STUDIES ON THE DIGESTIVE ENZYMES OF THE INDIAN WHITE PRAWN *PENAEUS INDICUS* H. MILNE EDWARDS

THESIS SUBMITTED
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
OF THE
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

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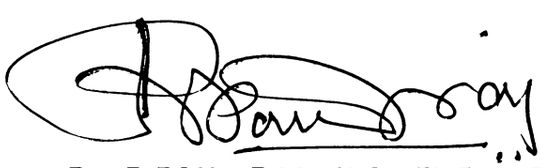
CERTIFICATE

This is to certify that the thesis entitled *"Studies on the digestive enzymes of the Indian white prawn Penaeus indicus H. Milne Edwards"* is the bonafide record of the work carried out by Smt. M. HEMAMBIKA under my guidance and supervision and that no part thereof has been presented for any other Degree.



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DECLARATION

I hereby declare that this thesis entitled ***"Studies on the digestive enzymes of the Indian white prawn Penaeus indicus H. Milne Edwards"*** has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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PREFACE

India is the largest exporter of prawns in the world. According to official sources out of India's annual export of about 80,000 tonnes of marine products, over sixty percent is contributed by prawns. The country's foreign exchange earning from export of marine products increased from Rs.4 crores in 1961 to Rs.400 crores in 1986 (The Hindu, April 18th 1987). The present plans are to raise the foreign exchange earning on this score to Rs.700 crores by 1990 (The Hindu, April 18th 1987). However, a point of concern is the fluctuations noticed in marine prawn landings, which necessitates identification of alternative methods of enhancing prawn production. Scientific farming of prawns in the low-saline coastal waters appears to be the best solution for not only increasing the production but also for generating gainful employment opportunities.

It is estimated that India has 26 million hectares of backwaters, lagoons and estuaries; out of which 3 lakh hectares can be utilized for culturing prawns (Muthu, 1978). But only about 30,000 ha are being currently utilized for culturing prawns employing the traditional extensive and semi-intensive methods. There is thus great scope for expanding prawn culture activities in India.

The penaeid prawns belonging to the genera *Penaeus* and *Metapenaeus* spawn in the sea but their postlarvae enter the estuaries and backwater areas in large numbers and grow rapidly. These areas serve as natural

nurseries for the juveniles. In the traditional culture operations, the naturally occurring postlarvae and juveniles are trapped in tidal impoundments constructed in suitable coastal brackishwater areas and allowed to grow for short periods before they are caught. In India this extensive type of prawn culture is practised in the brackishwater 'bheris' of West Bengal and in the 'pokkali fields' adjoining the Vembanad Lake in Kerala. In Singapore, Malaysia, Thailand and Vietnam, coastal mangrove swamps have been converted into prawn ponds. In Indonesia prawns are grown along with milkfish in the coastal brackishwater ponds called 'Tambaks'.

In semi-intensive culture practices, controlled stocking with known number of prawn juveniles, application of fertilizers and supplementary feeding are done. The yield is highly variable depending on the nature of the ponds, the fertilizers used, the food given and the species cultivated. This method is practised in Philippines, Japan, Korea, Taiwan, Indonesia, Australia, The Americas and to a certain extent in India.

In the most highly developed form of culture, all the stages from egg to the harvestable size are grown under controlled conditions. The prawn fry raised in hatcheries are grown to marketable size in large cement tanks with running water or in circular or rectangular concrete tanks fitted with false bottom and air-lift recirculating system. This method termed the intensive method of culture, is now being mainly practised in Taiwan and Japan.

In intensive culture operations, one of the foremost requirements is the availability of proper formulated feed for the different stages of prawns. The main aim of formulating artificial feeds is to obtain maximum gain in weight, survival and best conversion rate with economic rations. Essentially, the practical feeds should contain adequate levels of required nutrients.

The efficiency of a diet not only depends on its nutrient composition and nutrient balance but also on the effective utilization by the animal. In the utilization of dietary nutrients, the digestive enzymes play the crucial role of catalysing the hydrolytic reactions, splitting the macromolecules into simple absorbable molecules. The activity of these biocatalysts is regulated by alterations in pH, temperature, substrate type and concentrations, and also by the presence of activators and inhibitors. Thus any shift from the optimum conditions necessary for these enzymes may affect their activity, thereby correspondingly modify the digestibility of the nutrients supplied to the animals. Thus, investigations on the important digestive enzymes and their preferential conditions of activity are essential, so that the results obtained could be used in rationally adjusting the quality and quantity of feed supplied to the different stages of prawns.

Research concerning the digestive enzymes in shrimps and prawns, to date, remains mostly qualitative despite numerous investigations (Gopalakrishnan, 1957; Fugi *et al.*,1963; Devillez, 1965; Devillez and Buschlen,1967; Sather, 1969; Gates and Travis, 1969; Sather, 1969; Gates and Travis, 1967; Telford, 1970; Van Wormhoudt, 1973; Brockerhoff *et al.*,1970; Karunakaran and Dhage, 1977; Kulkarni *et al.*, 1979; Murthy and Saxena, 1979; Saxena and Murthy, 1980, 81, 82).

In India, directed research on nutritional physiology and biochemical approaches to digestion in commercially important prawns is taken up only recently, and the field is still in an infant stage. In view of its emerging importance it is identified as an area of priority and the present investigation has been carried out on the Indian white prawn *Penaeus indicus* with the following objectives:

- to delineate the morphology, histology and histochemistry of the digestive system, as well as the ultrastructure of the hepatopancreas of the adult prawn.

- to survey the distribution of digestive carbohydrases, proteases and lipases.

- to understand enzyme activity in different size groups - postlarvae, juveniles, and adults.

- to determine the effect of diet, starvation and eyestalk ablation on the activity of digestive enzymes.

- to characterise alpha amylase of *Penaeus indicus* with reference to the effect of temperature, pH, substrate concentration, enzyme concentration, incubation time, certain metallic ions, amino acids and vitamins on enzyme activity. Isolation and purification of α-amylase using gel-filtration chromatography was also attempted.

The present study contributes significantly to our knowledge of digestive organs and enzymes of *Penaeus indicus*.

The thesis is organised into three major Chapters for convenience:

Chapter 1 deals with the histology and histochemistry of the digestive tract and ultrastructure of the hepatopancreas of *Penaeus indicus*.

Chapter 2 presents the distribution and activity of digestive enzymes and includes a few experimental studies on enzyme activity in different size groups, in prawns fed varying levels of starch in the diet, and also the effect of starvation and eyestalk ablation.

Chapter 3 includes characterization and purification of α -amylase (α-1$\rightarrow$ 4, glucan -4 - glucanohydrolase E.C. 3.2-1.1) of *Penaeus indicus*.

A summary follows the three chapters and all the references are pooled and presented after the summary.

The tables, figures, and plates have been positioned in each of the relevant sections of the three chapters.

ACKNOWLEDGEMENTS

I am deeply indebted to my revered teacher, Dr. R. Paul Raj, Senior Scientist of Central Marine Fisheries Research Institute, Cochin, for suggesting this problem and for his invaluable guidance, thorough supervision, keen interest and constant encouragement during the period of investigation and the preparation of this thesis.

My sincere thanks are due to Dr. E.G. Silas, former Director, Central Marine Fisheries Research Institute and Sub-Project Co-ordinator, Centre of Advanced Studies in Mariculture, Cochin, for his helpful suggestions and for providing laboratory facilities to carry out the research work. I am also grateful to Dr. P.S.B.R. James, Director, Central Marine Fisheries Research Institute, Cochin for allowing me to utilise the various facilities at the Institute.

I have great pleasure in acknowledging my deep sense of gratitude to Dr. N.P. Jayashankar, Joint Director and Dr. J.J. Solomon, Scientist-S3 at Central Plantation Crops Research Institute, Kayamkulam for providing all the facilities to carry out electron-microscopical studies. My thanks are also due to Dr. P.V. Ramachandran Nair, Senior Scientist, C.M.F.R.I., for making necessary official arrangements for the electron-microscopical work at C.P.C.R.I., Kayamkulam.

Dr. A. Noble, Dr. P.V. Rao, Dr. A.D. Diwan, Shri. D.C.V. Easterson and Dr. Scariah, Senior Scientists of CMFRI have been helpful in several

ways and I record here my sincere appreciation for the same.

I acknowledge with thanks the invaluable help and kind co-operation of my junior and senior research colleagues and staff at C.A.S in Mariculture during the study.

I also wish to express my heartfelt thanks to my husband Mr. N. Venunath and my parents Mrs. and Mr. A.K.R. Nayar for the several courtesies extended to me during the course of this investigation.

I will be failing in my duty if I do not express my deep sense of gratitude to the Indian Council of Agricultural Research, New Delhi for awarding a Senior Research Fellowship throughout this investigation.

M. HEMAMBIKA

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CHAPTER 1

HISTOLOGY, HISTOCHEMISTRY AND ULTRASTRUCTURE STUDIES ON THE DIGESTIVE TRACT

1.1. HISTOLOGY AND HISTOCHEMISTRY OF DIGESTIVE TRACT

INTRODUCTION

The crustacean alimentary canal is an intricate system and in many cases, typically, consists of three major divisions: the foregut, the midgut and the hindgut. There are, however, certain variations in the histological organization of the digestive tract in the various groups. The order Decapoda has attracted the attention of few investigators as far as morphology and anatomy of the digestive system are concerned.

Studies on the alimentary canal of Decapoda date back to 1876 when Parker described the stomach of freshwater crayfish. Pearson (1908) in his monograph on the crab, cancer, dealt, in detail, with the anatomy of the alimentary canal. This was followed by the work of Farkas (1914) on the anatomy and histology of the oesophagus in some fresh water crabs. Yonge (1924, 1932) described the nature of the lining of the foregut, and the functional aspects of tegumental glands in the lobster, *Nephrops norvegicus*. The physiology and cytology of digestion in *Paratelphusa hydrodromus* was studied by Reddy (1937, 1938). The anatomy and histology of the digestive system of *Galathea* was worked out by Pike (1947). The digestive system of *Panulirus polyphagus* was worked out in detail by George *et al.* (1955). Van Weel (1955) made important contributions to the cytology and physiology of the digestive diverticula in *Astacus leptodactylus* and *Atya spinipes*. Pugh (1962) contributed to the knowledge of the hindgut of fiddler crabs.

Agarwal (1967) and Diwan (1972) have worked out the anatomy and histology of the digestive system of freshwater crabs, *Potamon martensi* and *Barytelphusa cunicularis* respectively. The functional morphology of the foregut in three species of crustaceans was given by Schaefer (1970). Barker and Gibson (1977, 1978) furnished information on the feeding mechanism, structure of the gut and digestive physiology of *Homarus gammarus* and *Scylla serrata*. Mykles (1979) furnished information on the ultrastructure of the alimentary epithelia of the lobsters, *Homarus americanus*, *H. gammarus* and the crab, *Cancer magister*.

Relatively little information is available on the digestive system of prawns. Patwardhan (1937) described in detail the digestive system of *Palaemon malcolmsonii* and Pillai (1960) studied the digestive system of the shrimp *Caridina leavis*. Some preliminary histological observations of *Palaemonetes* species have been carried out by Phill and Pugh (1973). However, knowledge about the structure and anatomy of the digestive system of penaeid prawns is very meagre. Young (1959) in his monograph on the morphology of *Penaeus setiferus* gave a brief account of the gut. The musculature and armature of the proventriculus in Penaeidae have been described by Kubo (1949) and Young (1959). Gopalakrishnan (1957) gave a brief account of the structure and histology of alimentary system of *Penaeus indicus*. Functional anatomy of the digestive tract of the shrimp *Metapenaeus bennettae* has been studied by Dall (1967b). Talbot *et al.* (1972) described the fine structure of the midgut epithelium in the developing brown shrimp *Penaeus aztecus*. Rigdon and Mensik (1976) carried out

studies on the histology of the gastrointestinal tract of *Penaeus aztecus*. An elaborate account on the comparative cell morphology of freshwater and marine shrimp midgut epithelia has been given by Cooke and Ahearn (1976).

Despite the foregoing literature on the structure and anatomy of the alimentary canal, there is no information on the histochemical nature of the various layers in the digestive tract of penaeid prawns. Histochemical studies of the different layers in the alimentary canal would reveal the functional nature of the various regions. This aspect, has been dealt with as an integral part of the present study. The interesting nature and structure of the hepatopancreas in itself has been made as a separate section where its histology, histochemistry and ultrastructure have been presented.

MATERIALS AND METHODS

Live specimens of *Penaeus indicus*, measuring 100-110 mm in total length, were collected from Narakkal Prawn Farm of the Central Marine Fisheries Research Institute and transported in collapsible polythene bags under oxygen. They were maintained in plastic pools with constantly aerated sea water (salinity 25-32‰) at ambient temperature for 2-3 days.

For histological study intermoult prawns were selected. Prawns were dissected alive over ice and the oesophagus, stomach, midgut, hepatopancreas and hindgut tissues were separately cut with a sharp blade, carefully washed and immediately fixed in Bouin's and Zenker's fixatives. All the tissues, except the

hepatopancreas, were fixed for 24 hours;hepatopancreatic pieces were fixed for more than 48 hours in 4 different changes of fresh fixative at 12 hrs interval, dehydrated in graded series of alcohol from 70% to 100%, cleared in xylene/methyl benzoate and embedded in paraffin wax in a hot air oven at 58-60°C. Stomach tissues fixed in Bouin's fluid were treated with 2% nitric acid-alcohol for 24 to 48 hours for decalcification, washed and then processed as above. The blocks prepared were sectioned at 8-10 μ.

For studying the histological details Heidenhain's iron haematoxylin, Eosin, Mallory's-triple and Delafield's haematoxylin-Eosin stains were employed. Best results were obtained with Heidenhain's iron haematoxylin and Mallory's triple. Sectioning was done using a microtome and spread over albumin coated micro-glass slides. Sections were stained after dewaxing with xylene, dehydrated in graded alcohol series, cleared in xylene and mounted in canada-balsam. The stained sections were phtographed in a Olympus (Model Vanox PM 10) microscope with automatic photomicrographic attachment, using Kodacolor 100 ASA and Ilford 100 ASA black and white films.

For histochemical studies, tissues were fixed in 10% neutral buffered formaldehyde and processed as in histological study. Cryocut sections were prepared from tissues fixed in cold 10% formalin for over 24 hours using American Opticals Histostat freezing microtome to study the fat localization in the tissues. Sections of 6-8 μ were cut at -10°C. The histochemical staining procedures given in Pearse (1968) were mainly followed.

Histochemical identification and characterization of proteins were made by (1) mercuric bromophenol blue test for proteins; (2) aqueous bromophenol blue test for basic proteins; (3) ninhydrin-schiff test for amino groups; (4) toluidine blue test for acidic groups; (5) ferric-ferricyanide method for - SH groups; (6) thioglycollate ferric-ferricyanide method for - SS groups; (7)Millon's test for tyrosine; (8) DMAB - nitrite method (Dimethylaminobenzaldehyde-nitrate) for tryptophan.

Histochemical staining procedures adopted for carbohydrates were (1) periodic acid-schiff technique for carbohydrates; (2) Best's carmine test for glycogen; (3) toluidine blue at different pH for acid mucopolysaccharides; (4) critical electrolyte concentration (CEC) method for acid mucopolysaccharides; (5) Alcian blue-PAS test for neutral and acid mucopolysaccharides.

Histochemical characterisation of lipids was made by (1) sudan black B test for lipids; (2) nile blue method for neutral and acidic lipids (3) nile blue sulphate method for phospholipids; (4) oil red '0' method for neutral lipids.

Histochemical characterization of nucleic acids was carried out using methyl green-pyronin G test.

Suitable blocking procedures were used for each test to prove the presence of specific reactive groups. The blocking procedures included deamination, methylation, demethylation, mercaptide iodination, formaldehyde thioglycollate reduction, acetylation, deacetylation, chloroform-methanol extraction and Taka-diastase treatment.

The histochemical observations for proteins, carbohydrates and lipids, obtained for each region of the gut layers and cell types of hepatopancreas, were tabulated indicating the intensity of reaction.

OBSERVATIONS AND DISCUSSION

The alimentary canal of *Penaeus indicus* has three distinct regions: the foregut, the midgut and the hindgut (Fig. 1). The foregut comprises the mouth, buccal cavity, oesophagus and stomach. The foregut and hindgut have an internal lining of cuticle (intima) but the midgut has a soft lining of endoderm.

