis (Bang 1970, 1971; Wood et al., 1971; Bauchau and De Brouwer, 1974). Further, they are of known importance in the defense reactions of crustacea towards invasive organisms (Rabin, 1970; Sinderman, 1971; Tyson et al., 1974; Fontaine and Lightner, 1975; Stewart, 1975; Paterson et al., 1976) which includes haemolymph coagulation and wound repair, phagocytosis, encapsulation and nodule formation. The presence and involvement of 'fibrinogen' in blood coagulation and the occurrence of heteroagglutinin which might aid in recognizing non-self has been reported in this context.

Hardy (1892) confirmed the presence of two blood cell types in the crayfish Astacus sp, reported earlier by Halliburton (1885). The first cell type was the explosive corpuscle, a spindle-shaped cell, highly thigmotactic; the other was the eosinophilic corpuscle, an avoid amoeboid cell, with granules and vacuoles in the cytoplasm. Hardy also reported the release of a clotting substance, similar to fibrin which is responsible for coagulation of plasma and further initiation of lysis in other explosive corpuscles. Yaeger and Tauber (1935) investigated the haemolymphs of 26 different species of marine invertebrates which included several crustaceans and reported the total haemolymph cell counts for each. George and Nichols (1948), on the basis of study of several species of decapods and few isopods reported the presence of lymphoid cells (lymphoblast and monocyte types), granular thigmotactic phagocytic cells, cells with large refractile eosinophilic granules and cells with small refractile granules and discussed the functions of these cells. Toney (1958) using phase and dark contrast microscopy

investigated the blood elements of Homarus. He also reported four types of cells as reported by George and Nichols (1948).

The clotting process and the internal defence reactions has been reviewed by several workers - Levin (1967); Rabin (1970); Sinderman (1971); Ravindranath (1980) and Bauchau (1986). In Limulidae, the factors involved in coagulation process are located inside the amoebocytes while in Decapoda they are found both in the cell-free plasma and haemocytes. A type of cellular coagulogen is reported in Decapods which is converted to a gel by serine protease clotting enzyme(Bauchau ,1986).

Dumont et al. (1966) attempted to study the characteristics of haemocytes of Limulus polyphemus during clotting. Kenny et al. (1972) investigated the aggregation of Limulus amoebocytes and reversible inhibition of aggregation by EDTA. Pistole and Britko (1972) and Furman and Pistole (1976) investigated the bactericidal activity of haemolymph from Limulus polyphemus and established that the serum bactericidal factor was released from the circulating amoebocyte during clotting since there was no activity in the 'plasma' portion of the blood. These studies were confirmed later by Johnson et al.(1973) and Murer et al.(1975) who established that blood of Limulus contains only one type of cell - the amoebocyte and the granules packed in the cytoplasm contain the clottable protein. Armstrong (1979, 1980 and 1982) studied the motility, adhesion and spreading of blood cells on artificial surfaces and endotoxin-induced degranulation of the Limulus amoebocyte.

Further investigation on the fine structure of amoebocytes and their role in coagulation was performed by Copeland and Levin (1985), Levin (1985a) and Tablin and Levin (1988). Holme and Solum (1973) conducted electron microscopy of the gel protein formed by the clotting of Limulus haemocyte extracts and concluded that the helical structure of the gel protein is stable over a wide range of pH and ionic strength. Nakamura et al.(1976) also studied the structural change of coagulogen polypeptide chain during gel formation. Sishikura et al. (1977) described and localized the clottable protein in two types of haemocytes in the Japanese horse shoe crab, Tachypleus tridentatus.

Among Isopods and Amphipods, haemocyte identification has not attracted much interest. Ravindranath (1974) studied the haemocytes of isopod Ligia exotica and described seven classes of haemocytes with distinct lineages while Benjamin and James (1987) conducted both light and electron microscopy of the haemocytes of the same species and proposed three cell types - hyaline, semi-granulocyte and granulocyte. This has been supported by recent study on Armadillidium vulgare (Dappen and Nickol 1981) as well as the hypothesis of Bauchau (1981). Hoarau (1976) has described four cell types of following ultrastructure study of blood cells in the Isopod Helleria brevicornis.

Almost all information on crustacean haemocytes has come from studies on Brachyurans. Bauchau et al. (1973, 1974, 1975, 1983) have conducted extensive studies on the haemocytes of Decapods. Bauchau and Brouwer (1972) studied the ultrastructure of haemocytes of Eriocheir sinensis

and described three major cell types - hyaline cell, semi-granulocyte and granulocyte. A fourth form, lipoprotein haemocyte detected in the haemolymph of mature females was also reported. Bauchau and Plaquet (1973) performed total and differential haemocyte count during the intermoult cycle and suggested their role in haemostasis and synthesis of new integuments in E. sinensis. Bauchau and De Brouwer (1974) studied haemolymph clotting in E. sinensis and Carcinus maenas by light and electron microscopy and revealed a two step reaction - direct agglutination of haemocytes and plasma gelation accompanied by release of material from hyaline and semigranulocyte. Bauchau et al. (1975) subjected the haemocytes of E. sinensis and C. maenas to different cytochemical reactions in order to bring out the nature of the granule's content, either by specific coloration or by enzymatic digestion. Bauchau and Passelco-Guerin (1983) subjected blood cells of E. sinensis, C. maenas and Homarus americanus to Cytochalasin B (known to interfere in a fairly specific way with normal cytoplasmic capabilities) and proved that morphological changes initiated by Cyto B are somehow related to the microtubule framework.

William and Lutz (1975) examined haemocytes of C. maenas by a variety of stains and distinguished two major cell types and discussed their role in carbohydratee mechanism. Evan (1972) described the free amino acid pool of C. maenas haemocytes. Ghiretti-Magaldi et al. (1972a,b and 1977) investigated the ultrastructure of haemocytes of C. maenas and reported the presence, origin and evolution of cyanoblasts and cyanocytes in the haematopoietic tissue. Chassard -

Bauchau and Hubert, 1975) described the ultrastructure of haemocyte present in the Y-organ of C. maenas. Smith and Ratcliff (1978) studied the host-defense reactions of C. maenas haemocytes in vitro and proposed that the phagocytic cells may provide a first line of defence against infection augmented by other defences. Smith and Soderall (1983) studied the effects of β-1-3-glucan activation of crustacean haemocytes in vitro and in vivo to determine the role of prophenoloxidase activating system in the defence reaction of C. maenas and Astacus astacus (crayfish) and suggested that certain proteins of the prophenol oxidase activating system may serve as opsonins and possibly constitute an important recognition system in crustaceans. Richard et al. (1980) attempted to explain the mechanism of carapace repair in C. maenas. Sewell (1955) and Paulus and Laufer (1987) described the presence of Lipo-protein cells in the blood of C. maenas and Libinia emarginata associated with moulting and vitellogenesis. Mix and sparks (1980) conducted differential counts and described three cells types in Cancer magister . Vacca and Fingerman (1975a,b, 1983) described the mechanism and role of haemocytes in tanning during the moulting cycle of fiddler crab Uca pugilator, based on histochemical study. Ravindranath (1975a,b) described the haemocytes of Emerita asiatia and studied the effect of pH and temperature on the morphology and coagulation process.

Bodammer (1978) described the ultrastructure of hyaline, intermediate cell and granulocyte of blue crab Callinectes sapidus while Wynder and Stanley (1973) described the haemocytes to be

of one basic type. Sizemone and Davis (1985) discussed the presence of Vibrio sp. in the haemolymph of C. sapidus . Ghidalia et al. (1977) studied the role of haemocytes and plasma in the haemolymph coagulation in Macropipus puber, while Madaras et al. (1979) investigated coagulation of haemolymph of sand crab, Ovalipes bipustulatus.

Tait and Gunn (1918) conducted the first study on crayfish blood, Astacus fluviatilis, with special reference to coagulation and phagocytosis. Wood and Visenten (1967) and Wood et al. (1971) conducted cytochemical observations on haemolymph cells of Orconectes virilis and described hyaline, small granulocyte and large granulocyte, and also demonstrated the presence of 'fibrin ferment' described by Hardy (1892). Unestam and Nylund (1972) described blood reactions in vitro in crayfish against a fungal parasite. Stang-Voss (1971) described cell types in Astacus astacus and suggested that the granules are the storage sites of hemagglutinin. Miller et al. (1972) characterized an agglutinin in the haemolymph of the freshwater crayfish Procambarus clarkii. Fontaine (1975) recorded observations on the wound repair process in Procambarus sp. Durliat and Vranckx (1976, 1983) identified a fibrinogen - like factor in the haemolymph of Astacus leptodactylus and described cellular and plasma coagulation. Sternshein and Burton (1980) using light and electron microscopy described three haemocyte types in Procambarus sp. and Orconectes sp.