Mouth

The mouth is a large slit like aperture situated midventrally below the anterior end of the head between the third and fourth segments. It is bounded by a shield-shaped labrum, on the sides by the incisor process of the mandibles and behind by the labium which is cleft to form two lobes of paragnatha.

Buccal cavity

The mouth leads into a short buccal cavity. It is antero-posteriorly compressed and has a thick chitinous lining which is thrown into irregular folds. The mandibular processes which are seen projecting into the buccal cavity, by their opposing dental plates help to crush the food materials.

Oesophagus

The oesophagus is a very short tube running vertically dorsalwards from the buccal cavity to the stomach (Fig. 1). The cellular arrangement (Fig.3) resembles the oesophagus of *Palaemon* (Patwardhan, 1937). The oesophageal walls of *Penaeus indicus* have four thick muscular longitudinal strands projecting into the lumen, separated from each other by two grooves on the inside. The inner walls of the strands are lined by cuticle as in allied decapods (Yonge, 1932; Richards, 1951). This cuticular layer consists of an epicuticle and an endocuticle, the latter with prominent longitudinal striations. Underlying the cuticle is the epithelial layer formed of columnar cells. Below the layer of epithelial cells is the basement membrane and the connective tissue layers comprising of reticular mass of tissue. Beneath the basement membrane circular and longitudinal muscle fibres are present. The dilator muscle fibres are connected to the cuticular layer.

The tegumental glands are found in the connective tissue beneath the epithelial cells (Fig.3). This globular gland is formed of conical cells with their apices directed towards the outerside. The tegumental glands in the foregut of decapods have been described in detail by Balss (1927) and Yonge (1932). This globular gland appears similar to the one found in other decapods.

The epithelium (Plate 1) is overlain by a lightly basophilic cuticular zone. The region between epicuticle and endocuticle is separated by a zone of strongly PAS - positive material (Plate 1). The tegumental glands are abundant in the connective tissues in the anterio-lateral walls of the oesophagus. These glands

stain intensely with periodic acid schiff and Alcian-blue-PAS reagent, indicating the presence of a considerable amount of acid mucopolysaccharides. The ducts of these glands also show positivity to PAS test.

Columnar cells in the epithelial layer showed faint and homogeneous reaction to mercury-bromophenol blue, toluidine blue, millon's test, and dimethyl amino benzaldehyde nitrite test. The nucleus and the nuclear sap stained intensely with pyronin-G. This layer showed absolute negative response to lipid tests. However, the membranes of the columnar cells showed faint blue colouration with nile blue sulphate indicating the presence of phospholipids.

Stomach

The oesophagus leads into the stomach, which has two divisions, the 'cardiac' and the 'pyloric' regions as in other penaeids. At the region of the opening of the oesophagus into the cardiac stomach are present six sharp spines, the oesophageal teeth (Fig. 2). The histology of the stomach wall is essentially the same as that of the oesophagus. The arrangement of epithelial layer, connective tissue layer, circular and longitudinal muscle fibres are all same as described for oesophagus. The epithelium of the cardiac stomach has a thick cuticular lining which at certain regions gets thickened into ossicles to form the gastric mill (Fig. 4).

As in *Palaemon*, the cardiac stomach of *Penaeus indicus* (Fig. 1) extends considerably and forms a broad chamber with a narrow floor area, longitudinal groove and a lateral fold, the latter with a row of sharp denticles, termed supra-lateral teeth by Reddy (1935).

The mesocardiac ossicle is a median ossicle on the dorsal wall of the cardiac foregut and has its apex pointing forwards (Fig.2) as described by Patwardhan (1935) and is fused posteriorly to the anterior end of the urocardiac ossicle. The posterior end of the plate like urocardiac ossicle projects into the chamber as a median tooth. On this median tooth are present lateral denticles. Attached to the base of the mesocardiac ossicle, the pterocardiac ossicles are present. The two zygocardiac ossicles which are attached to the pterocardiac ossicles anteriorly and pyloric ossicle posteriorly have in each a single median tooth and a row of curved small denticles projecting into the cavity of the cardiac stomach. A small triangular propyloric ossicle which is connected to the pyloric ossicle is present as shown by Gopalakrishnan (1957) and Patwardhan (1935). A valve like arrangement formed by a flap with long setae separates the cardiac and pyloric regions of the stomach.

The lateral walls of the pyloric portion are considerably thickened and divide the cavity into two regions, the dorsal and ventral. The lateral walls and floor of the ventral divisions have thick cuticular lining internally which constitute the characteristic filtering apparatus (Fig.2). The opening of the foregut into the midgut is provided with a single median and two pairs of lateral valves, together forming a sieve-like arrangement (Fig.2).

Stomach epithelial cells showed positive response to the histochemical tests (Table 1) for protein detection. The connective tissues are rich in basic and acidic proteins. Specific protein groups are moderately represented in the different layers. The apical region of the epithelial layer shows intense positivity to

PAS. Lipid substances are faintly distributed in the cell cytoplasm of the epithelial layer.

Midgut

The midgut is a long and narrow tube with slender walls, stretching from the thoracic region to almost the entire length of the abdomen. The structure of the wall is similar to that in *Palaemon*(Patwardhan, 1937). There is no cuticular lining. The epithelium is formed of columnar cells containing refractive bodies. Basal or replacing cells are present as in the midgut of other decapods (Plates 2 & 3). The epithelium of the midgut is thrown into villi-like longitudinal folds, which are more pronounced in the anterior region than in the posterior region. This layer is followed by a basement membrane and then the circular and longitudinal muscles and connective tissue.

The midgut comprises 40-50% of the post-gastric intestinal tract and is 1-2 mm in external diameter. It's epithelium in the anterior region histologically differ from that of posterior region. Anteriorly it is composed of cells containing lightly basophilic and homogeneous cytoplasm, in which the ovoid nuclei lie centrally. Posteriorly, however, the cell cytoplasm is proximally displaced by one or more vacuoles which flank the eccentric nucleus. The distal cytoplasm of the posterior midgut epithelial cells stains moderately and homogeneously with PAS. No inclusions can be discerned within the vacuoles.

The epithelium is covered externally by a PAS positive brush border which stains only weakly with Alcian-blue. The connective tissue stains deeply with mercury bromophenol blue and reacts faintly well to tests for - SH and - SS groups.

Glycogen could be seen distributed in the epithelial cells. Lipid inclusions are detected in some of the cells. Rich concentrations of DNA and RNA are found in the cell cytoplasm. Thick, acidophilic musculature is found beneath the basement membrane.

Hindgut

The hindgut is a very short tube with thick muscular walls, connecting the midgut and the anus (Fig.5). The cuticular lining of this region is thrown into folds and consists of the outer layer of epithelial cells and the inner pigmented cells with large nuclei. The epithelial layer shows only small longitudinal folds projecting into the lumen. The next layer is the basement membrane followed by the circular muscles, longitudinal muscle and connective tissue coat(Plate 4).

Cuticle is of a homogeneous nature; externally it is bounded by an acidophilic, PAS - positive epicuticle. The interface between the cuticle and epithelial tissue is marked by a narrow layer of acid mucopolysaccharide material. The epithelial layer responded moderately to protein tests and lipid tests. The cells exhibited slight positivity to pyronin-G test.

The anus is a slit like opening, the walls of which possess extensions of the longitudinal muscle fibres of the hindgut. In addition, the edges of the opening are provided with radiating muscle fibres.

The anatomical and histological features of the alimentary canal of *Penaeus indicus* resembles, in general, those of *Palaemon* (Patwardhan 1937). The structural differences which have been noted are probably due to the food and feeding habits of the animal. Considerable variations have been observed in the relative length of the midgut in different decapods. In many of the

forms that have been studied, the midgut is the shortest portion (Pearson, 1908; Reddy 1937). In *Penaeus indicus* the midgut is the longest portion of the alimentary canal as in *Nephrops* and *Neptunus* (Wallengren, 1901; Yonge, 1924; George, 1949).

The foregut of *P. indicus* includes the mouth, buccal cavity, oesophagus and stomach. The general structure of these regions is in close agreement to that of *Palaemon* (Patwardhan, 1937). The tegumental glands found in the connecting tissue of the oesophagus of *P. indicus* corresponds to those found in *Palaemon* (Patwardhan, 1937), *Nephrops norvegicus* (Yonge, 1924), *Paratelphusa hydrodromus* (Reddy 1937), species of *Uca* (Pugh, 1962), *Metapenaeus* (Dall, 1967), *Homarus gammarus* and in *Scylla serrata* (Barker and Gibson, 1977, 1978). The histochemical tests reveals that the tegumental glandular secretion contains neutral mucopolysaccharides in rich quantities. This indicates its role of secretion during digestion. These secretions may also be useful in the free passage of food material due to their mucous nature. Similar observation has been made by Erri Babu *et al.* (1982) in the crab, *Menippe rumphii*.

The histology of the midgut and hindgut is almost identical to that found in other crustaceans. The histochemical nature of the midgut epithelium shows that it is highly specialized for fluid secretion and absorption of food materials. The villi-like projections in the epithelial lining of the midgut aids in the absorption of digested food materials. Dall (1967b), using light microscopy, found that the midgut in *Metapenaeus* shrimps was lined by a dense columnar epithelium, and Talbot *et al.* (1972) using electron microscopy distinguished "light" and "dark" cells in the midgut of *Penaeus aztecus*,

Both cells had microvillous borders, suggesting absorptive functions. The epithelium is surrounded by a layer of circular muscle with bundles of longitudinal muscle embedded in connective tissue with numerous blood spaces. In *Penaeus indicus* light microscopic observations indicate that the anterior cells are quite different from the posterior cells in their staining ability to histological stains . Again, Ahearn and co-workers have shown that the isolated midgut of shrimps (*Penaeus marginatus* and *Macrobrachium rosenbergii*) are capable of absorbing amino acids (glycine or lysine) and glucose (Brick, 1973; Ahearn, 1974; 1976; Ahearn and Maginniss, 1977; Brick and Ahearn, 1978). According to Dall (1967 a,b) and Ahearn *et al.* (1978) water and electrolytic regulations may be more important roles for this part of the gut. Besides, secretion of a peritrophic membrane by the anterior epithelium of the midgut is a definite function in many decapods (Dall and Moriarty, 1983).

The diverticula or ceca found in association with the midgut in Brachyura (Smith, 1978) are totally absent in *Penaeus indicus*. The anterior diverticulum ranges from a rudimentary bulge in the crayfish (Huxley, 1884) to a well defined diverticulum lined with very tall columnar epithelium in shrimp (Dall, 1967b), and to the long coiled structure of the Brachyura (Pearson, 1908; Smith, 1978). In crabs, lobsters, penaeid shrimp, and some caridean shrimps a posterior diverticulum is also present (Pillai, 1960; Dall, 1967b; Barker and Gibson, 1977; Smith, 1978).

The cuticle-lined, short, hindgut observed here has internal longitudinal folds which serve to grasp the fecal pellet in its peritrophic membrane and

rhythmically expel it. This is in agreement to the smooth-surfaced pads containing spongy tissue found in *Metapenaeus* (Dall, 1967b), which also served for defecation and to pump water into the gut. Further research on the alimentary system at ultrastructural level would throw more light on the dynamic nature of the different layers.

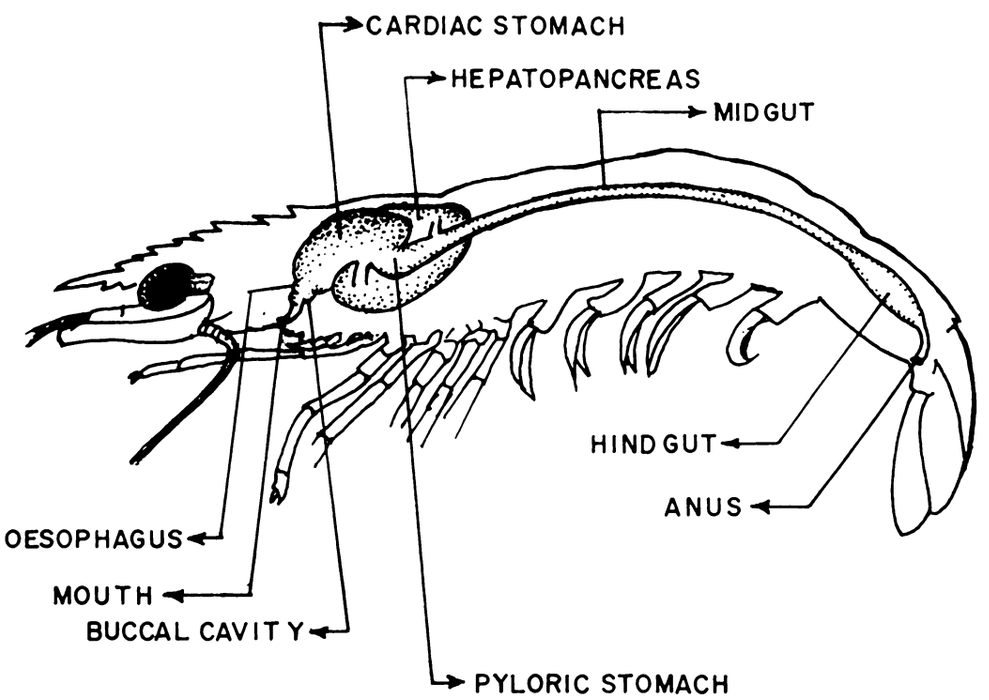
TABLE 1. Histochemical reactions of the epithelial layer at various regions of the alimentary canal of *Penaeus indicus*.

Histochemical tests	Oesophagus epithelial layer	Stomach epithelial layer	Midgut epithelial layer	Hindgut epithelial layer	Indicates
Proteins					
1. Mercury bromo phenol blue	++ B	++ B	+++ B	++ B	Proteins
2. a) Aqueous bromo phenol blue(ABB)	+ B	++ B	++ B	± -	Basic proteins
b) ABB after deamination	-	-	-	-	
3. a) Toluidine blue	+	++ P	++ P	++ P	Acidic groups
b) Toluidine blue after methylation	-	-	-	-	
4. a) Ferric-ferricy-anide test (FF)	-	+	+	+	- SH group
b) FF after mercaptide	-	-	-	-	
5. a) Thioglycollate ferric-ferricyanide-(TFF)	-	+ PB	++ PB	+ PB	- SS groups
b) TFF after thioglycollate reduction	-	-	-		
6. a) Millon's test	+ R	++ R	++ R	++ R	tyrosine
b) MT, after iodination	-	-	-	-	

TABLE 1. continued...

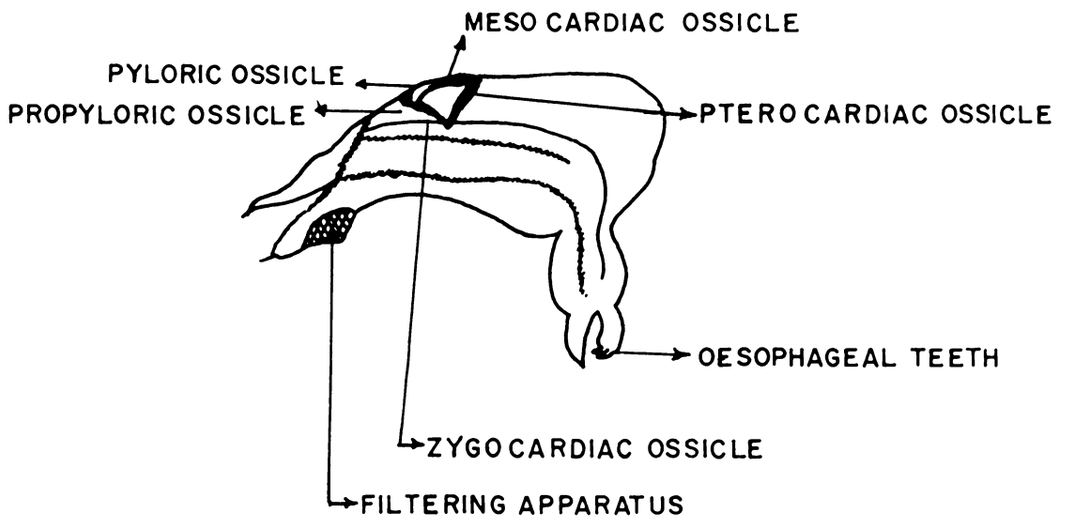
Histochemical tests			Oesophagus epithelial layer	Stomach epithelial layer	Midgut epithelial layer	Midgut epithelial layer	Indicates
7.	a)	DMAB - nitrite	±	+ B	+ B	+ B	trypto-phan
	b)	DMAB - nitrite after formaldehyde treatment	-	-	-	-	
Carbohydrates							
8.	a)	Periodic-Acid-Schiff(PAS)test	++ M	+++ M	+++ M	++ M	Glycogen
	b)	PAS after deacetylation	±	±	±	±	
9.		Alcian-blue PAS test	++ B	++ B	+++ B	+++ B	Acid and neutral AMP
Lipids							
10.	a)	Sudan black B(SBB)	-	-	±	±	Lipids
	b)	SBB after chloroform/ methanol extraction	-	-	-	-	
11.	a)	Nile blue sulphate method (NBS)	+ B	+ B	+ B	+ B	Phospho-lipids
	b)	NBS after chloroform/ methanol extraction	-	-	-	-	
12.		Pyronin G test	++ R	++ R	++ R	++ R	RNA & DNA

B	-	Blue	-	negative
P	-	Pink	±	weak
PB	-	Prussian blue	+	moderately positive
R	-	Red	++	positive
M	-	Magenta	+++	intensely positive



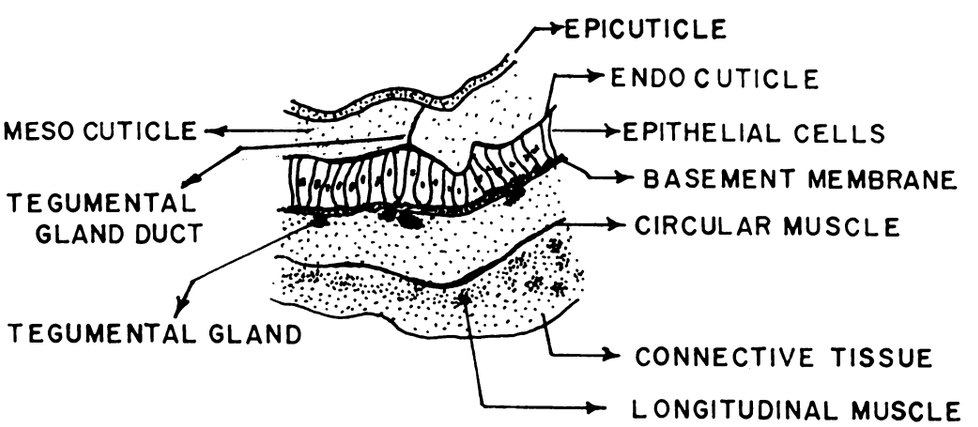
DIAGRAMMATIC SKETCH OF *PENAEUS INDICUS* SHOWING THE ALIMENTARY CANAL.

FIG. 1



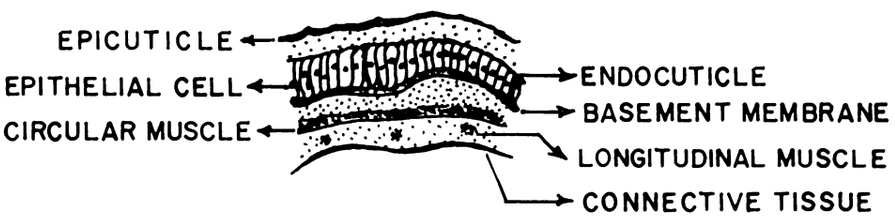
L.S. OF STOMACH SHOWING THE POSITION OF VARIOUS OSSICLES

FIG. 2



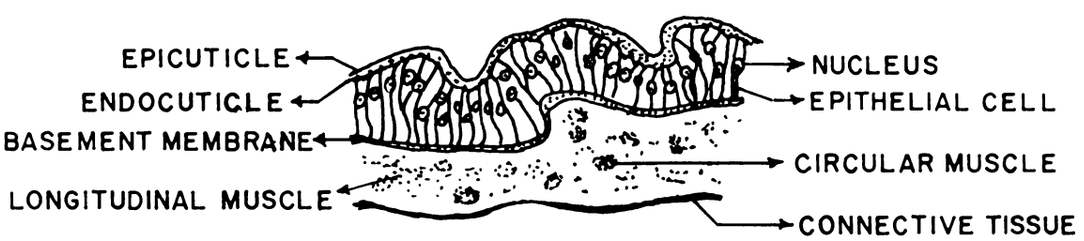
T.S OF OESOPHAGUS WALL (A PORTION ONLY)

FIG. 3



PORTION OF CARDIAC STOMACH (T.S)

FIG. 4



A PORTION OF HIND GUT WALL

FIG. 5

Plate 1: Oesophageal epithelial layers showing positive reaction to PAS test.

Plate 2: T.S. of midgut layer showing the villi like longitudinal folds.

Plate 3: T.S. of midgut layer showing the basement membrane and replacing cells.

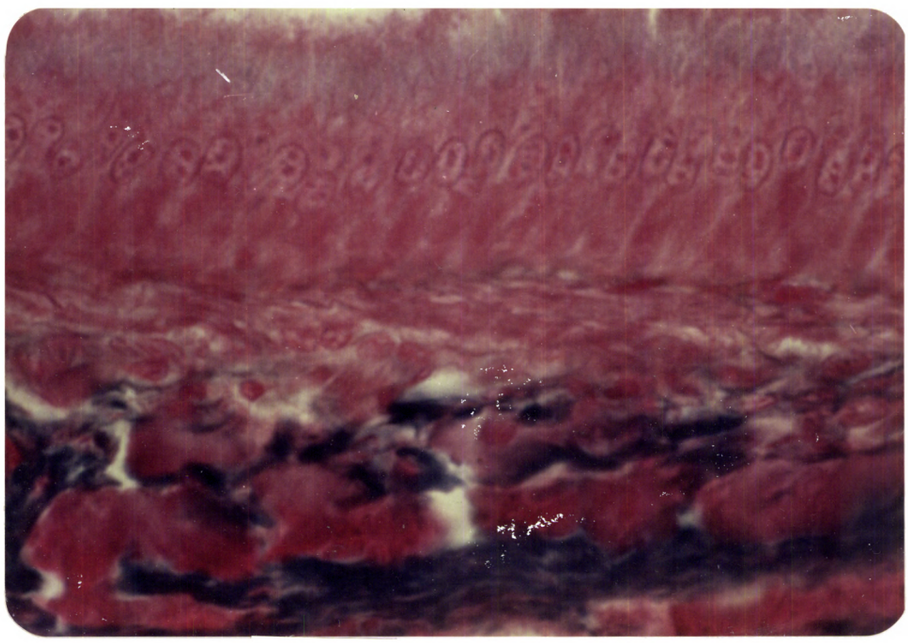


PLATE . 1

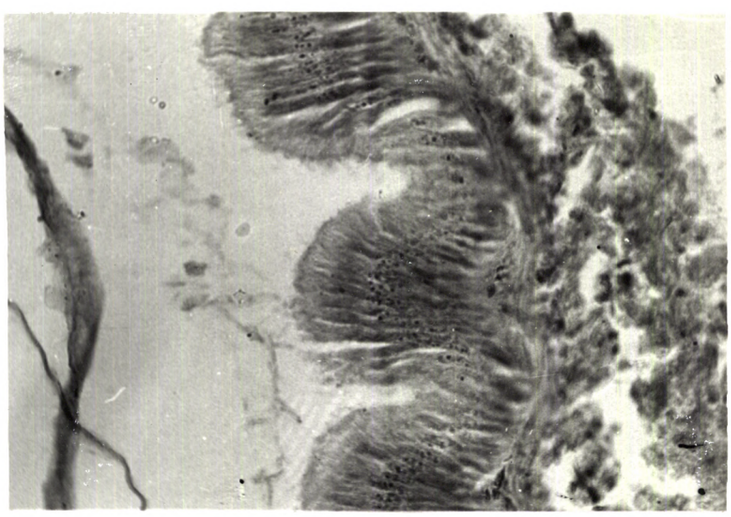


PLATE . 2

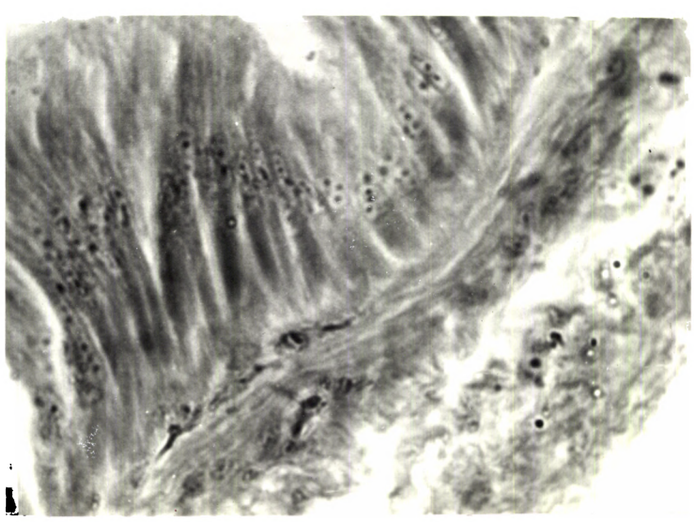
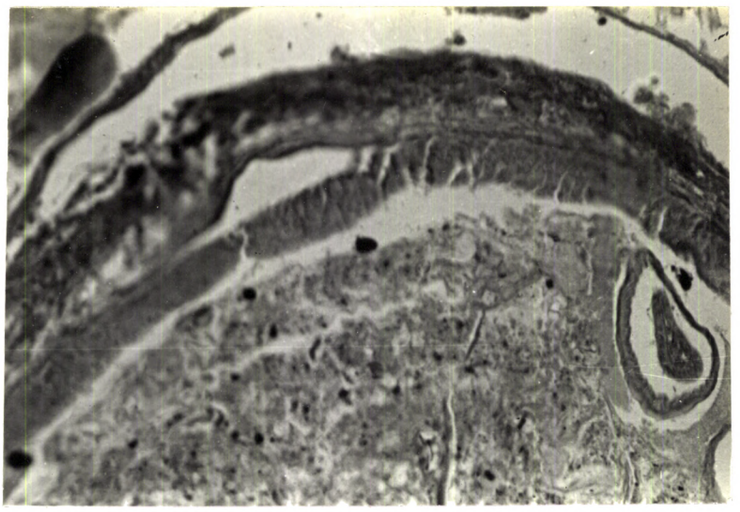


PLATE . 3

Plate 4: T.S. of hindgut layer showing the basement membrane and the longitudinal folds, and their positive reaction to PAS test.

Plate 5: C.S. of the hepatopancreatic tubule surrounded by tunica propria and the four-rayed appearance of the cells. Tubules stained with Heidenhein Azan.



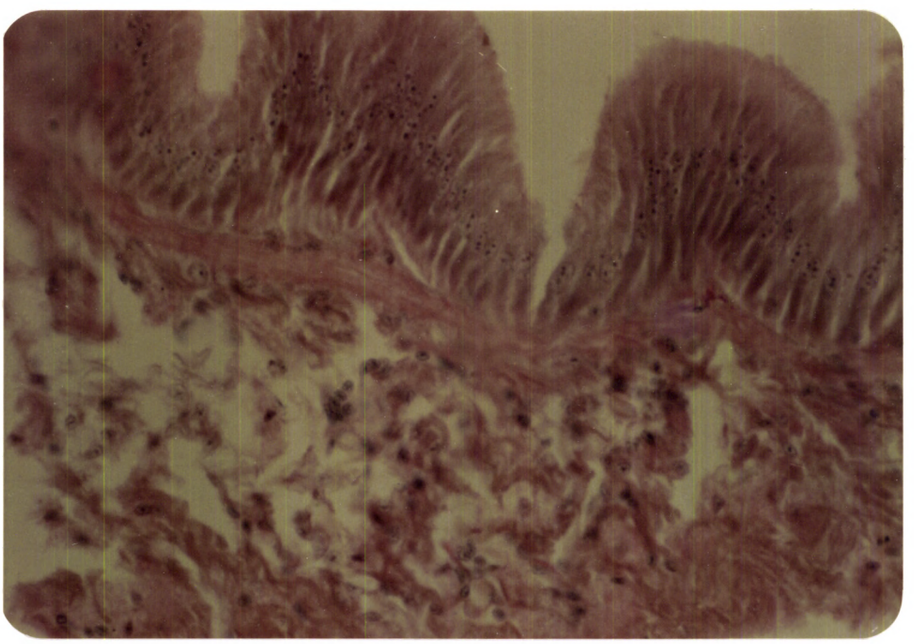


PLATE . 4

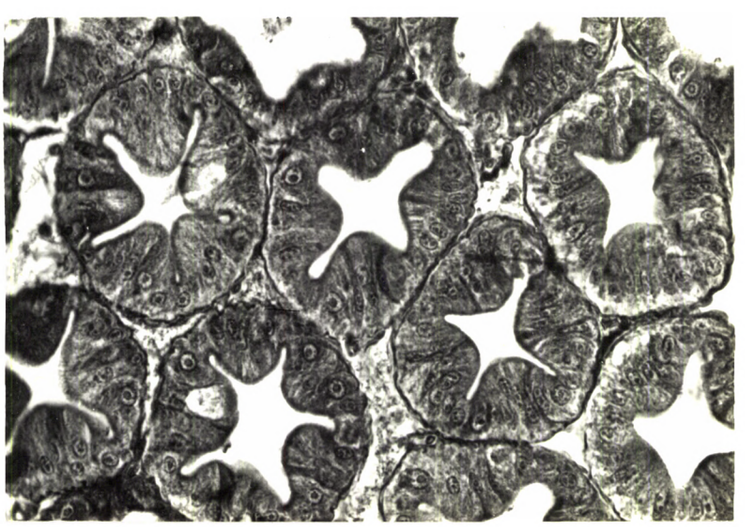


PLATE . 5

1.2. HISTOLOGY, HISTOCHEMISTRY AND ULTRASTRUCTURE OF HEPATOPANCREAS

INTRODUCTION

The crustacean hepatopancreas has attracted the interest of scientists for at least a century and a half, yet, as Loizzi (1966) commented, "the total information accumulated on this organ is meager, when compared to other areas of biology". That the hepatopancreas is a vital and major organ in the Crustacea is beyond doubt; involved in diverse metabolic activities, it is primarily responsible for the synthesis and secretion of digestive enzymes and subsequent uptake of nutrient materials, but is also implicated in excretion, the moulting cycle, the storage of inorganic reserves, and in the lipid and carbohydrate metabolism (Gibson and Barker, 1979).

Variously called the liver, pancreas, midgut gland, gastric gland, digestive gland, ceca anteriores, digestive diverticula, digestive organ, midintestinal gland or hepatopancreas, the present terminology established is the hepatopancreas because of its dual role as liver and pancreas of higher vertebrates (Gibson and Barker, 1979).

An in depth review of research on decapod 'midgut gland' has been presented by Gibson and Barker (1979). The cytology of the hepatopancreas of decapod crustaceans has been studied extensively by light and electron microscopy. Much of the work of earlier investigators was directed to discovering the function of each cell-type and the sequence of transformation

of one cell type into another. Much of the basic work on the histology of the hepatopancreas of the Decapoda was done between 1880 and 1930 (Gibson and Barker, 1979).

Apathy and Farkas (1908) were the first to distinguish four cell types in the hepatopancreas of *Astacus* which they named "Fibrillenzellen", "Blasenzellen", "Anfangzellen" and "Alveolenzellen". The first two were named according to their morphological characters one being fibrillar, the other containing a blister-like vacuole. Anfangzellen, believed to be resorptive in function and as site of active mitosis and "Alveolenzellen" was referred to as location of food storage.

Jacobs (1928) described four cell types from the tubule epithelium of *Astacus leptodactylus* and named them "Embryonalenzellen", "Fibrillenzellen", "Blasenzellen" and "Restzellen". It is from the nomenclature of Jacobs (1928) and Hirsch and Jacobs (1928) that the current labelling of hepatopancreatic cell types as E, F, B and R cells is based. Light microscopic studies by Vonk (1960) revealed two distinct cell types, the absorptive-storage cells (Restzellen, R-cells) and secretory cells (Blasenzellen, B-cells). Few others have reported four cell types, the embryonic (E-cells), absorptive (R-cells), secretory (B-cells) and fibrillar (F-cells) (Travis, 1955, 1957; Ogura, 1959; Miyawaki *et al*, 1961; Davis and Burnett, 1964). Furthermore, five different cell types were classified by Van weel (1955); the embryonic, transitory, "dark" secretory cells (D-cells), "light" absorptive cells (L-cells), and extrusion cells (E-cells).