Stewart et al. (1967) devised an electronic method for counting of lobster haemocytes, Homarus americanus and studied the influence of diet on haemocyte numbers. Hearing (1969) demonstrated acid phosphatase activity in the granules of blood cells of H. americanus while Mengeot et al. (1977) isolated the granules by a degranulation process in a hypotonic solution. Stewart and Zwicker (1972) and Cornick and Stewart (1978) performed differential count, classified and studied associated agglutinin activity of haemocytes in H. americanus. Paterson et al. (1976) and Goldenberg et al. (1984) studied phagocytosis in H. americanus. Vranckx and Durliat (1977) performed haemolymph withdrawals on different species of lobsters (Jasus frontalis, Jasus paulensis, Panulirus regius, H. vulgaris, Astacus lepto-dactylus) and brachyurans (Cancer pagurus, Maia squinado Xantho incisus) and anomuran (Galathea strigoes), with different anti-coagulants and stated that all circulating cells belong to a single lineage - the granulocyte. Cohen et al. (1983) examined blood cells of H. americanus for the presence of marginal bands of microtubules and their role in cell lysis. Hose et al. (1980) examined the morphology, cytochemistry and cell functions of haemocytes of H. americanus, Panulirus inter-ruptus and Loxorhynchus grandis and suggested a classification of these cells.

Haemocyte studies on penaeids has recently received much focus, due to their significnce in culture. Severe pathological

conditions arising out of intensive culture conditions has led to efforts to study the defence responses of these species. De backer (1961) studied the role of haemocytes in defence by inflammation of tissue in Palaemonetes varians, Dall (1964) distinguished three cell types, lymphocyte - like cell thigmocyte and a granular amoebocyte and discussed their role in carbohydrate metabolism. Fontaine and Lightner (1973, 1974, 1975) conducted extensive studies on the cellular responses to injury in penaeid shrimps, Penaeus aztecus and P. setiferus. They recorded the histopathological aspects of the response to injury of brown shrimp P. aztecus wounded with Petersen tag pin. The wound healing process showed pronounced haemocytic infiltration of the wounded area followed by encapsulation by fusiform haemocytes, thereby forming nodules and finally involution. In P. setiferus, phagocytosis and elimination of carmine particles injected into the abdominal musculature, followed by encapsulation in the necrotic area has been described. Tsing and Brehelin (1984) described three types of circulating cells - hyaline, semi-granulous and granulous haemocytes in Penaeus japonicus. Tsing et al. (1989) studied morphology, cytochemistry and hemograms of haemocytes of P. japonicus, P. monodon, Macrobrachium roseenbergii and Palaemon adspersus. In P. japonicus acid phosphatase activity in the granular haemocytes has been evidenced.

Structure of haemocytes from two species of penaeid shrimps, the ridge back prawn, Sicyoma ingentis and P. californiensis was examined by light and electron microscopy and three types (agranular, small granular and large granular haemocytes) have been identified. Hose et al. (1987) employed cytochemical techniques to develop a comprehensive description of haemocytes of S. ingentis described above. Martin et al. (1987) described the architecture and fine structure of the epigastric haematopoietic nodules of S. ingentis. Omori et al. (1988) studied the coagulation of haemolymph in the same species by light and electron microscopy. Mart in et al. (1989) described the fine structure of major arteries of the shrimp and indicated that the morphology is the same as that described for the tubules of haematopoietic nodules. Hose et al.(1989) evaluated the defence functions of granulocytes of S. ingentis, their role, in phagocytosis and encapsulation, supported by demonstration of prophenol oxidase in these cells.

Rearing of economically important crustacean species (especially shrimps) is now widespread and conducted on an intensive scale. This has triggered the development of epizootics which are sometimes explosive and lead to mass mortality. Monitoring health conditions under rearing is not easy due to lack of techniques to control such outbreaks. The study of haemocytes and hemograms is envisaged as a means to identify control measures, since it has been shown that infectious diseases are reflected in the haemolymph of crustaceans. Recent research efforts to study the haemocyte structure, particularly those of commercial importance, has been an outcome of this necessity.

The present study, identification and classification of haemocytes of the commercially important species of penaeid prawn, *Penaeus indicus* is an attempt to provide basic information for further studies on the immunological responses of the species to invasive organisms. Light and Electron microscopy studies were conducted to identify the different types of haemocytes in the haemolymph of *Penaeus indicus*.

MATERIALS AND METHODS

3.1. Light microscopy studies:

Haemolymph smears were prepared according to the procedure described by Mix and Sparks (1980). The smear of haemolymph was prepared as follows:- a drop of freshly extracted haemolymph was placed on a clean glass slide. A thin uniform smear was drawn by using a rectangular cover slip at 45°. The smear was air dried for few minutes, fixed in methanol for 5 minutes and stained with either Wrights' stain or Giemsa for 5-10 minutes, washed with double distilled water and mounted in DPX.

The smears were observed under American Opticals (AO) light microscope, under oil immersion and photographed on AGFA 125 ASA negative film.

3.2. Ultra structure studies:

Transmission electron microscopy:- The ultra-structural details of haemocytes in the haemolymph of *P.indicus* was studied with the help of the Transmission Electron Microscope (TEM) (Carl Zeiss) at the regional station of the Central Plantation Crops Research Institute (CPCRI, ICAR) at Kayamkulam, Kerala.

The procedure described by Martin and Graves (1985) was adopted with necessary modifications. Haemocytes were collected by inserting a 21 guage needle attached to a 1 ml syringe containing 0.4 ml of fixative (3% 0.1M glutraldehyde solution, buffered to pH 7.3 with 0.1 M cacodylate

buffer containing sucrose) (Papathanassiou, 1984) into the heart and withdrawing 100 µl of haemolymph. The sample was immediately transferred to a clean centrifuge tube to ensure mixing. The sample was allowed to fix for a minimum of 15 minutes at room temperature and maximum of 12 hrs. The fixed haemocytes were pelleted by brief centrifugation (3000 rpm) for 10 minutes. The supernatant was discarded and the pellet was removed and kept on a clean petri dish and trimmed into small rectangular pieces of 2 mm thickness using a fine sharp razor blade. The trimmed pieces were immersed in freshly prepared chilled glutaraldehyde solution and refrigerated for five hours. These fixed samples were given a fresh change of fixative and subjected to vaccum infiltration for one hour. They were then washed several times with ice cold 0.1 M cacodylate buffer to remove the excess aldehydes and then post-fixed in freshly prepared 1% osmium tetroxide solution in 0.1 M cacodylate buffer for two hours at 4°C. This was followed by repeated washings in buffer solution (15 minutes each) and the samples were left overnight in fresh buffer solution under refrigeration.

The fixed samples were stained with freshly prepared 0.5% aqueous uranyl acetate for three hours under refrigeration and excess stain was removed by repeated washings with double distilled water. They were then dehydrated through graded alcohol series (30%, 50%, 70%, 95% and 100%) at 4°C for fifteen minutes in each grade with final washing in absolute alcohol with 2 changes for 10 minutes at room temperature.

After dehydration, the samples were transferred to a 2:1 mixture of Alcohol - Spurr's resin for infiltration for 1 hour (Spurr, 1969). The

ingredients of Spurr's resin (Firm standard) include ERL-10 g, DER-6 g, NSA-26 g and DMAE-0.4 g, all ingredients were mixed together thoroughly. They were then transferred to a miture of 1:1 alcohol - Spurr's resin for one hour and finally into 1:2 mixture of alcohol - Spurr's resin for one hour. The samples were transferred to pure Spurr's resin and left overnight under refrigeration.

Fresh resin was prepared for embedding in the same ratio of firm standard as above. Each processed tissue sample was carefully transferred to small embedding capsules containing fresh resin. Care was taken to avoid air bubbles inside the capsules. These plastic capsules containing tissue and resin was allowed to remain at room temperature for one hour and subsequently incubated at 70°C for 48 hours to allow complete polymerization of the resin.

The polymerized resin blocks were removed from the capsules and trimmed with a glass knife using an ultramicrotome (LKB-2128, ultrome IV, Bromma, Sweden). Few semithin sections of 1 mm thickness were made using the glassknife. These sections were stained with methylene blue - Azure II/basic fuchsine staining technique (Humphrey and Pittman, 1974) and observed under a binocular compound microscope and the desired regions were located. The block heads were further trimmed and ultra thin sections (silver interference colour 600-700A°) were cut by using freshly cut glass knife. The ribbon of ultra thin sections were allowed to float on distilled water taken in a plastic boat shaped trough, attached to the glass knife with molten dental wax. The wrinkles in the sections were

smoothened out by exposing to chloroform vapour by waving a cotton roll dipped in chloroform over the sections.

Copper or nickel grids (300 mesh size, 3.00 mm dia, LADD, USA) prewashed in pure acetone was carefully introduced just below the ultra thin sections in the water, using a bent tipped forceps and gently lifted up, while directing the ribbon towards the grids, using a very fine pointed, quill feather, so that the sections adhered to the dull or matty surface of the grids. The sections on the grids were dried for a few minutes and then stained with 2% uranyl acetate in 50% ethanol (filtered before use) for 15 minutes (Hayat, 1970). They were then rinsed thrice in glass distilled water, stained with freshly prepared, filtered 0.4% lead citrate in 0.1 N NaoH for 5 minutes and treated with 0.02 N NaoH for 5 seconds. These stained ultra-thin sections were screened under a CARL ZEISS transmission electron microscope (EM 109 R) operated at 80 KW and observed under various magnifications. The required areas were photographed on AGFA Ortho 25 negative film and printed on high contrast glossy paper with accurately controlled magnification.