Patwardhan (1937) recorded two types of cells in *Palaemon*. George *et al.* (1955) working with *Panulirus polyphaqus* recorded two cell types

in the gland. Gopalakrishnan (1957) observed absorptive and secretory cells in the hepatopancreas of *Penaeus indicus*. Dall (1967) recorded two major cell types from the hepatic tubule of *Metapenaeus bennettae* and Madhyastha and Rangneker (1974) recorded four cell types (E, F, B & R) from *Metapenaeus monoceros*. Barker and Gibson (1977, 1978) observed four cell types in *Homarus gammarus* and *Scylla serrata*. Hopkin and Nott (1980) too observed four distinct types of cells in *Carcinus maenas*. Again among brachyurans, Erri Babu *et al.*(1982) recorded four cell types in *Menippe rumphii*.

Histochemical studies on the hepatopancreas have been very limited. Travis (1955, 1957) studied the hepatopancreas of the spiny lobster *Panulirus argus* in relation to histological and physiological changes associated with the moulting process. Madhyastha and Rangneker (1974), working with *Metapenaeus monoceros*, reported the histochemical changes in the hepatopancreatic cells during moulting.

Miyawaki *et al.* (1961) working with *Procambarus clarkii* histochemically demonstrated intracellular copper in the distal cytoplasm of the absorptive cells and iron in the fibrillar cells, contained in vacuoles which were rich in either mucopolysaccharide or mucoprotein material. F-cell cytoplasm contained ribonucleic acid and acid phosphatases. Davis and Burnett (1964) by employing histochemical techniques demonstrated the presence of esterases and phosphatases in the cells of the hepatic tubules in the crayfish.

Histochemical investigations on the hepatopancreatic tissues of *Scylla serrata* have been undertaken by Momin and Rangneker (1974, 1975a,b) and

Rangneker and Momin (1974) and their work was oriented towards the effects of eyestalk ablation on the distribution of enzymes, particularly phosphatases, lipases and esterases.

Miyawaki and Tanoue (1962) working with *Procambarus clarkii* carried out the first ultrastructural investigations of the decapod hepatopancreas, but their studies were mainly concerned with metal containing cells. The Fe-cells were characterized by their large electron-dense iron granule and an extensive endoplasmic reticulum. The cu-cells contained a copper 'granule' bounded by a double membrane which appeared to be a complex organelle possessing many membrane vesicles and particles.

Loizzi (1966) working on the changes during secretion in the hepatopancreas of *Orconectes virilis*, combined ultrastructural studies with physiological experimentation to the manner of Hirsch and Jacobs (1928a) and basically reaffirmed their observations.

Histological and ultrastructural studies (Loizzi, 1966) indicated that the B-cell vacuole was formed from the vacuole situated in the distal cytoplasm of the F-cells, which in *Orconectes* was rich in iron. The fibrillar cells displayed an elaborate endoplasmic reticulum, vesiculated golgi apparatus, high ribosome content and swollen intra-cisternal spaces, all of which Loizzi regarded as concerned with protein or enzyme synthesis. B-cells were found to contain an assortment of granules and the remnants of an endoplasmic reticulum in their vacuoles.

The R cell which functions in food absorption, is tall columnar in shape with a centrally or basally located nucleus and an apical striated border (Travis, 1955). That this cell functions also as a storage site may be concluded from its high content of lipid droplets; glycogen and calcium phosphate crystals.

The B-cell (extrusion secretory cell) is characterized by a basally located nucleus and large cytoplasmic vacuoles filled with acidophillc flocculant material (Van Weel, 1955; Davis and Burnett, 1964) and also an apical striated border (Travis, 1955). These cells store only moderate amounts of lipids, glycogen and calcium phosphate and are suggested to function in the formation and release of digestive enzymes.

The fibrillar cells ("dark")-immature secretory cells of Van Weel are described as scattered among the absorptive and secretory cells and characterized by basophilic striations of the cytoplasm due to a high content of RNA (Van Weel, 1955; Davis and Burnett, 1964). The nucleus of this cell type is basally located with an extremely large nucleolus.

Stanier *et al.* (1968) investigated the fine structure of hepatopancreas of *Carcinus maenas* and reported four cell types in the tubule. Bunt (1968) while elucidating the fine structure of hepatopancreas in *Procambarus clarkii* also revealed four cell types in the tubule.

Papathanassiou and King (1984) while studying the effects of starvation on the fine structure of the hepatopancreas in the common prawn *Palaemon serratus* reported four distinct cell types. Vogt *et al.*(1985) reported four different cell types in the tubule epithelium of *Penaeus monodon.*

Al-Mohanna *et al.*(1985) observed E, F, R & B cells in the hepatopancreas of *Penaeus semisulcatus* De Haan. They also described a fifth type known as 'M' or Midget cell as it appears in unfed animals at intermoult. Anger *et al.* (1985) while studying the effect of starvation on moult cycle and hepatopancreas of stage I lobster *Homarus americanus* clearly identified four different cell types in the epithelium of hepatopancreas.

In the present study, the hepatopancreas of *Penaeus indicus* has been subjected to detailed histological and histochemical observations to delineate the distribution and nature of the cells in the tubule. The gland has been examined with the electron microscope to study the characteristic of its cells. It was felt that the increased resolution offered by this instrument might help to determine more precisely the actual number of cell types in the hepatopancreas as well as indicating their functional roles.

MATERIALS AND METHODS

Histological and histochemical studies were made following the procedures given in the previous section (Pages 4-6). For ultrastructure studies *Penaeus indicus* of size 100-120 mm collected from the Narakkal ponds were maintained in the laboratory for 2 to 3 days. Animals in their intermoult stages were selected and dissected alive in ice. The gland as a whole was fixed in ice-cold 5% glutaraldehyde solution in phosphate buffer of pH 7.2. A few glands were also fixed in 5% glutaraldehyde solution in buffered sodium cacodylate (pH 7.2) for 1 hour at 0-4°C. The glands when found to be hard after 1 hour were cut into small pieces and fixed in fresh fixatives at 0-4°C for 1 hour. The tissues were then washed in several changes of buffer solution followed by post-fixation in 0.5%

osmium tetroxide solution for 1 hr at 0-4°C. After dehydration in graded levels of cold acetone(25%,75%,95%,100%) the material was embedded in spurr resin. Sections were cut with a ultramicrotome (LKB Bromma 8800 ultrotome) and mounted on copper grids. The ultrathin (600°A) sections were then double stained in 30% uranyl acetate (30 minutes) followed by lead citrate (10 minutes) and viewed in a Carl Zeiss Transmission Electron Microscope 109 R - at an accelerating voltage of 80 KV). Electron micrographs were taken using AGFA ortho 25 film.

OBSERVATIONS AND DISCUSSION

In *Penaeus indicus* the reddish brown hepatopancreas is a bilobed, multi-lobular gland, surrounded by a thin connective tissue, the tunica propria (Plate 5). This layer incorporates the circular and longitudinal muscle fibres and also the basement membrane. Each tubule in cross section shows a characteristic four rayed appearance (Plate 6). The lobulations are called 'acini' and each 'acinus' has a lumen in the centre (Gopalakrishnan, 1957). The lumen of different acini join together to form the duct of the hepatopancreas.

Histologically, the tubules consist of an epithelium composed of four cell types, the E, R, F and B-cells (Travis, 1955; Madhyastha and Rangnekar, 1974) lying on a thin basophilic membrane (Plates 6 & 7).

Embryonic cells (E-cells): Small, narrow, columnar cells of variable sizes (Plate 8). The size varies from 18-20 μ in height and between 6-8 μ in diameter. These small undifferentiated cells occur at the blind end of the

tubule. The nucleus of each cell is prominent and comparatively large with granular chromatin material, distributed evenly in the nucleoplasm. The cytoplasm is homogeneous and vacuoles are completely absent (Plate 9).

These cells show moderate positivity to protein test (Table 2). The various protein components detected have been found in small amounts in the cell cytoplasm. The cell border has been found to be PAS positive and the presence of neutral AMP has been weakly demonstrated by their reaction to Alcian blue PAS test. The cytoplasm show slight reaction to toluidine blue test indicating the presence of PO_4^- , SO_4^- and COOH groups of mucopolysaccharides. Majority of E-cells showed negative reaction to lipid tests, but very few cells recorded small amounts of phospholipids in the cell membrane.

Absorptive cells(R-cells): These are small columnar cells measuring 25-40 μ in length and 10-15 μ in width (Plate 10 & 11). Nucleus is situated either basally or centrally. The cytoplasm contains many small vacuoles and few cytoplasmic granules. These are the most abundant cell types and occur throughout the hepatopancreatic tissues.

R-cells revealed heavy concentrations of glycogen and lipid droplets. Their brush border stained deeply with nile blue sulphate reagent indicating the presence of phospholipid (Plate 12 & 13). Their cytoplasm reacted positively to all the protein tests. The cytoplasm and nucleus stained uniformly and vividly with pyronin G and toluidine blue indicating

a marked affinity for these dyes. The microvilli and basement membrane stained intensely with PAS both before and after acetylation, which suggests that they contain a neutral mucopolysaccharide or mucoprotein component.

Fibrillar cells (F-cells): Measuring about 50-80 μ in height and 15 μ in width the F-cells are distributed in the middle region of the tubule. The cell possess strongly basophilic cytoplasm and a large nucleus. Cytoplasm present a granular appearance. This cell is seen sometimes with a small vacuole at its distal end(Plate 14).

F-cells, characterized by their supranuclear vacuole (Plates 15, 16 & 17) showed intense staining to mercury bromophenol tests, indicating the presence of proteins (Table-2). The cytoplasm showed faint reaction to the - SH, - SS groups of proteins. They generally lack glycogen and lipid in the cytoplasm. But a few of them showed feeble staining abilities to nile blue sulphate test indicating minimal amounts of phospholipid in the cytoplasm. The cell membrane and basement membrane stained intensely with periodic acid schiff reagent.

Secretory cells (B-cells): Large swollen cells measuring 60-70 μ in width, with a prominent vacuole towards the distal end (Plate 18). The vacuole invariably contains stained material. The roughly spherical nucleus is pushed towards the base and has a little cytoplasm (Plates 19 & 20). These cells are abundant and found in the middle portion of the tubule.

B-cells showed intense staining reaction with protein tests (Table 2). Rich amounts of proteins were seen uniformly distributed in the cytoplasm. Fairly all the other groups of proteins with - SS, and - SH groups were seen localized around the vacuoles (Plate 21). Tyrosine and tryptophan groups were also seen in the cytoplasm. Nucleus and nuclear sap stained positively to Pyronin G and protein tests. The brush border and the basement membrane stained deep pink with PAS. Acid mucopolysaccharides, both sulphated and non-sulphated are localized in the cell border and around vacuoles. Neutral mucopolysaccharides occur in the intertubular connective tissues.

Gopalakrishnan (1957) reported only two types of cells in *Penaeus indicus* based on light microscopy. Dall (1967) also reported two types of cells in *Metapenaeus bennettae.* Secretory and storage cells have been reported by Travis (1955) in *Panulirus* and George *et al.* (1955) in *Panulirus polyphaqus.* However, Madhyastha and Rangnekar (1974) working with *Metapenaeus monoceros* clearly distinguished four cell types in the epithelium. The present study reveals that the hepatopancreas of *Penaeus indicus* contains four types of cells-embryonic, secretory, fibrillar and absorptive cells.

The embryonic-cell (E-cells) observed here correspond to the E-cells of the *Metapenaeus monoceros* (Madhyastha and Ranganekar 1974), *Procambarus* (Bunt, 1968), and that of *Carcinus maenas* (Stanier *et al.*, 1968). The secretory cell (B-cell) corresponds to the B-Cell reported in *Metapenaeus monoceros* (Madhyastha and Rangnekar, 1974), *Carcinus maenas* (Stanier *et al.*,

1968), and *Panulirus* (Travis, 1955). Absorptive cell (R-cell) in *P. indicus* resembles the R-cell of *Metapenaeus monoceros* (Madhyastha and Rangnekar, 1974), *Panulirus* (Travis, 1955) and *Procambarus* (Bunt, 1968). Fibrillar cell observed in *P. indicus* corresponds to the F- cell of *Metapenaeus monoceros* (Madhyastha and Rangnekar, 1974) and F-cells of *Carcinus maenas* (Stanier *et al.*,1968).

R-cells are the absorptive cells, which contain large quantities of lipid globules and glycogen granules. They combine the function of absorption and storage of digested food materials. This is in agreement to the observations of Loizzi (1971) on *Procambarus*. Loizzi (1971) detected fatty acids, neutral lipids and phospholipids in the R-cells. Momin and Rangnekar (1975) also identified the lipid substances in the R-cells. Rangnekar and Momin (1974) identified glycogen, while granules presumed to be glycogen were also seen by Loizzi (1971).

Histochemical studies on the cell types is carried out for the first time in *Penaeus indicus*. The characteristic reaction of E-cell to the tests indicate that during the intermoult, the cell is getting ready for differentiation into other cell types which would participate in the construction and growth of tissues. The rich amounts of proteinaceous material detected in the secretory and fibrillar cells is an indication of its synthesizing role of digestive enzymes.

Histochemically, it has been observed that all epithelial cell types bear microvilli on their luminal borders. The microvilli, material in the

secretion vacuole, and the basement membrane react positively when stained by the PAS method, both before and after saliva treatment, which may suggest that they contain a neutral mucopolysaccharide or mucoprotein component. The microvilli are also stained by alcian-blue which may indicate the presence of an acid mucopolysaccharide.

Ultrastructure:

Four distinct cell types are seen in electron micrographs of the hepatopancreas (Plates 22, 23, 24, 25, 26) .

E-Cell (Embryonic cell):

These are undifferentiated cells (Plate 27) and have high nuclear: cytoplasmic ratio. They contain few mitochondria having randomly oriented cristae. Proximal to the nucleus there are Golgi complexes and moderate amounts of rough endoplasmic reticulum(Plate 27). The cytoplasm possess abundant free ribosomes and single membrane reticulum. Microvilli are present on the border adjacent to the lumen (Plates 27 & 28).

The E-cells at the blind ends of the tubule in decapods are generally believed to be involved in mitotic activity (Gibson and Barker, 1979) for the production of the other cell types which comprise the tubule epithelium. The E-cell of *Penaeus indicus* as observed in the present study is similar to the E-cells observed in other crustaceans (Van weel, 1955; Davis and Burnett, 1964; Bunt, 1968; Stanier *et al.*, *1968;* Gibson and Barker, 1979; Papathanassiou and King, 1984; Al-Mohanna *et al.*, 1985).

2. F-Cells (Fibrillar cell)

These cells (Plate 29) contain extensive rough endoplasmic reticulum with long, narrow ribosome-studded cisternae. The Golgi cisternae in the cells are always dilated and the entire complex is ringed by small dense vesicles (Plates 29 & 30). The cytoplasm contains abundant free ribosomes but fewer mitochondria (Plate 31). The structure of these cells resembles that of the F-cells in the crayfish hepatopancreas(Loizzi, 1971). Extremely electron dense cytoplasmic inclusions are occasionally observable in the fibrillar cells. Each inclusion is enclosed within a membranous vacuole and consists of granular material which is concentrated centrally to form a rather solid core surrounded by irregular clumps of the dense material. These are the zymogen granules (Plates 32 & 33). The F-cells manufacture the digestive enzymes (Van weel, 1970; Loizzi 1971) and in *P. indicus* are probably derived from the E-cells. This is supported by the increased amount of rough endoplasmic reticulum in later stages of E-cell development. Similar observations have been made by Bunt (1968) and Stanier *et al.* (1968) for the hepatopancreas of other crustaceans.

The sequence of cell transformation may be similar to that observed by Jacobs (1928) for *Astacus* who gave the sequence of epithelial cell stages as: E-cell → F-cell → B-cell. The F-cells may become secretory cells, secreting their products into the lumen. Observations made by Stanier *et al.* (1968) and Loizzi (1971) working on *C. maenas* and *Procambarus clarkii* respectively also support the sequence of cell transformation putforth by Jacobs (1928).

Loizzi (1971) suggested that F-cells in crayfish are involved in synthesis of digestive enzymes because of their rich concentrations of rough endoplasmic reticulum. F-cells in *P. indicus* also showed abundant rough endoplasmic reticulum, their cisternae being filled with a flocculent material (Plate 33). These cells may be actively involved in digestive enzyme synthesis and sequester them in a supranuclear vacuole which enlarges by pinocytic intake of luminal nutrients and fluids. The exocrine cell of the vertebrate pancreas, specialised for secretion of digestive enzymes, is characterized by a large nucleus containing a prominent nucleolus, numerous profiles of rough endoplasmic reticulum and a prominent Golgi complex (Fawcett, 1966). These fine structural characteristics are also shared by other cells like Vertebrate plasma cells which are engaged in active protein synthesis. Within the F-cells of *Penaeus indicus* also active endoplasmic reticulum and Golgi complex are seen supporting their role in enzyme secretion. Inside the Golgi vesicles are also seen certain moderately electron dense, somewhat flocculent material which may be interpreted as secretory product of proteinaceous nature, again indicative of active protein synthesis within the F-cell.

B-cells (Secretory cells):

These cells contain large number of vesicles, which initially occupy a considerable part of the cell and eventually occlude it (Plates 34 & 35) or sometimes contain a single large vacuole enclosed by a thin shell of cytoplasm. Separating the vacuole from the lumen is an apical complex consisting of a microvillar border, dense cytoplasm, small mitochondria

and a meager surface enteric coat. These are the secretory cells and resemble the B-cells of Davis and Burnett (1964) and Stanier *et al.* (1968).

These cells are sometimes characterised by a single large vacuole, and may be considered to be essentially an F-cell in which the small secretory vacuoles have coalesced (Plate 36) to form a single large vacuole and a compressed basal nucleus. The contents of the vacuole have obviously been affected by solvents used during dehydration and embedding but the presence of myelin figures may indicate a phospholipid component. Loizzi (1971) has attributed that in crayfish these cells are secretory in nature and B-cell secretion involves pinching off the apical complex followed by extrusion of the enzyme-rich vacuolar contents. Some what similar structural components are also observed in the B-cell of *Penaeus indicus* indicating their functional significance in secretion (Plate 37).

R-cell (Absorptive cells):

These cells are characterized by the presence of lipid material (Plates 38,39,40). The cytoplasm contains a number of mitochondria, some of which are concentrated below the apical plasma membrane (Plate 41). The nucleus is centrally or basally located and nucleolus is present. The apical membrane of the cells has microvilli, the surface of which are covered by short filaments. Rough endoplasmic reticulum and golgi complex occur throughout the cell (Plates 42, 43).

Smooth endoplasmic reticulum is restricted to the basal region of the cell. Apically, the cell resembles a vertebrate intestinal absorptive epithelium

with a dense brush border and an organelle-free region lying below the microvilli. Numerous basal invaginations occur in cells of this type. Autophagic vacuoles containing material of different configurations with low electron density are also present in the cytoplasm (Plate 42). These cells are similar to those described as R-cells in the crayfish hepatopancreas (Loizzi, 1971).

The cell possesses a striated apical border strikingly similar to that of vertebrate cells known to function in food absorption (Yamada, 1955; Ito, 1965). The function of lipid storage may be attributed to this cell from light microscopic observation of histochemical tests applied, where dense concentration of lipids were observed in membrane bounded vacuoles. Hence these cells are involved in storage and metabolisation of glycogen and lipids. To a certain extent, R-cells of *P. indicus* may combine the functions of vertebrate's intestinal absorptive and hepatic parenchymal cells. The R-cells may be derived independently from E-cells as in the hepatopancreas of other crustaceans (Stanier *et al.*, 1968; Loizzi, 1971).

TABLE 2. Histochemical characterization of Hepatopancreatic cell types of *Penaeus indicus*.

Histochemical Tests			E Cell	F Cell	B Cell	R Cell	Indicates
Proteins							
1.	a)	Mercuric bromo phenol blue(MBBS)	++ DB	++ DB	+++ DB	+++ DB	Proteins
2.	a)	Aqueous bromo phenol blue (ABB)	++ B	++ B	+++ B	+++ B	Basic proteins
	b)	ABB after deamination	-	-	-	-	
3.	a)	Ninhydrin - Schiff test (NS)	+ R	+ R	+ R	+ R	Amino Groups.
	b)	NS after deamination	-	-	-	-	
4.	a)	Toluidine blue test(TB)	+ R	-	+ R	+ R	Acidic groups
	b)	TB after methylation	-	-	-	-	
5.	a)	Ferric-ferricyanide test(FF)	+ B	+ B	+ B	+ B	-SH group
6.	a)	Thioglycollate FF Test(TFF)	+	+	++	++	-SS group
	b)	TFF test after thioglycollate reduction	-	-	-	-	
7.	a)	Millon's test (MT)	+	+	+	+	tyrosine
	b)	MT after Iodination	P -	P -	P -	P -	
8.	a)	DMAB-nitrite method	+ B	+ B	++ DB	++ DB	tryptophan
	b)	DMAB - nitrite method after formaldehyde treatment	-	-	-	-	

TABLE 2. continued....

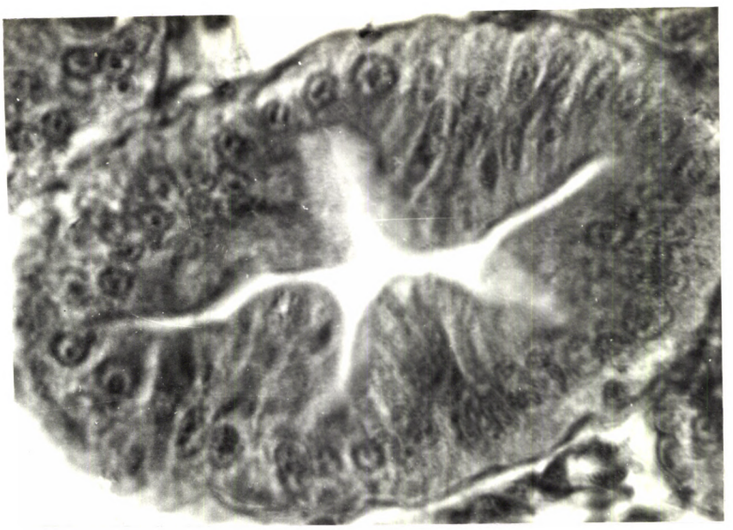
Histochemical tests		E Cell	F Cell	B Cell	R Cell	Indicates
Carbohydrates						
1.	a) Periodic-acid Schiff test (PAS)	+ M	-	+++ M	+++ M	Glycogen
	b) PAS after deacetylation	+	+	+	+	
2.	a) Best's carmine test (BC)	+ R	-	++ R	++ R	Glycogen
	b) BC after Taka-diastase	-	-	-	-	
3.	Toluidine blue at					
	pH 1.09	+ B	-	+ B	+ B	$-SO_4$ group of AMP
	pH 2.01	+ B	+ B	+ B	+ B	$-SO_4$ "
	pH 3.09	+ B	+ B	+ B	+ B	$-PO_4$ & $-SO_4$ "
	pH 4.09	+ B	+ B	+ B	+ B	-COOH "
4.	Critical electrolyte concentration (CEC) method					
	0.1 M	+	+	+	+	-COOH group AMP
	0.2 M	-	-	-	-	-COOH "
	0.6 M	-	-	-	-	-COOH "
	0.8 M	-	-	-	-	$-SO_4$ "
	1.0 M	-	-	-	-	$-SO_4$ "
5.	Alcian-blue-PAS test	+ B	++ B	+++ B	+++ B	Acid and Neutral AMPS

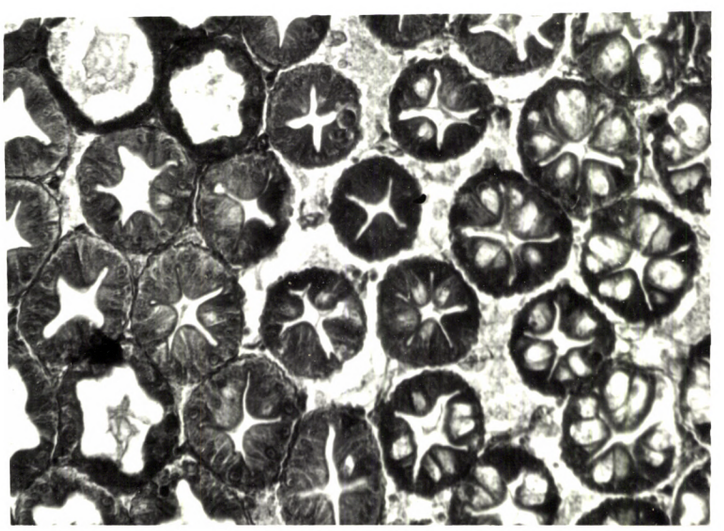
TABLE 2. Continued.....

Histochemical tests			E Cell	F Cell	B Cell	R Cell	Indicates
Lipids							
1.	a)	Sudan black B test(SBB)	+ B	+ B	+++ DB	+++ DB	Lipids
	b)	SBB after chloroform methanol extraction	-	-	-	-	
2.	a)	Nile blue method (NB)	-	± R	++ R	++ R	Neutral and Acidic lipids
	b)	NB after chloroform/ methanol extraction	-	-	-	-	
3.	a)	Nile blue sulphate (NBS) method	-	-	++ b	++ b	Phospholipids
	b)	NBS after chloroform/ methanol extraction	-	-	-	-	
4.	a)	Oil-Red 'O' method	-	+ R	+ R	+ R	Neutral lipids
	b)	Oil Red 'O' after Pyridine extraction	-	-	-	-	
5.		Pyronin G-test	++ R	++ R	++ R	++ R	RNA and DNA

B	-	blue	-	negative
DB	-	dark blue	±	weak
R	-	red	+	moderately positive
P	-	pink	++	positive
M	-	Magenta	+++	intensely positive

Plate 6: C.S. of the hepatopancreatic tubule showing the cells with a lumen in the centre. The same region under higher magnification indicating the cell types (Embryonic cell - E-Cell and Secretory - B-cell).





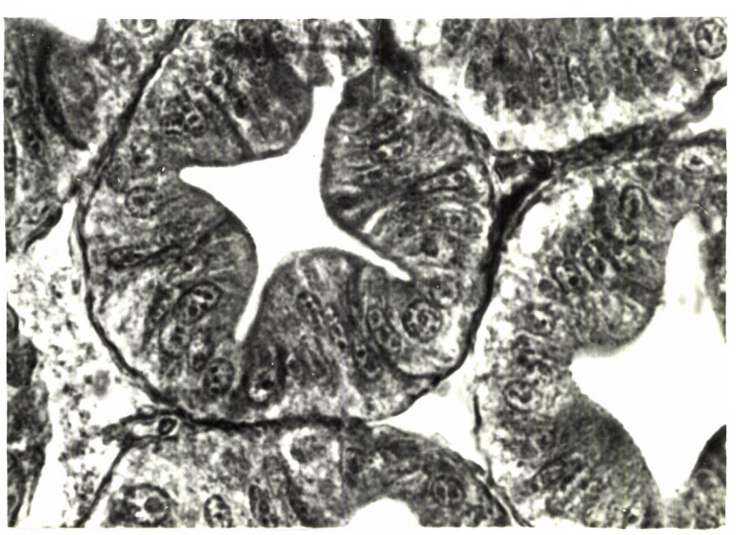


PLATE . 6

Plate 7: C.S. of a hepatopancreatic tubule showing the R and F cells.

Plate 8: L.S. of a hepatopancreatic tubule showing the E-cell (Embryonic cells) with prominent nucleus.

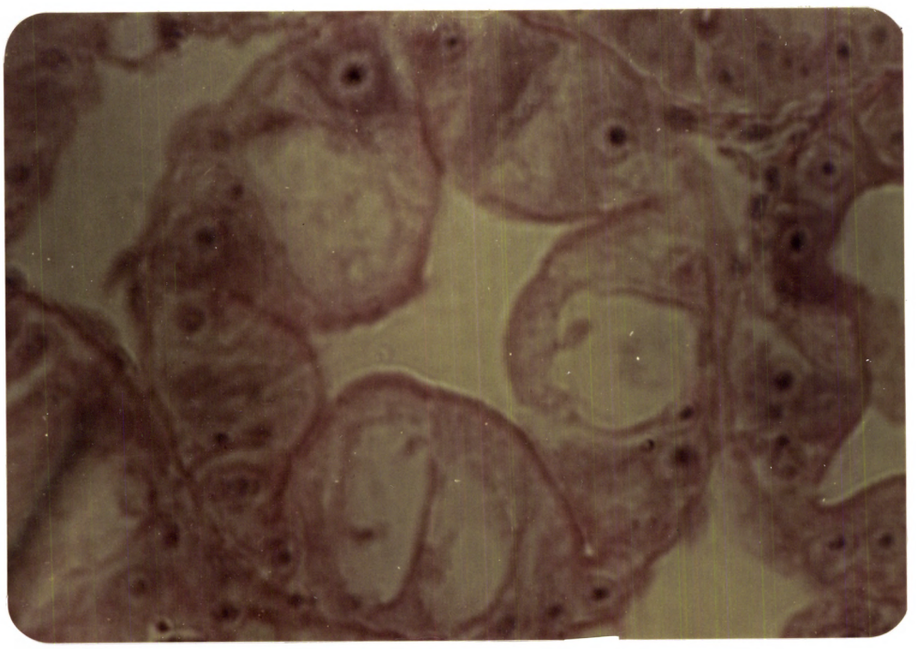


PLATE . 7

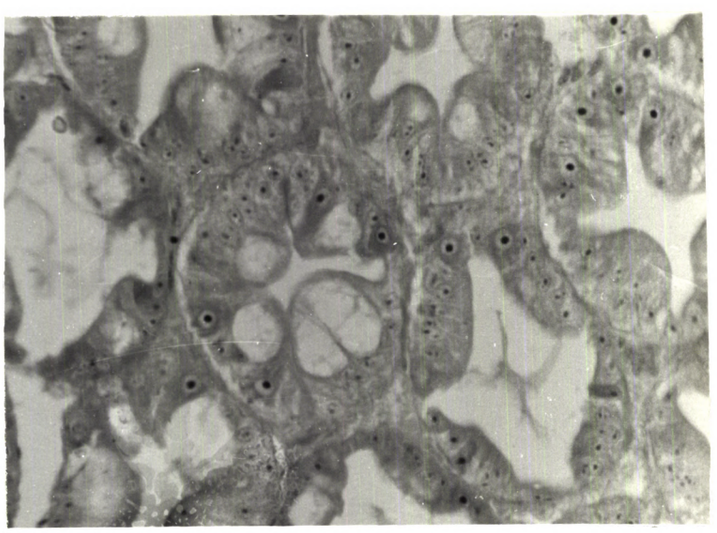


PLATE . 8

Plate 9: C.S. of a hepatopancreatic tubule showing the Embryonic cell with a positive response to the Alcian-blue PAS test.

Plate 10: C.S. of a tubule showing the R-cell (Absorptive cell) with a basally located nucleus.

Plate 11: C.S. of a tubule showing the R-cell with a nucleus and few vacuoles with cytoplasmic granules. Note the darkly stained brush border of the cells.