The flowchart of the procedure followed is presented overleaf.

TISSUE SAMPLES
Haemocytes in the Haemolymph extracted from *P.indicus*

↓

FIXATION
aemocytes are collected by inserting a 21-guage needle attached to a 1 ml syringe containing 0.4 ml, 3% (0.1M) glutaraldehyde solution ffered to pH 7.3 with 0.1M cacodylate buffer containing sucrose, into the heart and withdrawing 100 µl haemolymph

↓

The sample is transferred immediately into a centrifuge tube and allowed to fix at room temperature for 15 minutes

↓

CENTRIFUGATION
he fixed haemocytes are pelleted by centrifugation for 10 minutes at 3000 rpm. The pellet is removed for trimming

↓

TRIMMING
(Pellet size : rectangular pieces of 2 mm thick)

↓

REFIXATION
(In chilled glutaraldehyde solution; 5 hours under refrigeration)

↓

VACUUM INFILTRATION
(In fresh fixative for one hour, followed by repeated washing in ice-cold cacodylate buffer)

↓

POST - FIXATION
reshly prepared 1 % osmium tetroxide in 0.1 M cacodylate buffer; 2 hours at 4 ° C)

↓

WASHING & REFRIGERATION
(In cacodylate buffer, 5 times, 15 minutes each. Followed by refrigeration of tissues overnight in fresh buffer)

↓

STAINING
fresh 0.5 % aqueous uranyl acetate - 3 hours under refrigeration followed by repeated washing in double distilled water)

↓

DEHYDRATION
ough graded alcohol series 30 %, 50 %, 70 %, 95 % and 100 % - 15 inutes each, at 4 ° C followed by 2 changes in pure acetone - 10 minutes each, at room temperature)

↓

INFILTRATION
:1 acetone - Spurr's resin (1 hour), 1 : 1 acetone - Spurr's ** resin ur), 1 : 2 acetone - Spurr's resin (1 hour) and in 100 % Spurr's resin (overnight under refrigeration))

↑

EMBEDDING
(In fresh resin taken in plastic capsules - one hour at room temperature followed by incubation at 70 ° C for 48 hours)

↑

TRIMMING
(Resin blocks were trimmed using a glass knife fitted to an ultramicrotome - LKB 2128, Ultrotome IV, Bromma, Sweden)

↑

SECTIONING (FOR SEMITHIN SECTIONS)
(Using ultramicrotome with a new glass knife)

↑

Semithin sections, 1 µ m thick

↑

STAINING (Humphrey and Pittman, 1974)
(Methylene blue - Azure II / Basic fuchsine technique)

↑

Observation under a binocular compound microscope to locate desired regions

↑

PHOTOGRAPHY (LIGHT MICROSCOPY)
(Semithin sections ; 'Microstar' (American Opticals, USA) binocular compound microscope with camera unit; various magnifications; ORWO NP22 (125 ASA, Panchromatic, 24 x 36 mm) black and white negative film; soft, glossy, single weight contrast printing paper)

↑

Further trimming of block heads

↑

SECTIONING (FOR ULTRATHIN SECTIONS)
(Using fresh glass knives)

↑

Ultrathin sections - (Silver interference colour, 600 - 700 ° A)

(Ultrathin ribbon - floating on distilled water taken in a small plastic boat - Wrinkles on the ribbon, removed by exposing them carefully to chloroform vapours - Sections collected on the dull or matty surface of copper or nickel grids prewashed in acetone (300 mesh size, 3.00 mm dia., LADD, USA); Grids with sections dried in air for few seconds)

↑

STAINING (Hayat, 1970)
(Fresh, filtered 2 % uranyl acetate in 50 % ethanol (15min.); Rinse in glass distilled water (3 times) – Fresh, filtered 0.4 % lead citrate in 0.1 N NaOH (5 - 10 min.) – 0.02 N NaOH (5 sec.)

↑

SCREENING AND PHOTOGRAPHY
(CARL ZEISS EM 109 R Transmission Electron Microscope; Current 80 KW; Various magnifications; Agfa Ortho25 negative film; High contrast, glossy printing paper)

References : - Hayat (1970), Weakley (1981), Papathanassiou & King (1984), Martin & Graves (1985)
*** Spurr's resin - 'firm standard' : ratio - ERL 10 g; DER 6 g; NSA 26 g and DMAE 0.4 g (Spurr, 1969)**

RESULTS

LIGHT MICROSCOPY STUDIES

Thin smears of haemolymph prepared to identify the haemocytes of *Penaeus indicus* did not yield satisfactory results. Plate 1 A, B, C, D, E & F and Plate IIA shows the different types of haemocytes occurring in the haemolymph of *P. indicus*. In Plate 1A and B, the agranulocyte with hardly any granules can be seen. The nucleus appears to occupy the entire cytoplasmic space. In Plate 1C and D, the semi-dense granulocytes with very few small granules can be noticed; Plate 1E and F represents the dense granulocyte; Plate IIA, represents the 'exploding cell' as described in the case of crayfish Astacus by Hardy as early as 1892. Plate II B, C, D, E and F are micrographs of haemocytes from mature and spent female *P. indicus*. Clear differentiation between agranulocytes and granulocytes was not possible. The size and shape of the haemocytes varied greatly. The granules present within the granulocytes could not be distinguished clearly, based on which classification is done. Hence, preparation of haemolymph smears did not facilitate haemocyte classification.

ELECTRON MICROSCOPY STUDY

Based on the ultrastructural study, the haemocytes of *Penaeus indicus* can be divided or classified into three main types - Agranulocytes (AG), Semi-dense granulocyte (SG) and Dense granulocyte (DG).

AGRANULOCYTE (AG)

Agranulocytes are the smallest type of haemocytes found in the haemolymph. The average size of these haemocytes is 3.35 /um x 4.76 /um. They are ovoid to spherical, with nuclear chromatin dispersed. They are otherwise known as hyaline cells. The nucleus occupies much of the cell, and is generally ovoid (Plate III A, B, C). The nucleus/cytoplasm ratio is generally high. The nuclear envelope is smooth. Cytoplasmic inclusions are few or almost absent (Plate III A, B, C and Plate IV B). Rough and smooth endoplasmic reticulum are rarely seen in this type of haemocyte. Free ribosomes, Golgi bodies are rarely found in these haemocytes. Granules are totally lacking in this type of haemocytes.

The agranulocytes show little signs of differentiation. In some cases, the nucleus is seen dividing, being filled with mitochondria (Plate III, B, C, D). The nuclear material appears dispersed and the agranulocyte appears to be dividing. Such differentiation has not been recorded elsewhere. (Plate III C, D and Plate IV A). In these haemocytes also no cytoplasmic granules can be seen (Plate IV A, B). Very rarely one or two granules are noticed (Plate IV C, D and Plate VA). In Plate IV D, at one end, a peculiar structure probably 'marginal band' quoted by few others, is noticed In Plate V A, the nucleus is horse-shoe shaped. The agranulocytes thus lack cellular differentiation and are also termed as undifferentiated haemocytes.

DENSE GRANULOCYTE (DG)

The haemocytes are well differentiated and possess large number of dense granules and therefore termed granulocytes or Dense granulocytes (Plate VC). These haemocytes are generally ovoid and larger than the other haemocytes, the average size is 4.72 /um x 5.85 /um. The nucleus does not occupy the complete cytoplasmic space and also possess masses of dense heterochromatin close to the nuclear envelope. The nucleus may be variously shaped (Plate V C and Plate VI C). The most distinct and distinguishing feature of these haemocytes is the presence of thick or dense granules (Plate V D and Plate VI A, B, C, D). The granules vary from 0.1 /um to 0.57 /um. In some cases two types of granules are noticed within the cytoplasm - very dense granules (DG) and less dense granules (LG) (PLate VI C and Plate VII A). The granules are always membrane bounded and generally possess a homogenous electron-dense content but sometimes they exhibit an internal heterogenous structure made up of electron -dense and electron-lucent areas (Plate VI C).

These dense granules form the basis for identification of different haemocyte types in crustaceans. The cytoplasm of these haemocytes possess few ribosomes and endoplasmic reticulum. Vacuoles are also present. The development of pseudopodia - like extensions on contact with any surface is seen in Plate VC.

SEMI-DENSE GRANULOCYTE (SG)

These haemocytes are intermediate type of blood cells (Plate VII B, C, D and Plate VIII A). They are ovoid or spindle shaped, sometimes

irregular, with an average size of 4.16 /um x 7.18 /um. They are characterised by the presence of cytoplasmic granules, more numerous than in the agranulocytes, always of round shape. These inclusions are usually smaller than those found in the dense granulocytes, measuring 0.043 /um. The nucleus in this type of haemocyte is variously shaped (Plate VII B, C, D), but does not occupy the entire cellular volume. Regions of very dense heterochromatin are numerous and packed around the inside of the nuclear envelope (Plate VII B and Plate VIII A). In few cases, the nucleus is greatly reduced (Plate VII C). Free ribosomes are abundant and rough endoplasmic reticulum is more developed with narrow but elongated cristae, compared to the dense granulocyte and agranulocyte. Secretory vescicles with dense electron content and mitochondriae are also noticed in these haemocytes.