PLATE . 9

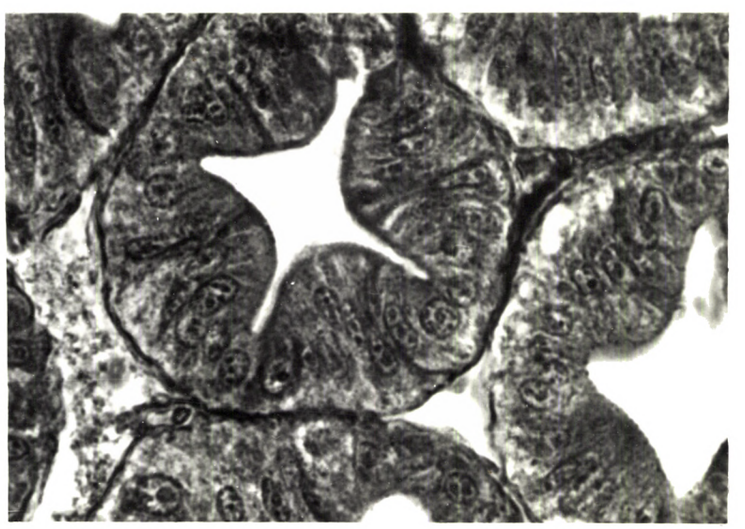


PLATE . 10

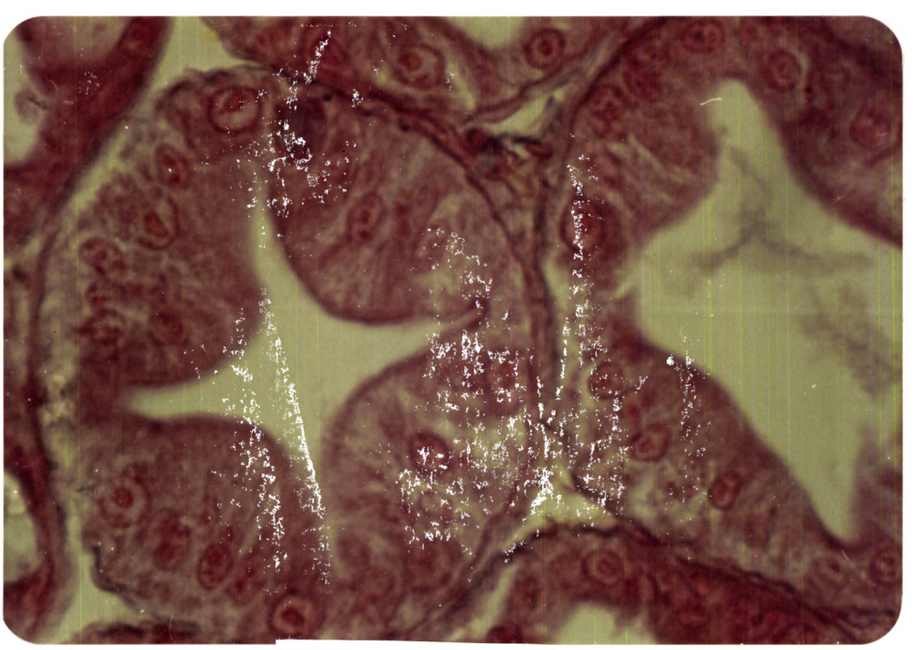


Plate 12: Photomicrograph of R-cell showing positive reaction to glycogen and lipid tests.

Plate 13: Photomicrograph of R-cell showing positive reaction to phospholipid test and their microvilli for PAS test.

Plate 14: L.S. of a tubule showing the F-cells (Fibrillar cells) with a large nucleus and vacuole.

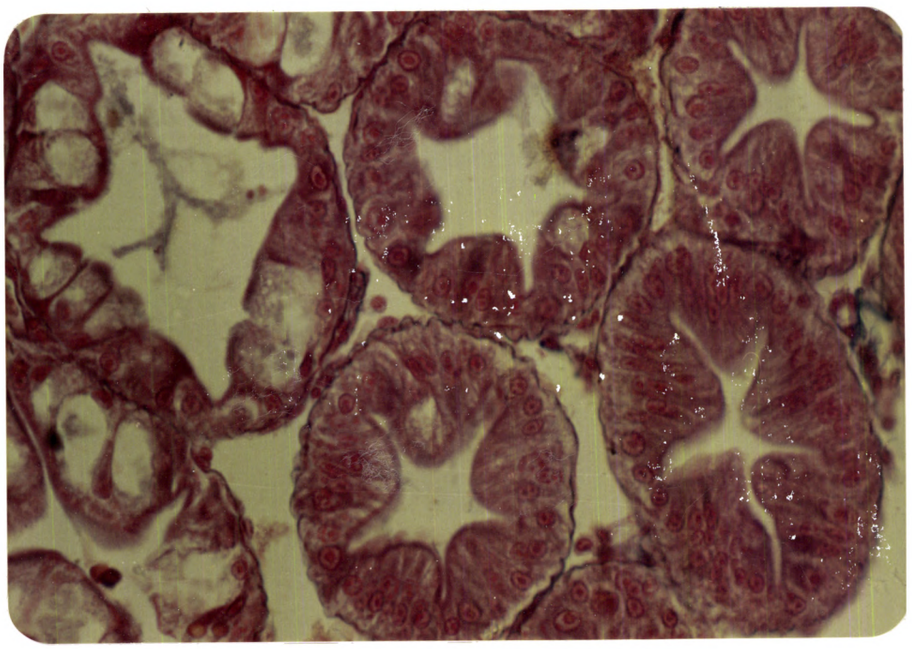


PLATE . 12

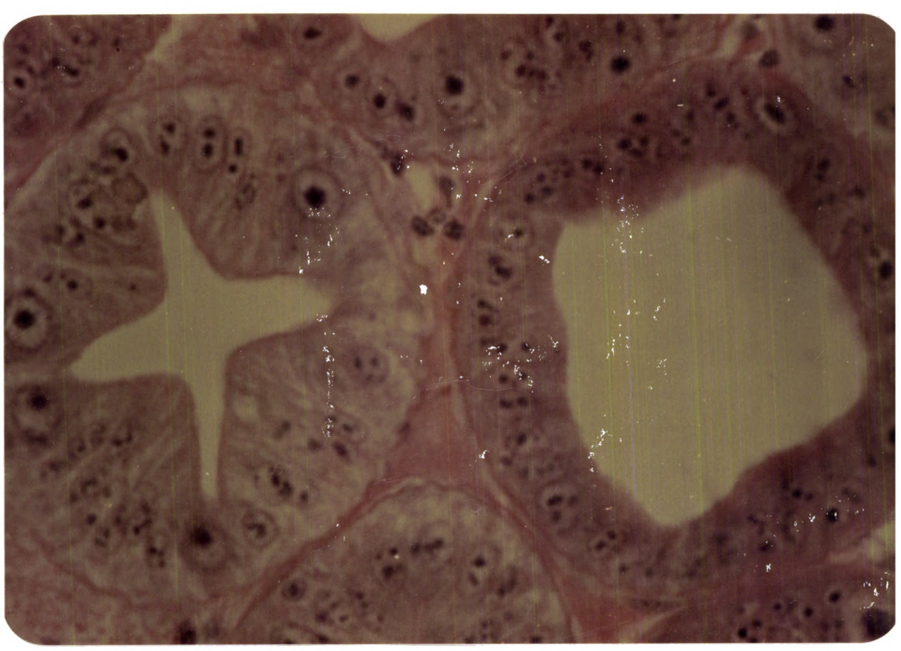


PLATE . 13

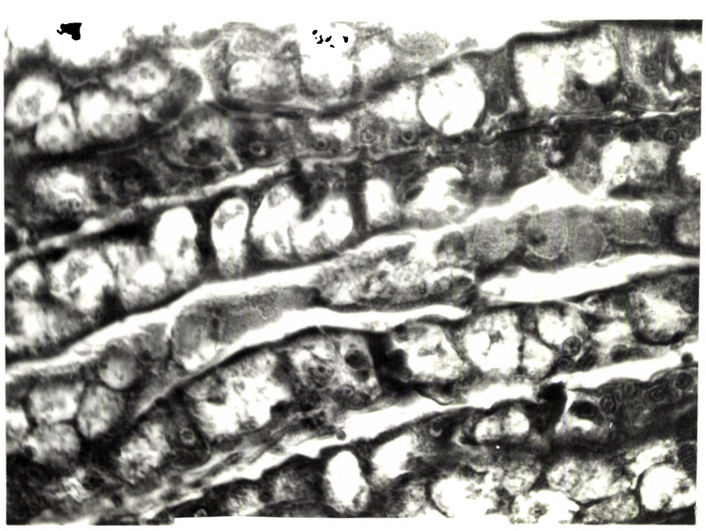
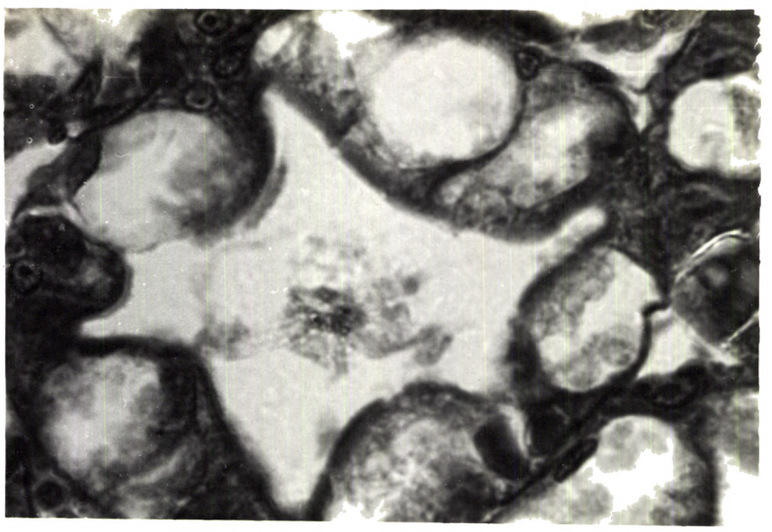


Plate 15: Photomicrograph of F-cells showing a supranuclear vacuole and a basal nucleus.

Plate 16: Photomicrograph of F-cells showing response to mercury bromophenol blue test.



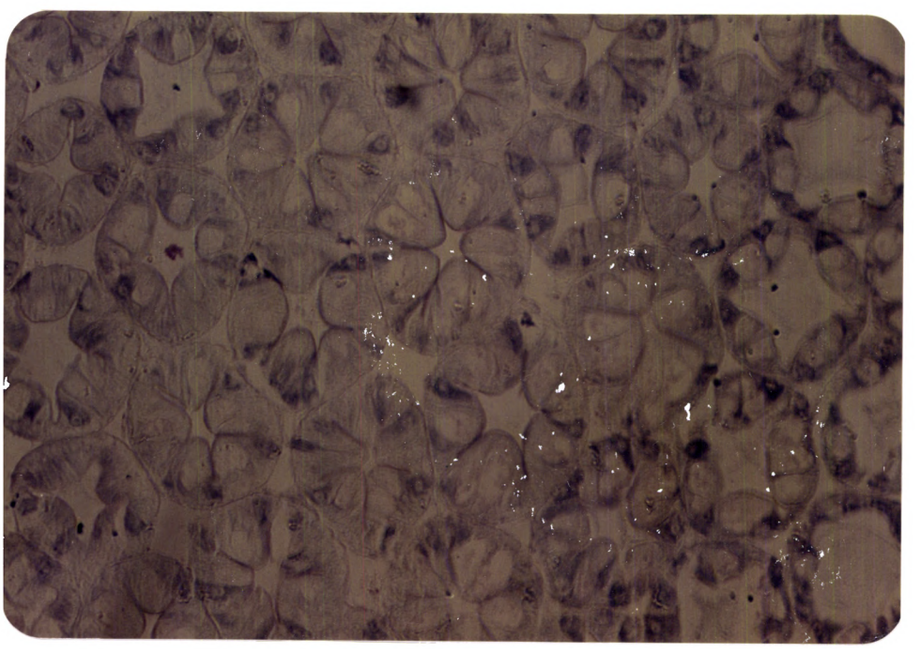


PLATE . 15

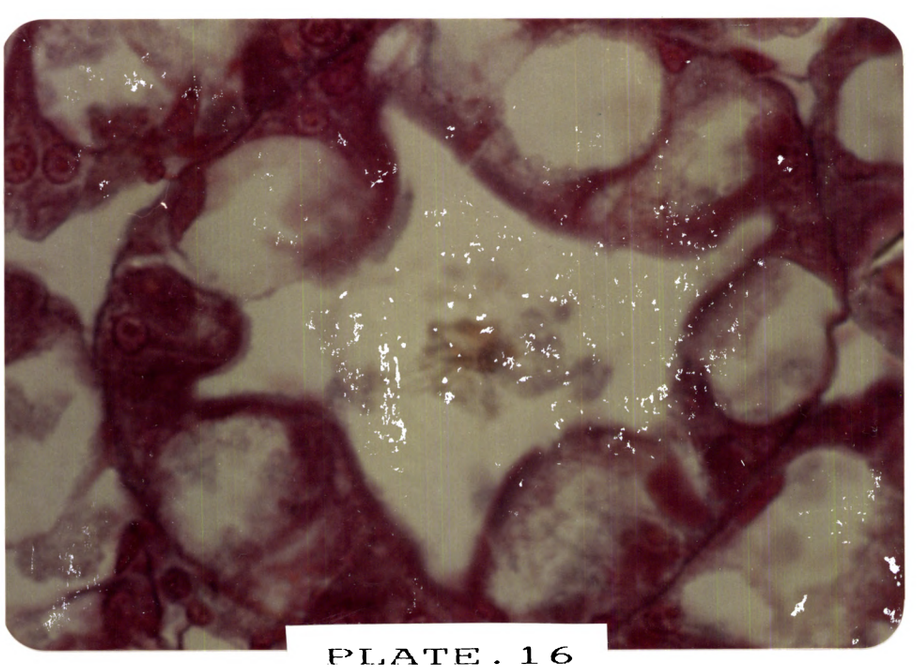


PLATE . 16

Plate 17: A portion of F-cell with a supranuclear vacuole showing strong positivity towards PAS test in the brush border and basement membrane region.

Plate 18: C.S. of a hepatopancreatic tubule showing Secretory cell (B-cell)

Plate 19: Photomicrograph of B-cell showing spherical nucleus and vacuolated cytoplasm.

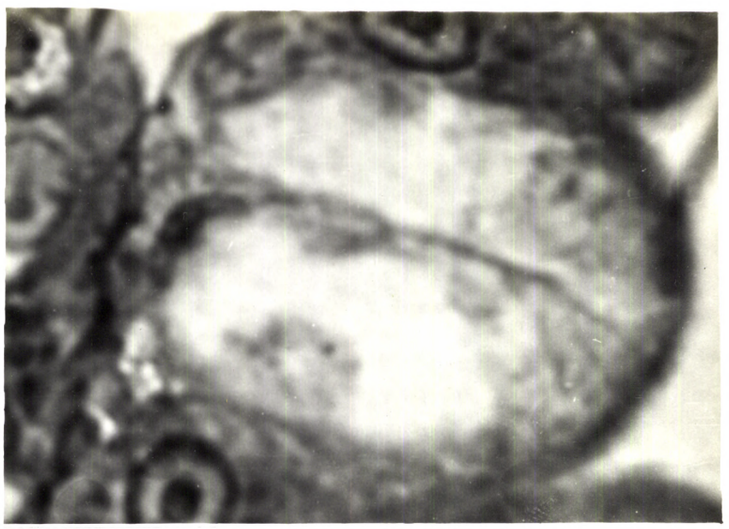


PLATE . 17

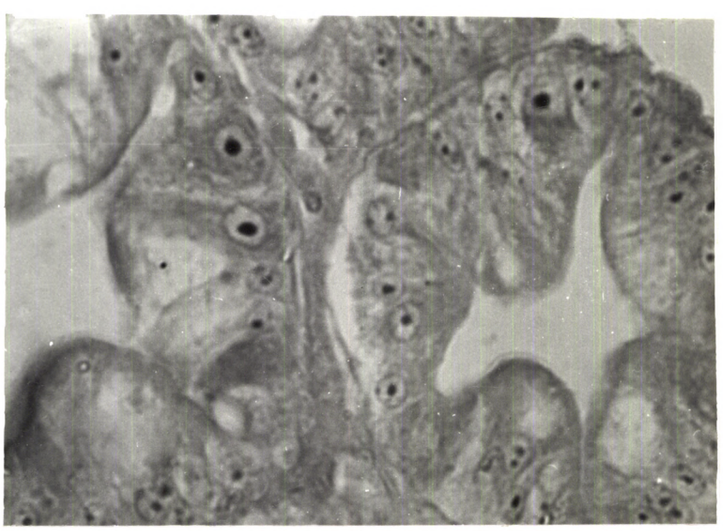


PLATE . 18

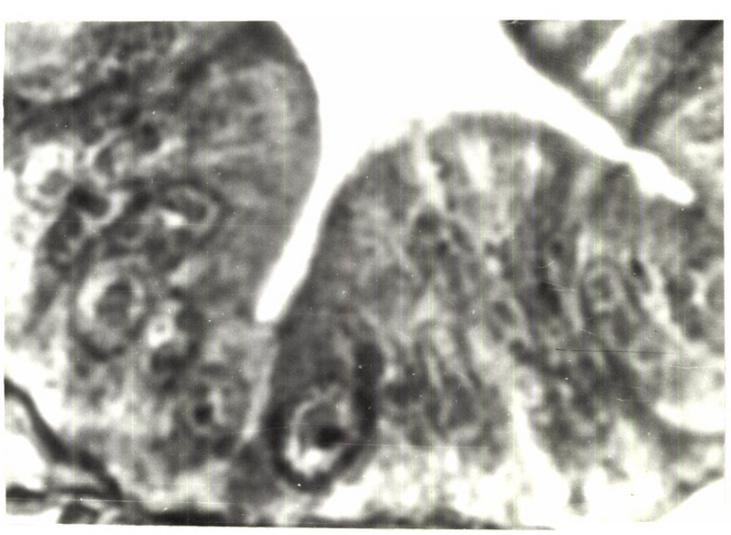


PLATE . 19

Plate 20: A portion of R-cell (Absorptive cell) magnified showing the spherical nucleus with a prominent nucleolus and vacuolated cytoplasm.

Plate 21: Photomicrograph of B-cell and R cell showing positivity to PAS reaction and nuclear test.

PLATE . 20

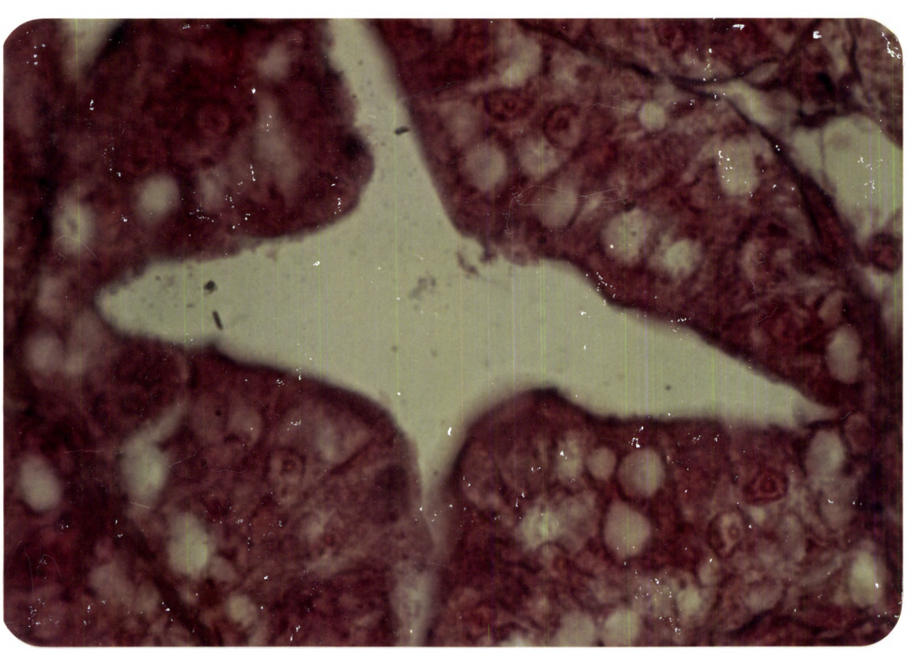


PLATE . 21

Plate 22. Electron-micrograph of hepatopancreas of Penaeus indicus showing the different cell types X 3000.

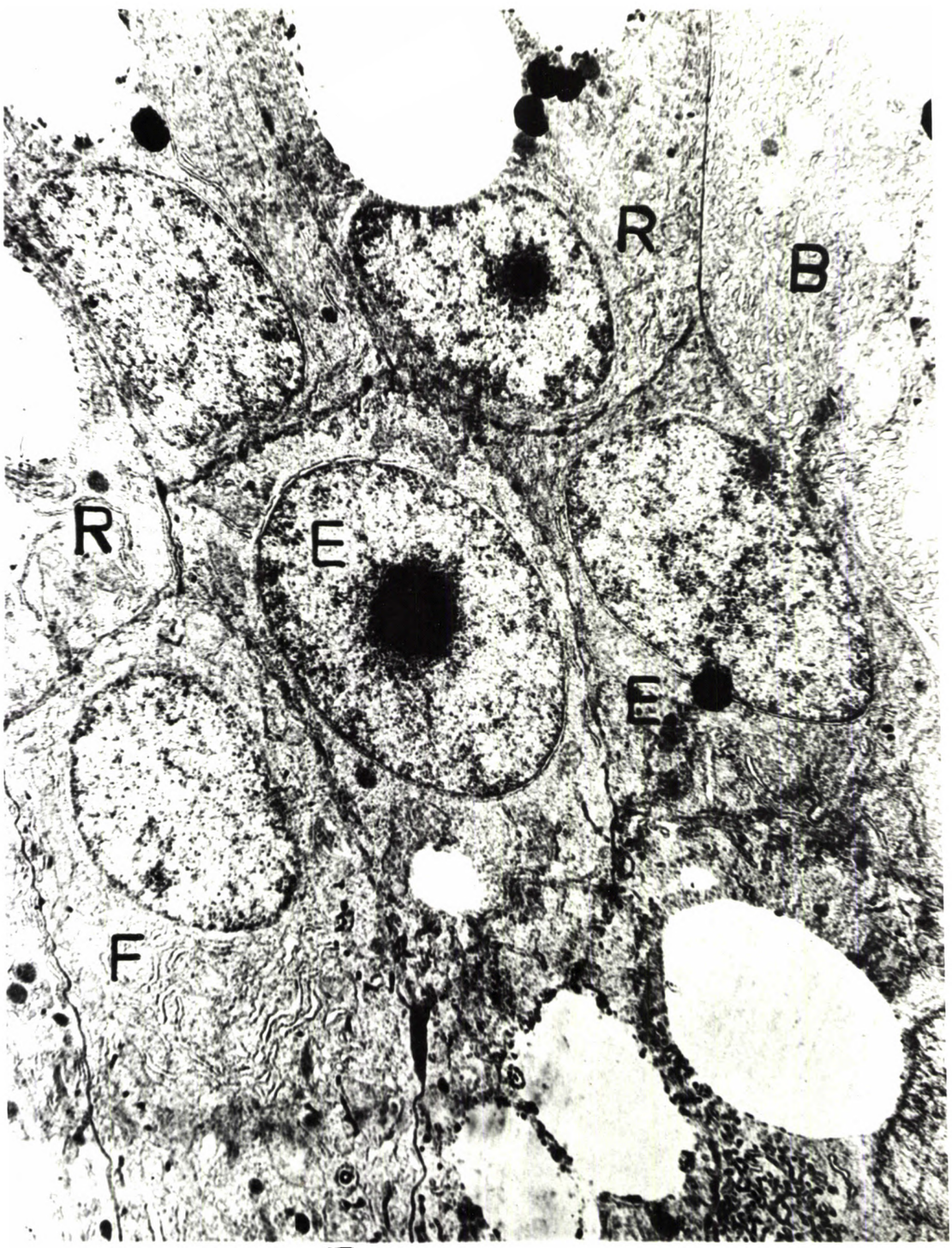


Plate 22

Plate 23: Electron-micrograph showing the E-cell (E) with its nucleus. F-cell(F) with nuclear components and golgi complexes. B-cell (B) with its numerous mitochondria. R-cells (R) with enormous vacuoles X 3000.

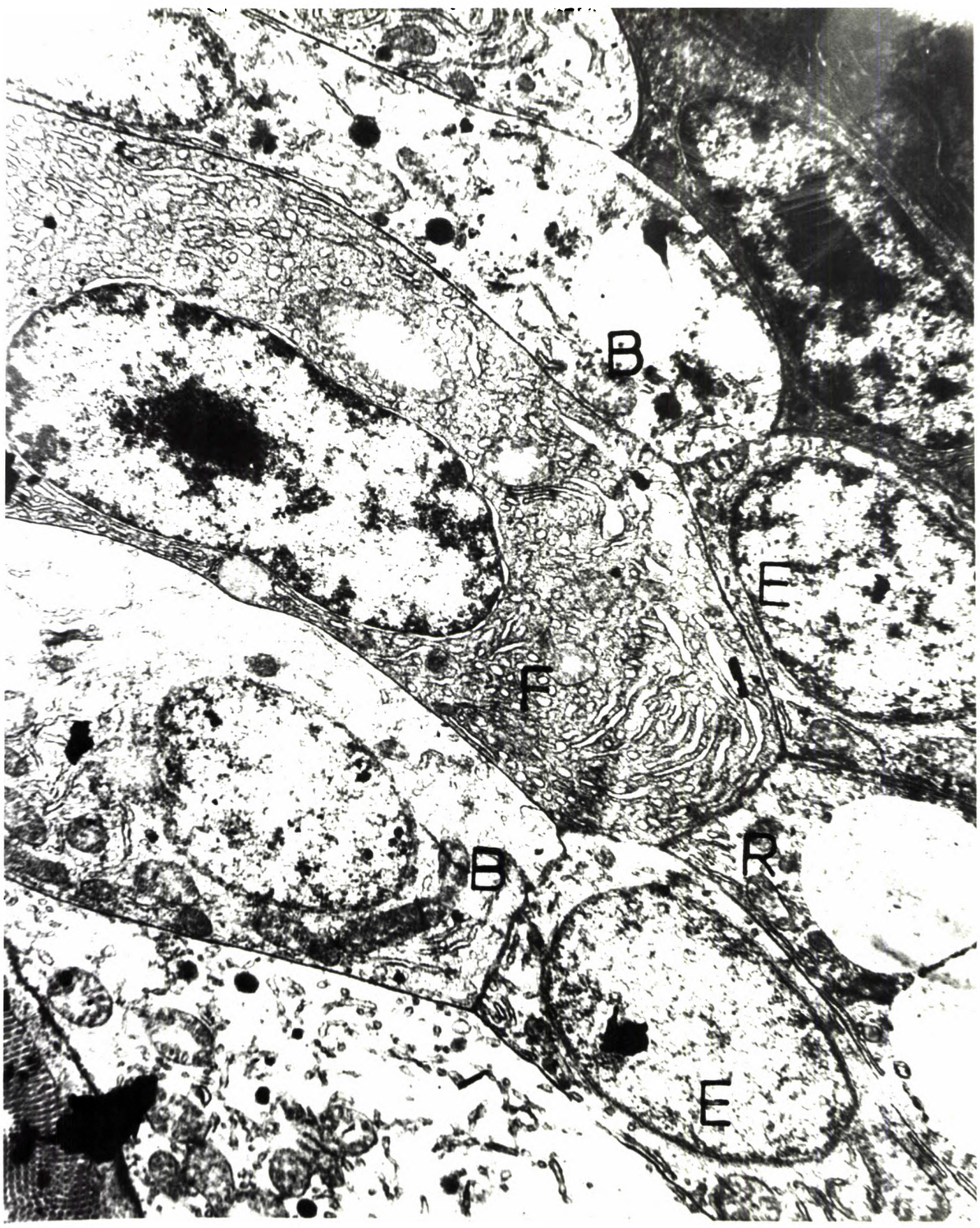


Plate 23

Plate 24: Electron-micrograph of R-cell, F cell and E-Cell X 3000. The dark region shows the hemolymph space between the tubules.

N - Nucleus; m - Mitochondria; gc - Golgi complex

rer - rough endoplasmic reticulum.

Plate 24

Plate 25: Electron-micrograph of hepatopancreatic cells X 7000

F-cell, R-cell, E-cell

F- cell with rough endoplasmic reticulum and golgi cisternae

N- Nucleus; L - lipid globule; m - mitochondria

Plate 25

Plate 26: Electron-micrograph showing the special membrane of the B & R cells x 7000.

mV	microvilli
m	mitochondria
db	dense bodies
lu	lumen between the adjacent cells

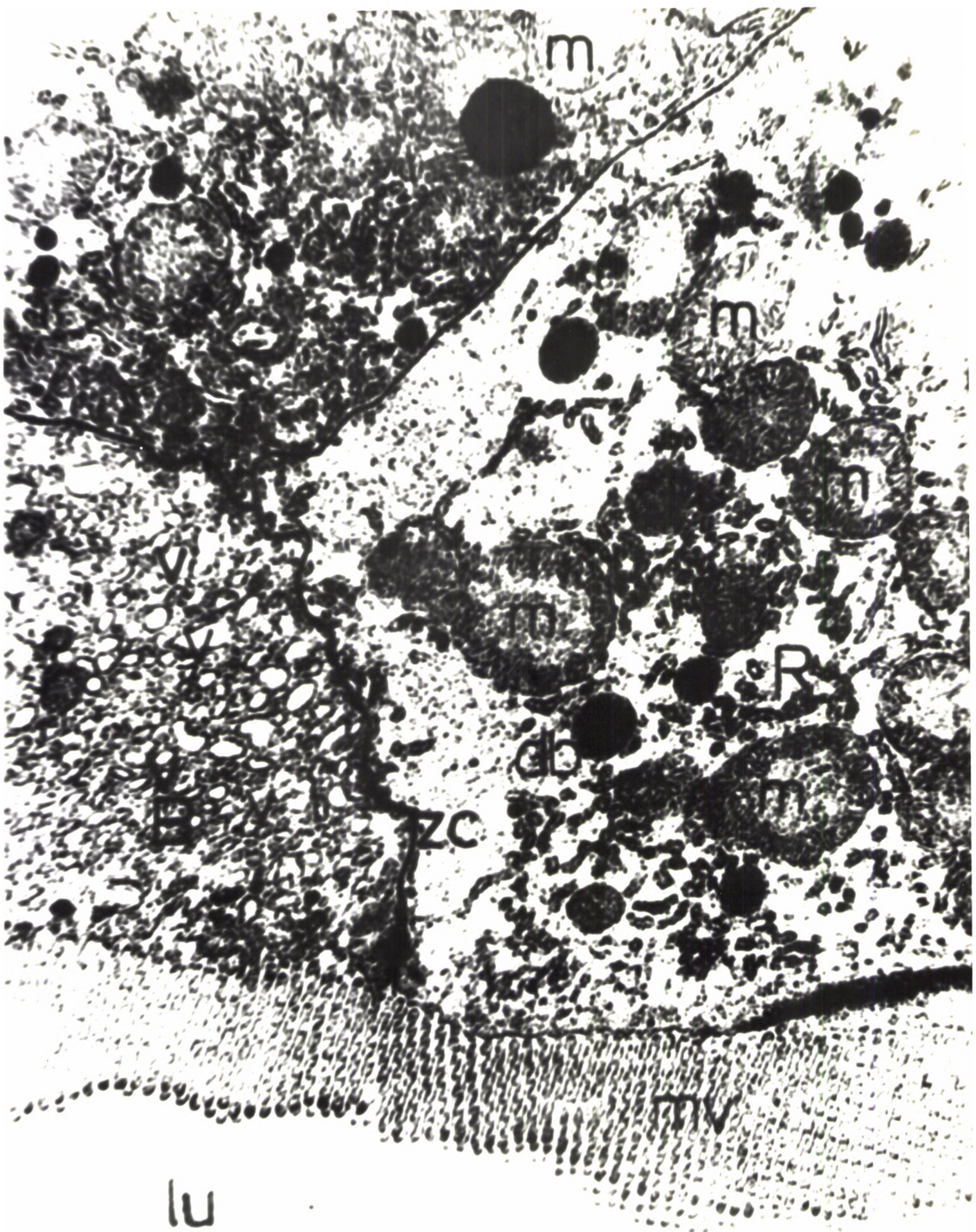


Plate 26

Plate 27: Electron-micrograph showing an embryonic cell (E) and secretory cell (B) X 3000. Note the small number of mitochondria(m), nucleus (N), golgi complex (ge) and granules (gr) in the E-cell

mV-microvilli, Cy - cytoplasm

rer - rough endoplasmic reticulum.

Plate 27

Plate 28: Electron-micrograph showing the E-cell (E) with golgi complexes (gc) rough endoplasmic reticulum (rer) in the cytoplasm and vesicles of smooth endoplasmic reticulum (ser) X 4400.

mb - myelin bodies.

Plate 28

Plate 29: Electron micrograph of Fibrillar cell (F) with vesicles (V), golgi cisternae (gc), few mitochondria (m) and a centrally located nucleus (N). Note the abundant rough endoplasmic rticulum (rer) db - dense bodies. L - lipid inclusion mv - microvilli X 3000.