The semi-dense granulocytes occur in such varied forms with number and size of the granules varying greatly such that, it is often difficult to assign or classify them as agranulocytes or granulocytes. Numerous intermediate forms are present (Plate VIII B, C). Other transient forms that occur in the haemolymph are represented (Plate VII C, D and Plate VIII A, B, C). Plate IX A, B, C also represent transient haemocytes, which probably are developing or maturing agranulocytes. Their function and role is unknown.

It is believed that the dense granulocytes develop from the semi-dense granulocytes. Probably the agranulocytes develop into semi-dense granulocytes which develop into dense granulocytes as reflected by the size, structure and number of the granules occurring in them.

The three types of haemocytes - viz. agranulocyte, semi-dense granulocyte and dense granulocyte are seen generally in the haemolymph of *Penaeus indicus*. In the haemolymph of females with developing ovary, an entirely different type of haemocyte is noticed. This haemocyte is at least three times larger than the other haemocytes (Plate IX D, and Plate XI B). This haemocyte is seen in all stages of maturity as well as spent stage. This haemocyte has plenty of glycogen granules in the cytoplasm. Several different types of lipoprotein granules are also present (Plate IX D). Probably these different granules are of different nature and function(Plate IX D,a,b,c,d,e,f) indicating different types of cell inclusions. Highly electron dense granules of homogenous as well as heterogenous nature are present. Mitochondria are abundant in this type of haemocytes. Plate XI A represents the kidney shaped structure within which is abundant mitochondria. The immense energy requirement during vitellogenesis and subsequent spawning is met by the mitochondria. Plate X B, D and Plate IX A represents another type of granule present in this haemocyte. A crystalloid structure is present within it. This structure is also of two types. While in Plate X B, the structure appears ribbed; the crystalline structure in Plate X D and Plate XI A is homogenous. The role of these granular structures in vitellogenesis is unknown. Apart from these, the haemocyte has numerous vacuoles and other homogenous granules. In Plate XI C, degenerate lipid bodies are noticed. Plate IX B represents a developing mature haemocyte, with abundant glycogen granules.

Apart from the above mentioned haemocytes Plate XI D represents a fifth type of haemocyte. This appears to be similar to "cyanocyte" -copper

containing haemocyte reported by few others, although it is yet to be confirmed. Probably, this cell type does not circulate in the haemolymph but is found in the haematopoietic nodules. The 'cyanocyte' has a large central granule with plenty of rough endoplasmic reticulum and mitohondria. Golgi bodies are also present. Probably this haemocyte is involved in the synthesis and release of hemocyanin into the haemolymph.

PLATE I

A) Light micrograph of agranulocytes, from Penaeus indicus; Leishman stain; X 1000.

B) Light micrograph of agranulocyte from P. indicus; Leishman stain; 1000.

C) Light micrograph of semi-dense granulocyte from P. indicus; Leishman stain; X 1000.

D) Light micrograph of semi-dense granulocyte from P. indicus; Leishman stain; X 1000.

E) Light micrograph of dense granulocyte from P. indicus; Leishman stain; X 1000.

F) Light micrograph of dense granulocyte from P. indicus; Wright stain; X 1000.

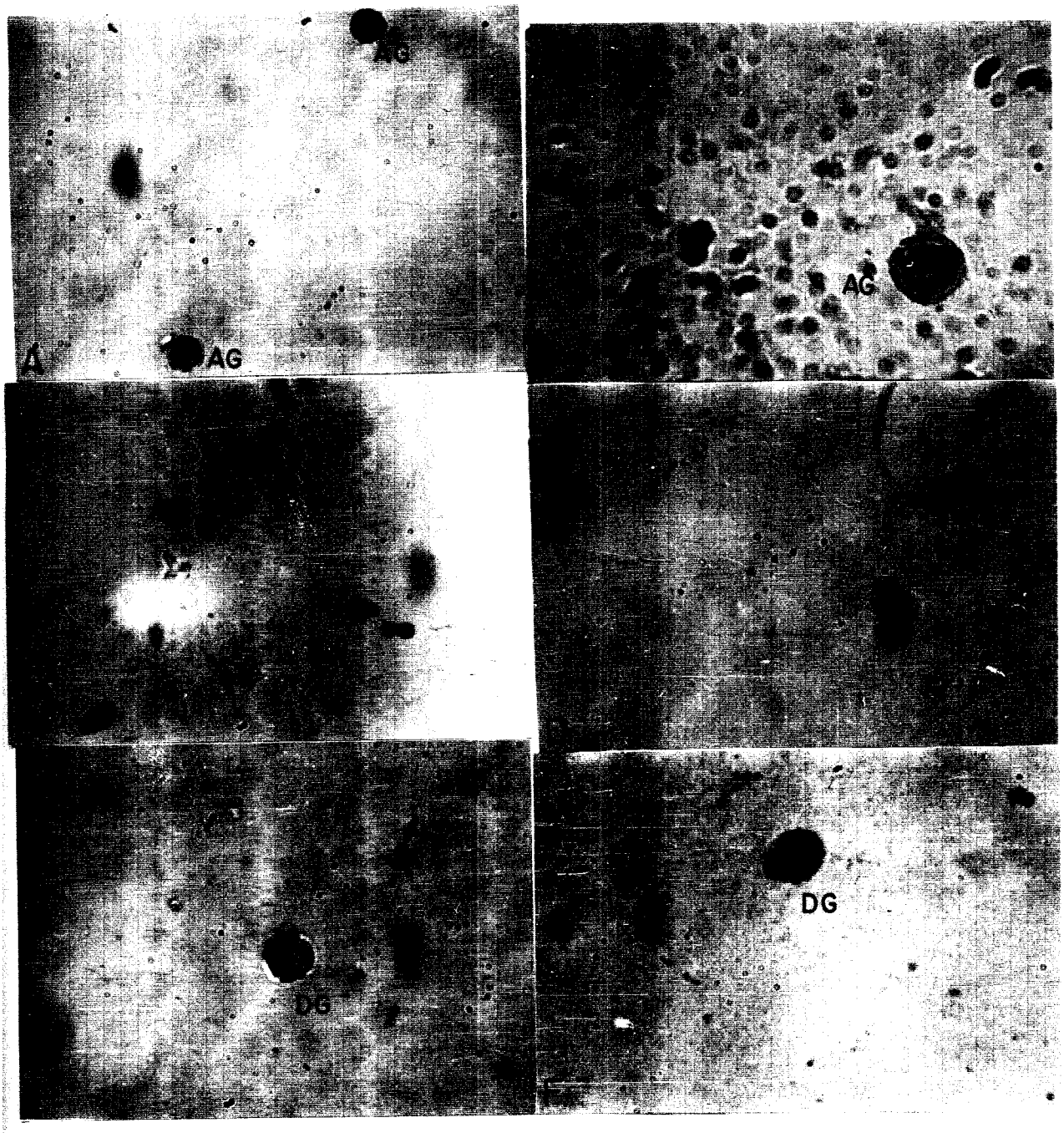

PLATE I

PLATE II

A) Light micrograph of a exploding cell from P. indicus; Leishman stain; X 1000.

B) Light micrograph of a cluser of haemocytes from mature female P. indicus (Semithin section); Leishman stain; X 1000.

C) Light micrograph of haemocyte from mature female P. indicus; Leishman stain; X 1000.

D) E & F - Light micrograph of haemocytes from spent female P. indicus (Semithin); Leishman stain; X 1000.

PLATE II

PLATE III

A) Transmission Electron micrograph of agranular haemocyte from Penaeus indicus.
Note the small amount of cytoplasm and large nucleus (N); X 10804

B) Electron micrograph of another agranulocyte from P. indicus.
Note the divided nucleus with dense mitochondria N - Nucleus, M - Mitochondria; X 28572.

C) Electron micrograph of another agranulocyte.
This agranulocyte also appears to be dividing.
N - Nucleus; X 11000.

D) Electron micrograph of the upper region of the above agranulocyte; X 18000.

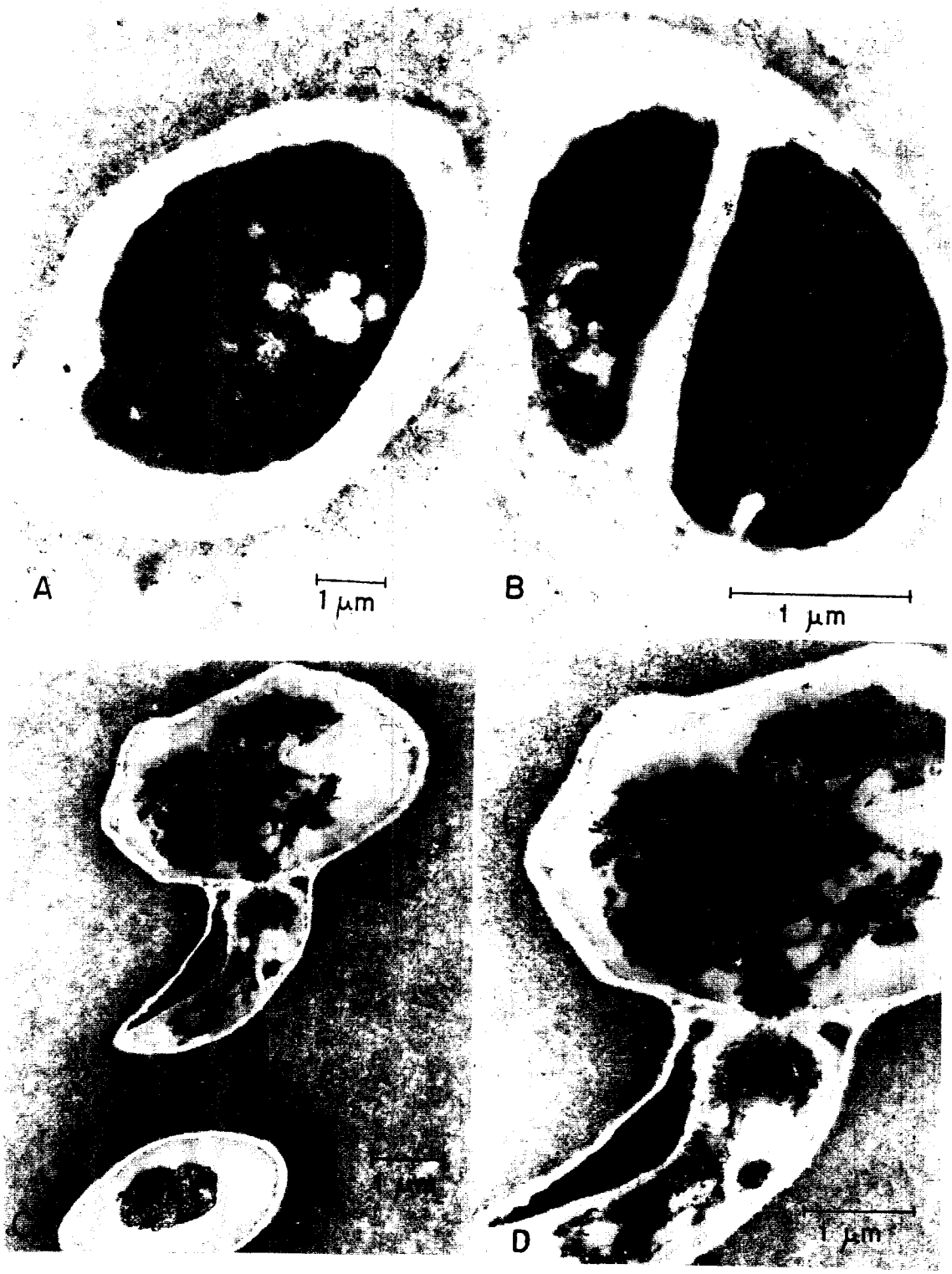

PLATE III

PLATE IV

A) Electron micrograph of lower region of the agranulocyte (in Plate ID); X 29851.

B) Electron micrograph of another elipsoidal agranulocyte; X 17778.

C) Electron micrograph of ovoid agranulocyte with scarce cytoplasm. Note the presence of a few granules also.
N. Nucleus; X 14000.

D) Electron micrograph of a smaller agranulocyte, with scarce cytoplasm Nucleus (N) occupies entire cytoplasm; X 12333.

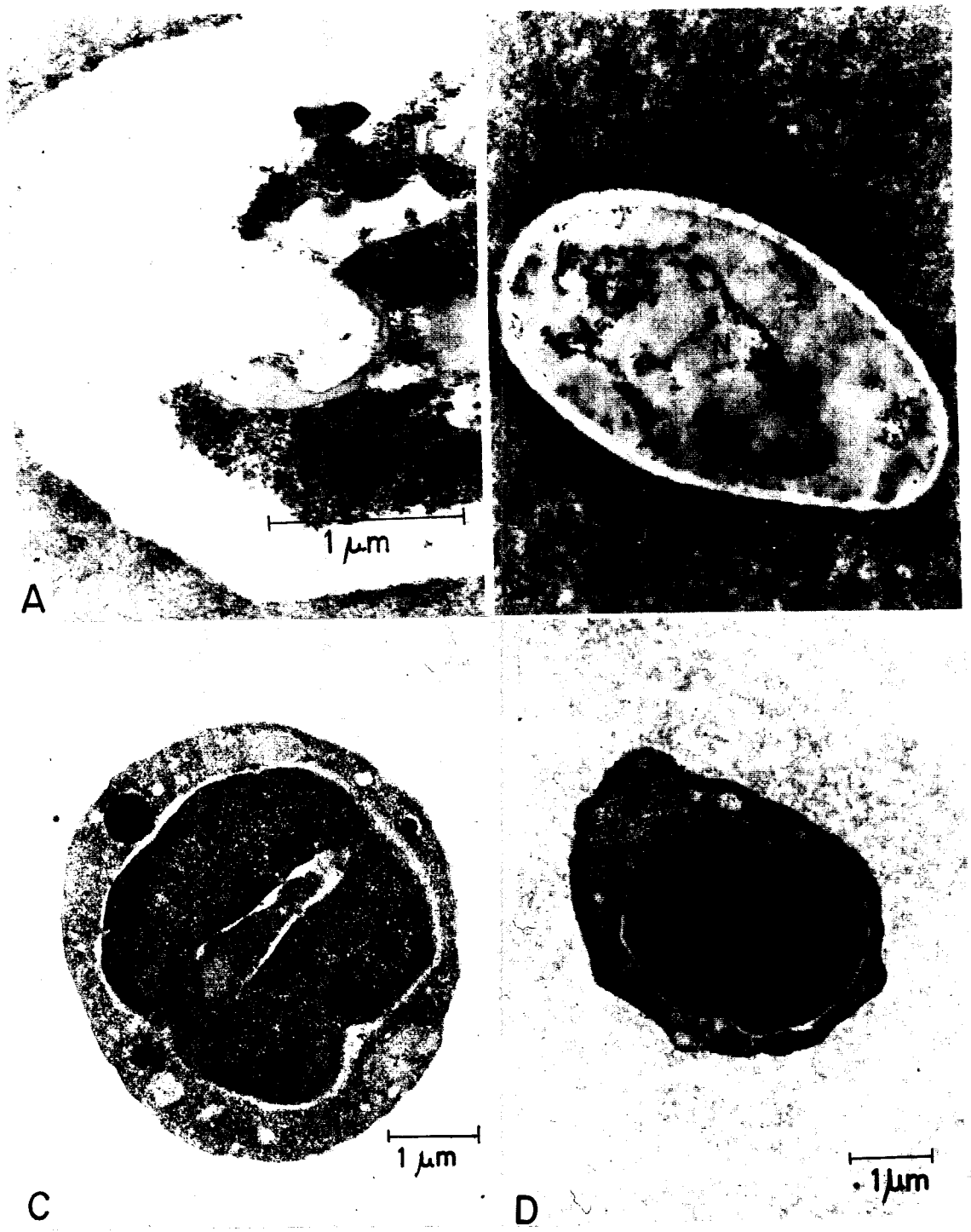

PLATE IV

PLATE V

A) Electron micrograph of small agranulocyte.
Note the horse-shoe shaped nucleus (N) and two small granules.
X 14206.

B) Electron micrograph of yet another agranulocyte.
Note the large nucleus (N); X 13253.

C) Electron micrograph of dense Granulocyte (DG).
Note the presence of dense granules (G) Vacuoles (V), Mitochondria (M), Hetrochromatin (Hc) in the nucleus (N) and the psuedopodia like extensions (Ps); X 10585.

D) Electron micrograph of lower region of the above granulocyte.
Note the dense granules (G) and vacuoles (V); X 29302.

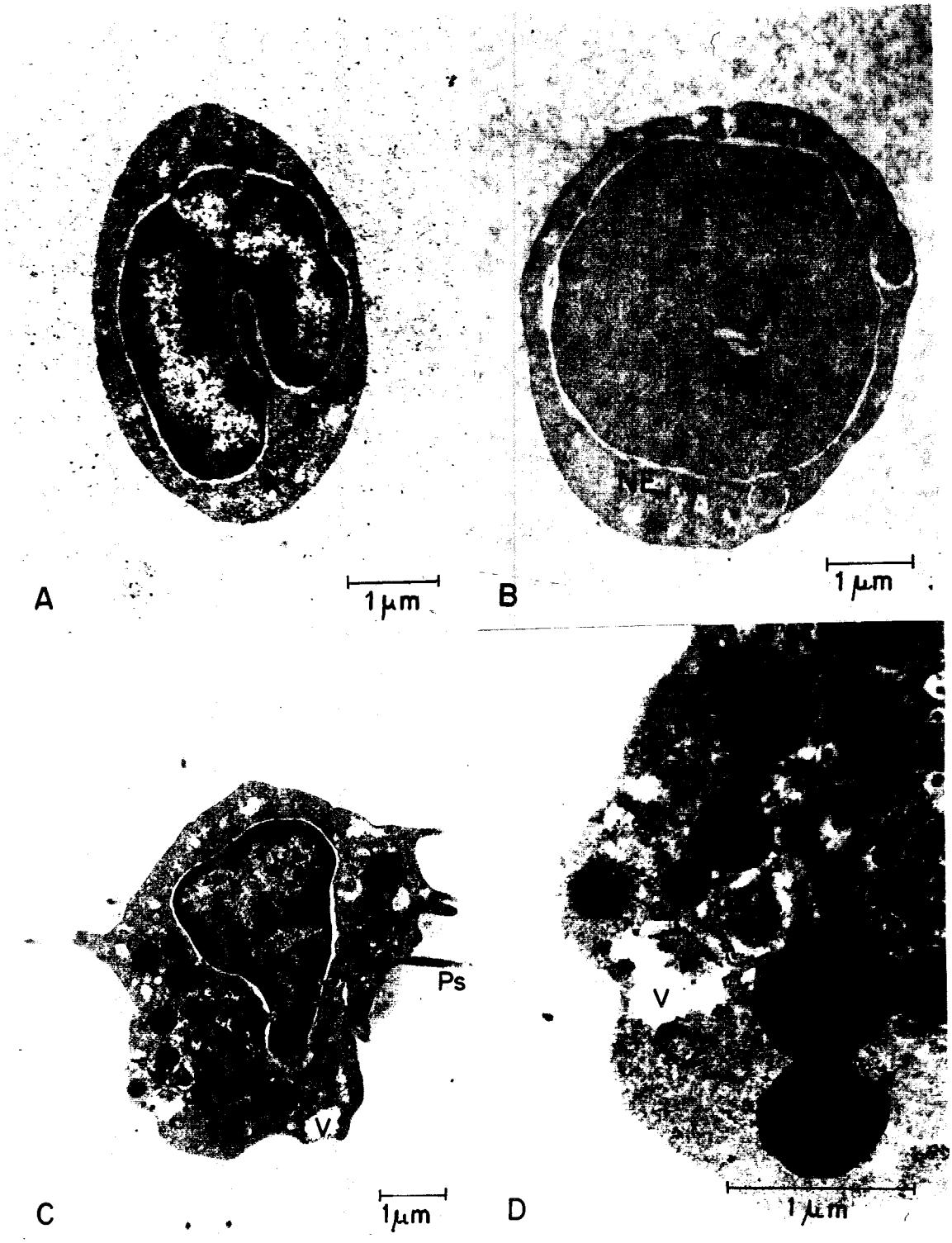

PLATE V

DISCUSSION

Investigations on crustacean haemocytes have consistently distinguished between agranular cells and granular cells. Among granular cells, two types have been recognised, those containing small granules and those containing large granules, termed semi-granulocyte and dense-granulocyte respectively. (Bauchau and De Brouwer 1972; Bodammer, 1978; Mix and Sparks, 1980; Martin and Graves, 1985; Benjamin and James 1987). However, classification schemes have placed importance on other features also, such as, size, shape or staining characteristics of the cells. In shrimps, as well as other crustaceans, these differences are too slight to be of practical use. Cell shape is also an inadequate basis for classification since in vivo most cells are ovoid and in vitro the cells rapidly alter their shapes by extending filopodia and pseudopodia (Johnson 1980). Features identified using routine staining procedures (Wright's stain, Giemsa, Leishman's Hematoxylin-Eosin) are not considered useful because, besides being subtle and ambiguous, are not based on properties that allow one to distinguish granules in different stages of maturation or with different physiological function. Also, these methods are not reproducible. In addition, when staining is performed on haemolymph smears, the resultant cell morphology is highly distorted. These inadequacies are being overcome by classification based on electron microscopy study of haemocyte structure.

The haemocyte ultra-structure described for *Penaeus indicus* can be observed in most of the decapod crustaceans described to date. The main

differences are in the terminology adopted by various authors and possible affiliations between haemocyte types (George and Nichols, 1948; Wood & Visentin, 1971; Stangvoss, 1971; Bauchau and De Brouwer, 1972; Ravindranath, 1974; Cornick and Stewart, 1978; Smith and Ratcliff, 1978; Bodammer, 1978; Martin et al., 1988).

The three types of haemocytes found in *Penaeus indicus* can be compared with those reported in few others. In *P. indicus* based on ultrastructure, the agranular, (hyaline), the semi-granular and dense-granular haemocytes have been identified. Dall (1864) distinguished three types of cells in Metapenaeus mastersii based on light microscopy: the lymphocyte, thigmocyte and large granulocyte, based on cell shape. These cells are comparable to the agranular, semi-granular and dense-granular cells of *P. indicus* respectively. The variety of shapes reported by Dall resulted from examination of cells without proper fixation. Fontaine (1978) based on examination of fine structure blood cells of white shrimp P. setiferus, generally classified them into agranular and granular haemocytes according to cytoplasmic inclusions. Among the granular haemocytes, two types have been described - those which possess large numerous lipid inclusions and those with inclusions that displayed a definite texture or structure unlike that of the large lipid like inclusions. These are similar to the semi-granular and granular haemocytes of *Penaeus indicus*.

Tsing and Brehelin (1984) and Tsing et al. (1984) described the ultrastructure of circulating cells of Penaeus japonicus, P. monodon, Macrobrachium rosenbergii and Palaemon adspersus. The hyaline haemocyte

has a high nucleocytoplasmic ratio and lacks granules. The semi-granular haemocyte has few round, membrane-bound granules in the cytoplasm and the granulocytes possess numerous, variously shaped granules along with other cytoplasmic inclusions. Under phase contrast microscope only two cells are described owing to their transformed nature. Tsing et al. (1989) preferred to name the hyaline cells of P. japonicus and P. monodon as undifferentiated cells, since it was not possible to affirm that the hyaline or agranular cells do not possess any granular inclusions. In *P. indicus* also this condition is noticed and hence the agranular cells may be considered to be undifferentiated cells similar to that of P. japonicus and P. monodon The absence of true agranular cells is in agreement with Hearing and Vernick (1967), Stang-Voss (1971) and Hoarau (1976).

Martin et al. (1987) have found that mitosis occurs both within the haematopoietic nodules (HPN) and to a lesser degree within the haemolymph as described by Fontaine (1978) and Johnson (1980). They have observed cell division only in the agranular and small granular haemocytes that lack deposits. In *P. indicus* only the agranular cells appear to be dividing, which suggests that the agranular haemocytes can mature after release into the haemolymph. The progression of small to large granule haemocytes as suggested by Martin et al. (1987) in Sicyonia ingentis, has been repeatedly postulated since the early work of Cuenot (1893) and confirmed by Bodammer's (1978) observation of fusion of small electron-dense granules into larger structures. In *P. indicus* larger number of intermediate small granule haemocytes has been observed indicating the progression of agranular to granular through intermediate forms. However,

elucidation of the exact lineage and maturation sequence of haemocytes of *P. indicus* necessitates definitive autoradiographic analysis. The utility of such haemocyte classification scheme will be demonstrated through morphologic examination of haemocytes participating in physiological and pathological processes.

The differentiation of crustacean haemocytes is still on open subject for discussion. The group of authors have proposed the evolution "from the hyaline cells to the granular cells". (Bodammer 1978; Bauchau and Mengeot 1978; Benjamin and James 1987; Martin et al. (1987). This theory was first proposed by Cuenot in 1893. Few others have proposed the reverse order of evolution, starting from granular haemocytes towards hyaline haemocytes (Vranckx and Durliat, 1977). On the other hand, Sternshein and Burton (1980) failed to find evidence for the above theories, suggesting that, in crayfish the observed haemocyte types are functional or developmental stages of one cell type. In *P. indicus* from the micrographs, it is evident that some apparently hyaline cells do possess few small granules indicating the possible differentiation of the agranular to semi-granular to dense-granular stage. Since the shape of the granules within the cells does not vary much (ie. they are round in most cases), it adds credence to the theory proposed by Bauchau et al. (1978). But the total lack of these haemocytes during the period of vitellogenesis and the occurrence of special kind of heavily granule packed haemocyte in the haemolymph of mature females suggests that probably only one haemocyte type exists and all other functional or developmental stages of this haemocyte type as proposed by Sternshein and Burton (1980). However, more detailed studies are needed to arrive at such a conclusion.

The haemocytes of *Penaeus indicus* similar to those recorded in cray fish and lobsters, although the terminology used varies widely. Wood and Visentin (1967) and Wood et al. (1971) based on cytochemical observations described hyaline, small granulocyte and large granulocyte in Orconectes virilis. They also demonstrated the presence of 'fibrin-ferment' described earlier by Hardy 91892) in the granulocyte indicating that the granulocyte is involved in the clotting process. This hypothesis was supported by Unestam and Nylund (1972) in Pacifastacus leniusculus and Astacus astacus. Stang-Voss (1971) described only one cell type in A. astacus - the amoebocyte However, three functional forms - the clotting cell, the phagocyte and granular amoebocyte are described. The clotting cell is characterised by large number of granules containing internal tubular structure. This appears to be analogous to the granulocyte described in other forms. It is suggested that these granules are the storage site of haemagglutinin. The clotting cells are very fragile, their cytolysis accelerates the clotting process. In the phagocytes, membrane bound granules in initial stages are found and in the granulocytes they occupy the complete cytoplasmic space. The granules in these are supposed to be involved in hemocyanin synthesis. The existence of three functional forms of the amoebocyte supports the hypothesis put forward by Bauchau et al (1978). Sternshein and Burton (1980) using light and electron microscopy described the coagulocyte, granulocyte and amoebocyte in Procambarus sp. and Orconectes sp. comparable to the semigranulocyte, dense granulocyte and agranulocyte of *P. indicus* The coagulocyte is involved in coagulation by releasing the contents of the granules into the haemolymph after rapid cytolysis. The amoebocyte and granulocyte are capable of phagocytosis.

In Homarus sp. George & Nichols (1948) and Toney (1958) described four types of blood cells while Hearing (1969) had identified three types based on ultrastructure in H. americanus - the eosinophil, the ovoid basophil and spindular basophil. All these cells contain large granules exhibiting acid - phosphatase activity,but was not exhibited by the cytoplasm and other organelles. Due to concentration of reactive sites on the limiting membrane of the granules and on the stroma, it is suggested that the enzyme is bound to or associated with the membrane or else the membrane is the specific site of action of the enzyme. In Homarus, the normal blood cells, the granules appear homogenous while they become striated during the coagulative process, fibrillar stroma becomes visible and the limiting membrane ruptures. Thus lysosomic nature of the granules in the lobster blood cells appear to be important in cell lysis prior to the coagulation process. Mengeot et al. (1977) isolated the granules of the haemocytes of H. vulgaris and concluded that these granules form a gel under certain conditions. In *P. indicus* it is probable that the granules present in the granulocyte as well as semi-granulocyte are involved in the process of coagulation and enzymes are involved in cell lysis and further gelation. In *P. indicus* it is observed that the haemolymph clots within seconds and forms a thick gelly when extracted from the heart in the absence of any anticoagulant, implying that the haemocytes are definitely involved in coagulation and heteroagglutinin is certainly present either within the granules of the haemocytes or in the plasma. A two step process - cell lysis followed by gelatin as suggested by Tait (1911) and Bauchau and Mengeot (1978) probably takes place in *P. indicus*. Further enzyme studies are needed to explain this phenomenon.

Ravindranath (1974) described seven haemocyte types with distinct lineages in the isopod, Ligia exotica. He classified them into prohaemocytes of hyaline cells (which was further subdivided into five classes) plasmatocytes, granulocytes, cytocytes, oenocytoids, spherule cells and adipohaemocytes as suggested for insects (Jones, 1962). This was based on central or eccentric nucleus, staining of cytoplasm and granules, kinds of granules and are not valid enough. Benjamin and James (1987) described, in the same species, three cell types - hyaline, semi-granulocyte and granulocyte based on their ultrastructure. This has been supported by Dappen and Nikol (1981) in Armadillidium vulgare. Hoarau (1976) described four cell types following study of blood cells in isopod Helleria brevicornis.

The blood of Limulus polyphemus contains only one cell type - the amoebocyte and the granules packed in the cytoplasm contain the clottable protein. The presence of a bactericidal factor released by the amoebocyte has also been reported. (Dumont et al. 1966; Kenny et al., 1972; Pistole and Britko, 1972; Holme and Solum, 1973; Murer et al., 1975; Furman and Pistole, 1976; Armstrong, 1989, 1980, 1982; Copeland and Levin,1985a; Fablin and Levin, 1988). Thus, when apparently different functional forms exist in other crustaceans, including *P. indicus*, in Limulus, the single amoebocyte is involved in both coagulation and phagocytosis for over 300 million years. Fabrenbach (1970), and Sherman (1981) reported the occurrence of cyanoblasts and cyanocytes in Limulus suggesting their involvement in synthesis and storage of hemocyanin. Suhr-Jessen et al. (1989) recently explored the immune defence system of L. polyphemus by transmission electron microscopy combined with immuno-gold labelling

and described two type of haemocytes - the amoebocytes (granulocytes) and plasmatocytes. In Japanese horse-shoe crab, Tachypleus tridentatus, two types of haemocytes with clottable protein localized within them, has been localized by Sishikura (1977).

Among the Brachyurans, exhaustive studies have provided conclusive evidence of the existence of three major haemocyte types. Preliminary studies based on staining characteristics of haemocytes of C. maenas, William & Lutz (1975) described two major cells while Ghiretti-Magaldi (1975a,b, & 1977) described three types apart from the cyanocytes based on ultrastructure. Bodammer (1978) described three cell types - hyaline, intermediate cell and granulocyte of blue crab, Callinectes sapidus comparable to those in *P. indicus* while Synder and Stanley (1973) described the haemocytes to be of one basic type.

Bauchau and De Brouwer (1972) described three cell types in Eriocheir sinensis - hyaline, semi-granulocyte and granulocyte, corresponding to the agranulocyte, semi-granulocyte and dense granulocyte in *P. indicus* A fourth form, the lipo-protein haemocyte detected in the haemolymph of mature females was also reported. This type of haemocyte has been reported only in this species so far, and is filled with abundant mitochondria, granular reticulum, Golgi apparatus, abundant small granules and abundant myelin membranes accumulated in the form of large bundles. A comparable cell has been detected in *P. indicus* in the haemolymph of females in all stages of ovarian maturity. This haemocyte is several times larger than the normal circulating haemocytes, filled with granules of different shapes and sizes and nature, unlike in the case of E. sinensis. Large membrane

bound bundles of mitochondria, lipid granules and protein granules are seen in the cytoplasm which is filled with abundant glycogen granules. Few of the membrane-bound protein granules have a rod-like crystalline structure within them. This type of haemocyte has not been studied in other species and probably holds great functional significance. The occurrence of this special kind of haemocyte in *P. indicus*, almost obliterating all the other haemocytes, generates great interest. It is probable that this haemocyte could have developed from the granulocytes. Their large size, abundant granules of various kinds prove that the special requirements during ovarian development necessitates the transformation of the granulocytes into special carrier cells. These cells, apart from providing for the great energy demand (as justified by the presence of enormous mitochondria) during vitellogenesis and subsequent spawning, may also be transporting nutrients in the form of protein, lipid and glycogen. The haemocytes seen in normal circulation are totally absent during all stages of ovarian development thus indicating that probably the agranular and semi-granular haemocytes also transform into these special carrier cells. This again questions the actual lineage and mode of differentiation as well as the specific functional role of the individual haemocytes, necessitating detailed and specific studies.

Sewell (1955) and Paulus and Laufer (1987) described the presence of a lipo-protein cell associated with moulting in the blood of C. maenas and Libinia amarginatus. They believe that it is a derivative of the hyaline or semi-granular cells and could be responsible for secretion of non-chitinous epicuticle prior to moulting. Such a haemocyte could not be identified in *P. indicus*. This is probably due to the shorter moult cycle of penaeids

compared to crabs. The moult cycle of penaeids is not a high-energy demanding process unlike the process of vitellogenesis and spawning. Moreover endocrine control and release of moult related secretions has been well justified by the x-organ-sinus gland complex in penaeids (Hamann, 1974). The lipo-protein haemocyte described by Sewell (1955) could probably be an adipohaemocyte described by Ravindranath (1974) (Bauchau, 1981).

The cyanocyte described by Fahrenbach (1970) in Limulus polyphemus and Ghiretti-Magaldi et al. (1977) in C. maenas appears to be similar to the one present in *Penaeus indicus*. This haemocyte could not be located in the general circulation. These cells are believed to synthesise and store hemocyanin as crystalline cytoplasmic granules. The proteinaceous material is confined within membrane-bound granules and probably has a crystalline substructure. As in the case of crabs, the cyanocyte in *P. indicus* probably stems from haemoblasts and leaves the haemotopoietic nodules (HPN) or organ, but does not enter into circulation instead becomes attached to connective tissue strands surrounding the ophthalmic artery, the dorsolateral surface of the foregut and dorsal surface of the hepatopancreas as suggested in the penaeid ridgeback prawn, Sicyonia ingentis (Martin et al., 1987). The hemocyanin may be released by the plasma membrane into the blood by a kind of holocrine secretory (Ghiretti-Magaldi et al., 1977).

The presence of copper in the granulocytes has been advocated by Bauchau and De Brouwer (1972) Chassard-Bouchaud and Hubert (1975), Durliat and Vranckx (1976a), Mengeot et al. (1977), and Martin et al. (1987) These observations based on electrophoretic, immuno-electrophoretic and

electron microscopy studies makes the circulating granulocytes plausible candidates for synthesis of hemocyanin. In *P. indicus* this aspect could not be confirmed. Whether the cyanocyte is involved in synthesis and storage of hemocyanin or whether the granulocytes are also involved in this function cannot be decided at this stage.

The haemocytes of crustacea are reported to participate in numerous physiological functions. Their role in the defence reactions towards invasive organisms and foreign bodies has been documented in several species but the specific mechanisms are still not very clear. The secretion of 'fibrin-ferment', which initiates or participates in the formation of clot was discovered by Halliburton and Hardy (1892), supported by Wood et al. (1971), which is believed to be released from the blebs of the hyaline cells as well as from the granulocytes. The presence of 'fibrinogen' in the plasma or within the haemocytes is still unconfirmed. In some cases, the clottable protein is reported to be present in the plasma as demonstrated by Duchateau and Florkin, 1954; (Homarus sp); Stewar et al. 1966, (H. americanus); Fuller and Doolittle (1971a,b) (P. interruptus); Doolittle and Fuller (1972); Jazdzewski et al. 1975 (Gammarus sp., Asellus s. and Oniscus sp.), Durliat and Vrankx 1976a (A. leptodactylus) Vendrely et al. 1977 (M. puber). In other cases, it could not be traced by electrophoresis. (Wood and Karpawich (1962) in O. virilis), Stutman and Dolliver (1968) in Gecarcinus lateralis, Stewart and Dingle, (1968) in C. irroratus, C. borealis and Hyas coaretatus. In *P. indicus* a slow moving fraction or rather with no mobility, probably the fibrinogen (as identified by others in other species) was separated by electrophoresis. Whether it is stored within the haemocytes in unknown.

Durliat & Vranckx (1976b) and Durliat (1985) identified the clottable protein in the plasma of A. leptodactylus and also found a monomeric form in the haemocytes, which are converted into dimers or polymers when secreted. In *P. indicus* also probably the same phenomenon occurs.

Haemolymph coagulation is an important adaptive mechanism of survival, particularly for organisms with open vascular system. Tait (1971) has classified coagulation in crustaceans into three basic types. In the first type the haemocytes simply agglutinate without any subsequent gelation of the plasma. (C. pagurus, Maia squinado). In the second type - cell agglutination is followed by a general gelation of the plasma (Palaemon serratus, H. vulgaris, Macropipus puber, C. maenas). In the third type, the gelation process is first limited to the periphery of a few insignificant cell clots but subsequently affects the whole of the remaining plasma (Isopoda, A. fluviatilis, P. vulgaris). In *P. indicus*, it is suggested that the second type of coagulation takes place. This is indicated by the occurrence of degenerate cells which probably have released the content of their granules (probably fibrinogen) into the plasma, initiating coagulation and resulting in gellification. This can be confirmed only by identifying and isolating the clottable protein.

Phagocytosis is a more common cellular defence reaction recorded in crustacea. Damaged or decaying cell debris, microorganisms or particulate matter introduced into the body cavity are soon engulfed by the haemocytes (Chassard-Bouchaud and Hubert, 1975; Shivers, 1977; Bauchau, 1986). However, few exceptions to this blood clearing process are also recorded. The bacterium Aerococcus viridens in H. americanus, the ciliate Anophrys

sp. in C. maenas and M. squinado are not phagocytosed but multiply and induce fatal septicemia (Cornick and Stewart, 1968; Bang, 1967). Each type of haemocyte has the capacity to phagocytose although hyaline and semigranular haemocytes appear to be more active than the granulocytes. Functional specialization is also reported as in C. maenas in which Paramoeba pernicosa is engulfed by hyaline cells while gram-negative bacteria are enveloped by both hyaline and granulocytes (Johnson, 1976,1977b) Phagocytosis also appears to be affected by external and internal factors such as pH and temperature (Ravindranath 1975a,b). The presence of hetero-agglutinins in the haemolymph to aid in recognising non-self is also reported in several species. (Ghidalia et al., 1975, Shapiro, 1975). In *P. indicus* the heteroagglutinin seems to be present, as identified by electrophoresis and plays an important role in phagocytosis as well as coagulation. Naturally occurring haemogglutinins with opsonic activity in the haemolymph has been recorded in crayfish and lobsters (Peterson and Stewart 1974, Stang-Voss, 1971; Peterson et al. 1976). They are probably synthesised by the haemocytes and released into the blood (Cornick and Stewart, 1973, 1978). There has been no record of haemagglutinins in shrimps particularly penaeids, but the possibility cannot be ruled out.

Encapsulation and nodule formation is a third line of defence adopted by the haemocytes in crustaceans. A capsule is formed around any foreign body introduced into its tissue, by concentric layers of blood cells, which are held together by dense intercellular collagen-like substance. Necrosis and melanization takes place swiftly. The melanin-forming enzyme, a polyphenoloxidase as well as its substrate originate from the haemocytes (De Backer, 1961, 1962; Fontaine and Lightner, 1975; Solangi and Lightner,

1976; Unestam and Weiss, 1970; Unestam and Nylund, 1972; Smith and Ratcliff 1980a,b).

The haemocytes in crustaceans are also apparently involved in synthesis, storage, transport and release of various kinds of polysaccharides. The neutral or acidic nucopolysaccharides are packed in membrane-bound granules while glycogen stores, although scattered in the cytoplasm of all the haemocytes types are more abundantly found in the semigranular and granular cells (Johnston et al., 1973; Bauchau et al., 1975; Bodammer, 1978). Haemocytes may regulate the blood sugar levels according to particular needs as in the case of starvation, moulting, vitellogenesis. In *P. indicus* the presence of specialised cells during vitellogenesis justified the role of haemocytes in synthesis storage and transport of metabolites required for ovarian development and spawning. The altered levels of protein, glucose, lipid and various other metabolites recorded during the moult cycle, may be regulated by haemocytes in *P. indicus*. Chitin or chitin-precursor has been proposed as one of the other polysaccharides stored in the haemocytes (Dall 1965, Johnston et al., 1973) which may be released during moult for synthesis of new skeleton. Bauchau and Palquet (1973) and Tsing et al. (1989) have recorded the increase in haemocytes number prior to ecdysis. The haemocytes may also be involved in the process of synthesis and release of hemocyanin as mentioned earlier. The involvement of haemocytes in endocrine activity has also been observed by few authors (Chassard-Bouchaud and Hubert, 1975; Madyastha and Rangnekar, 1972).

From the foregoing discussion it is clear that haemocytes perform a large number of specific functions. The present study involved the identification of haemocytes of *P. indicus* and classification based on their ultrastructure, and provides a basis for future investigation which will no doubt underline their specific and significant role in various physiological processes and defence responses.

SUMMARY

1) Biochemical constituents of the haemolymph of *Penaeus indicus* with respect to sex, size group, moult and reproductive cycle was estimated. The constituents studies were total protein, free amino acids, glucose, total lipid, free cholesterol, copper, calcium, iron, zinc and manganese. There was no significant difference in the level of the biochemical constituents between male and female. The content of the biochemical constituents showed a progressive increase with increase in size of the species ie. the content of biochemical constituent registered a progressive increase from group I (60-80 mm) to group IV (120-140 mm). All the constituents exhibited a cyclic pattern during the moult cycle. During the ovarian development also, all the constituents studied except manganese and zinc recorded an increase from stage I to stage IV (fully mature stage) and declined in the spent stage. Thus physiological processes such as growth, moulting and reproduction are reflected by the altered levels of the biochemical constituents in the haemolymph of *Penaeus indicus*.

2) Electrophoretic separation of the haemolymph proteins of *Penaeus indicus* with respect to sex, size moult and reproductive cycle was carried out by SDS-PAGE. The species-specific pattern revealed four major protein fractions - viz. slow and fast hemocyanin, heteroagglutinin and fibrinogen. These along with several other simple

protein fractions was present in all samples analysed. Wide intra-individual variation in the pattern was noticed. Sex and moult stage, although result in variations in the simple protein fractions, did not produce or reveal any significant variation in the haemolymph protein pattern. Quantitative variation in the protein content due to size and other factors was reflected by the density and thickness of the fast haemocyanin fraction. The slow and fast hemocyanin together contribute to over 60-80% of the total protein content of the haemolymph. The molecular weights of these were determined to be 2,80,000 daltons and 1,37,184 daltons respectively. The haemolymph protein pattern of mature females indicated the presence of female specific proteins (vitellogenins). The copper fractions also revealed peculiar alterations in the haemolymph protein pattern during the maturity stages.

3) The circulating haemocytes in the haemolymph of *Penaeus indicus* was identified and classified based on ultrastructure study. Three types of circulating haemocytes are observed - agranulocyte, semidense granulocyte and dense granulocyte. The agranulocytes are the smallest type of haemocytes generally ovoid in shape, with large nucleus/cytoplasmic ratio. These cells show little signs of differentiation and lack cytoplasmic inclusions, these haemocytes are therefore also known as undifferented cells. The dense granulocytes are the largest circulating haemocytes with high degree of differentiation and large, dense granules in the cytoplasm. The nucleus/cytoplasm ratio is low. The semidense granulocyte is the intermediate form with moderate

degree of differentiation. Dense granules, smaller in size than those found in the dense granulocyte are seen in these haemocytes. These haemocytes are ovoid to spindle shaped. Apart from these circulating haemocytes, a fourth type of haemocyte is found in the haemolymph of females with developing ovary, in all stages of maturity. This haemocyte is three times larger than the normal haemocytes with large dense granules of different nature and abundant glycogen granules in the cytoplasm. The functional significance is discussed. Another haemocyte, the cyanocyte is also observed in the haemolymph which probably synthesizes and releases hemocyanin in the haemolymph. The need for further study of granule composition, the response of haemocytes in injury and moulting and of the origin of haemocytes is stressed.

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