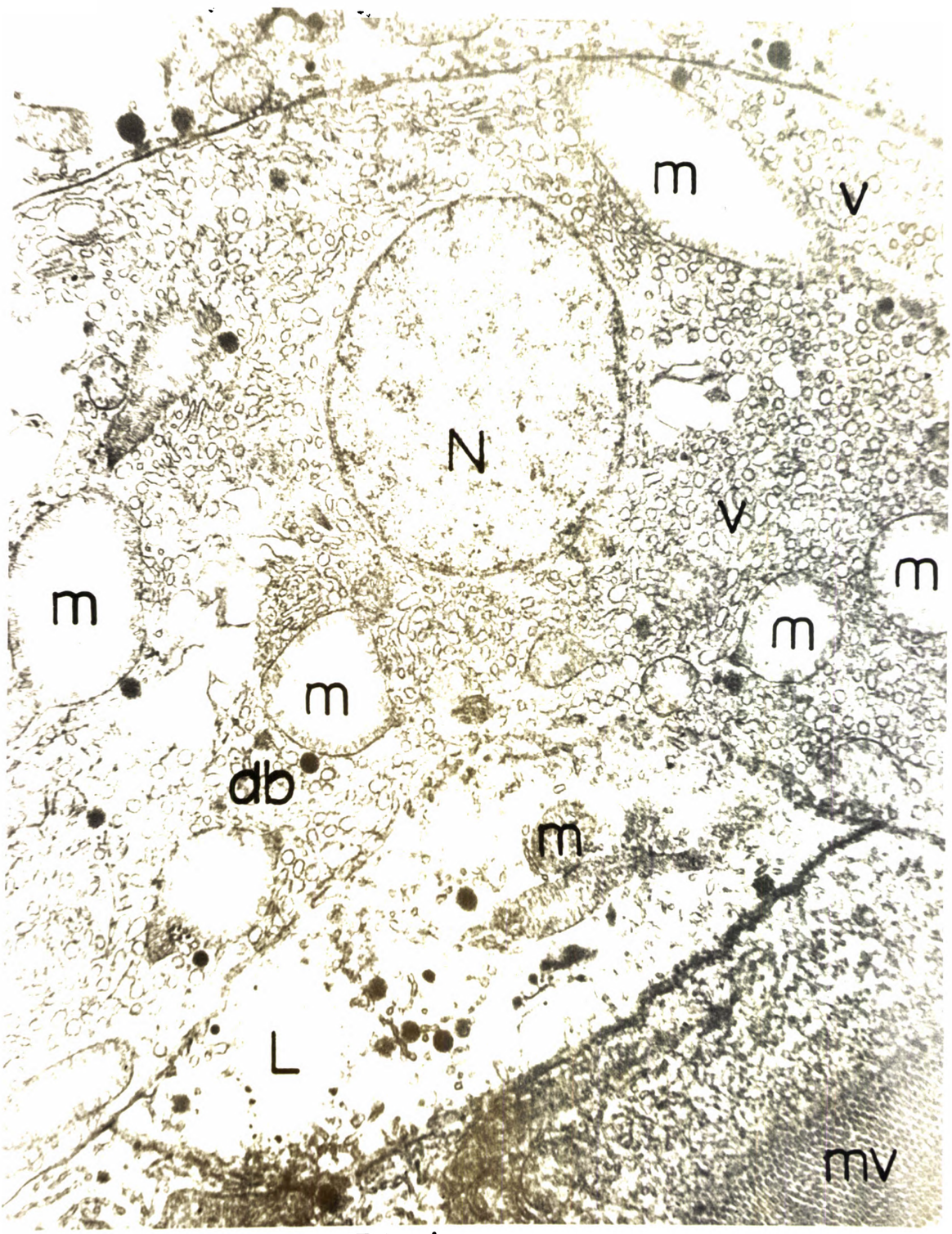


Plate 29

Plate 30: Electron-micrograph showing active golgi complex (gc) in association with rough endoplasmic reticulum (rer) in a Fibrillar cell (F) X 7000

av - autophagic vacuole; rer - rough endoplasmic reticulum, L - lipid droplet, db - dense bodies m - mitochondria.

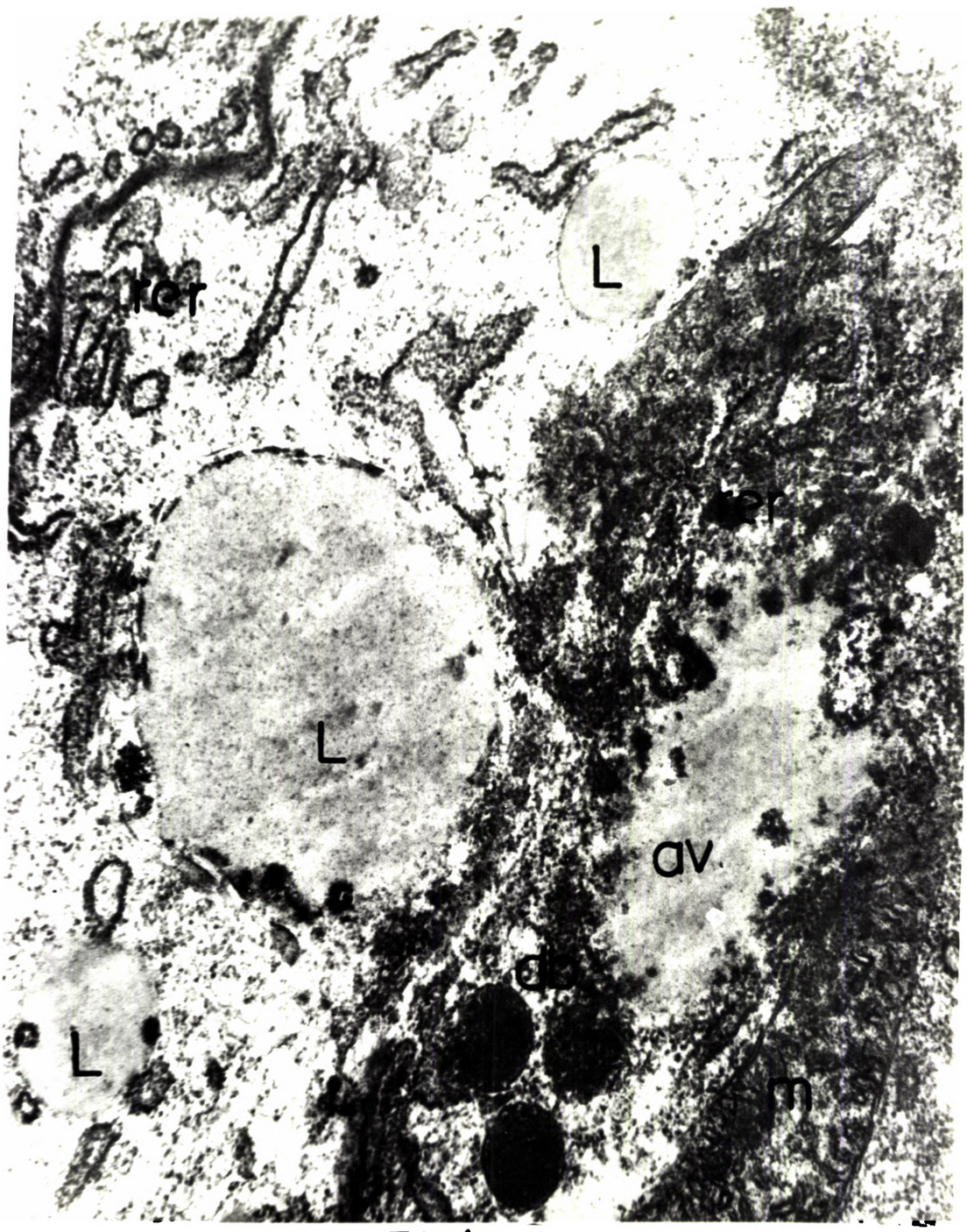


Plate 30

Plate 31: Electron micrograph of a Fibrillar cell (F) illustrating a portion of the large vacuole (V) abundant rought endoplasmic reticulum (rer) and active golgi complex (gc) X 7000

ed - electron dense bodies.

Plate 31

Plate 32: Electron-micrograph of Fibrillar cell (F) with dense granules (gr) surrounded by vesicles of rough endoplasmic reticulum Note the lateral cell attachments X 7000.

db - dense bodies; m - mitochondria

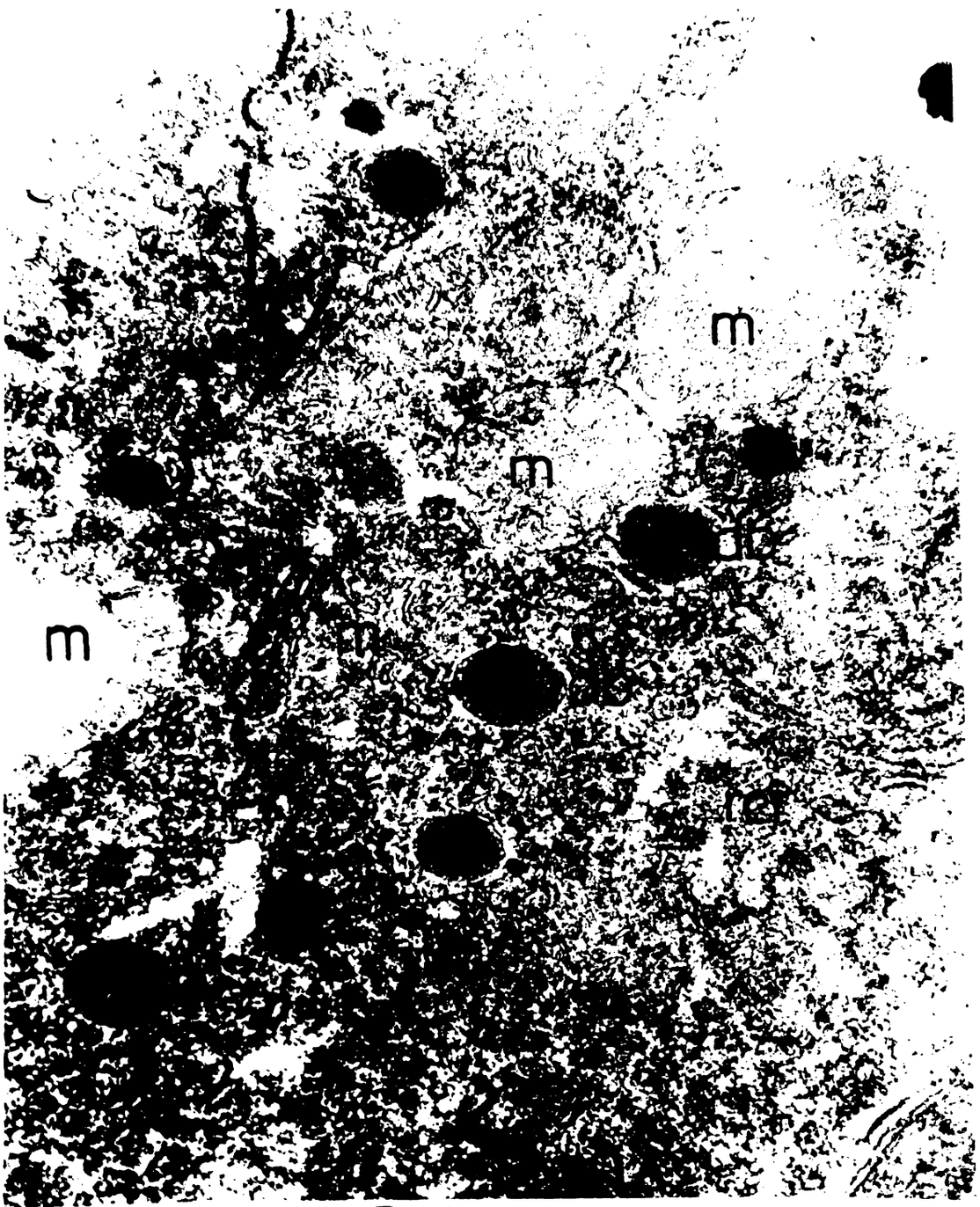


Plate 32

Plate 33: Electron-micrograph showing a Secretory cell (B-cell) Note the numerous vesicles (V) within the cell and the undulating appearance of the apical membrane (am) and microvilli (mv) X 4400.

Plate 33

Plate 34: Electron-micrograph showing cytoplasm of a Secretory cell(B). Note the vesicles filled with floculent material and small spherical mitochondria (m) X 3000

rer - rough endoplasmic reticulum

gr - granules.

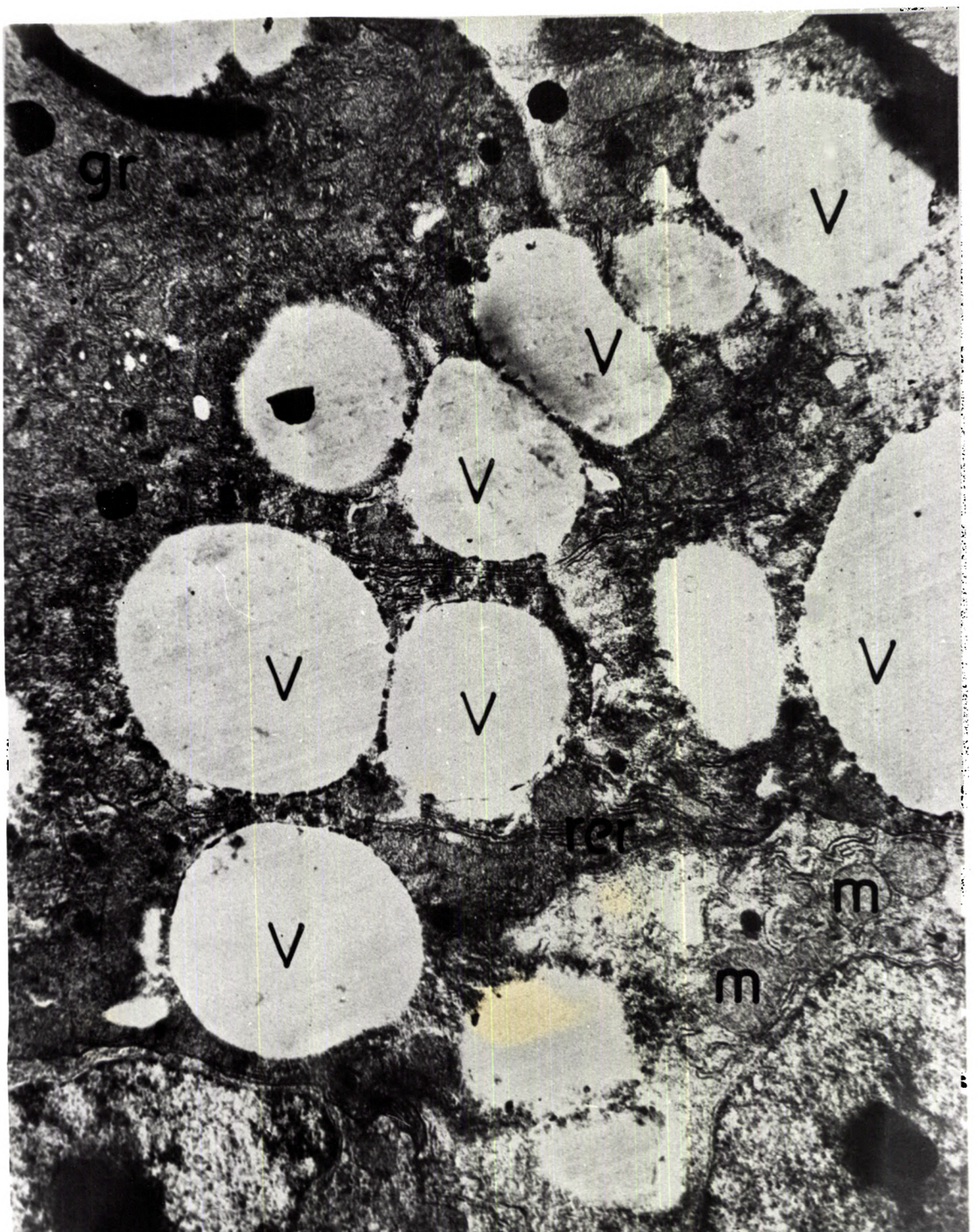


Plate 34

Plate 35: Higher magnification of a portion of Secretory cell (B-cell) showing the vesicles filled with proteinaceous substances at the periphery X 12000

sv - secretory vesicles; Lu - lumen; mb - myelin bodies

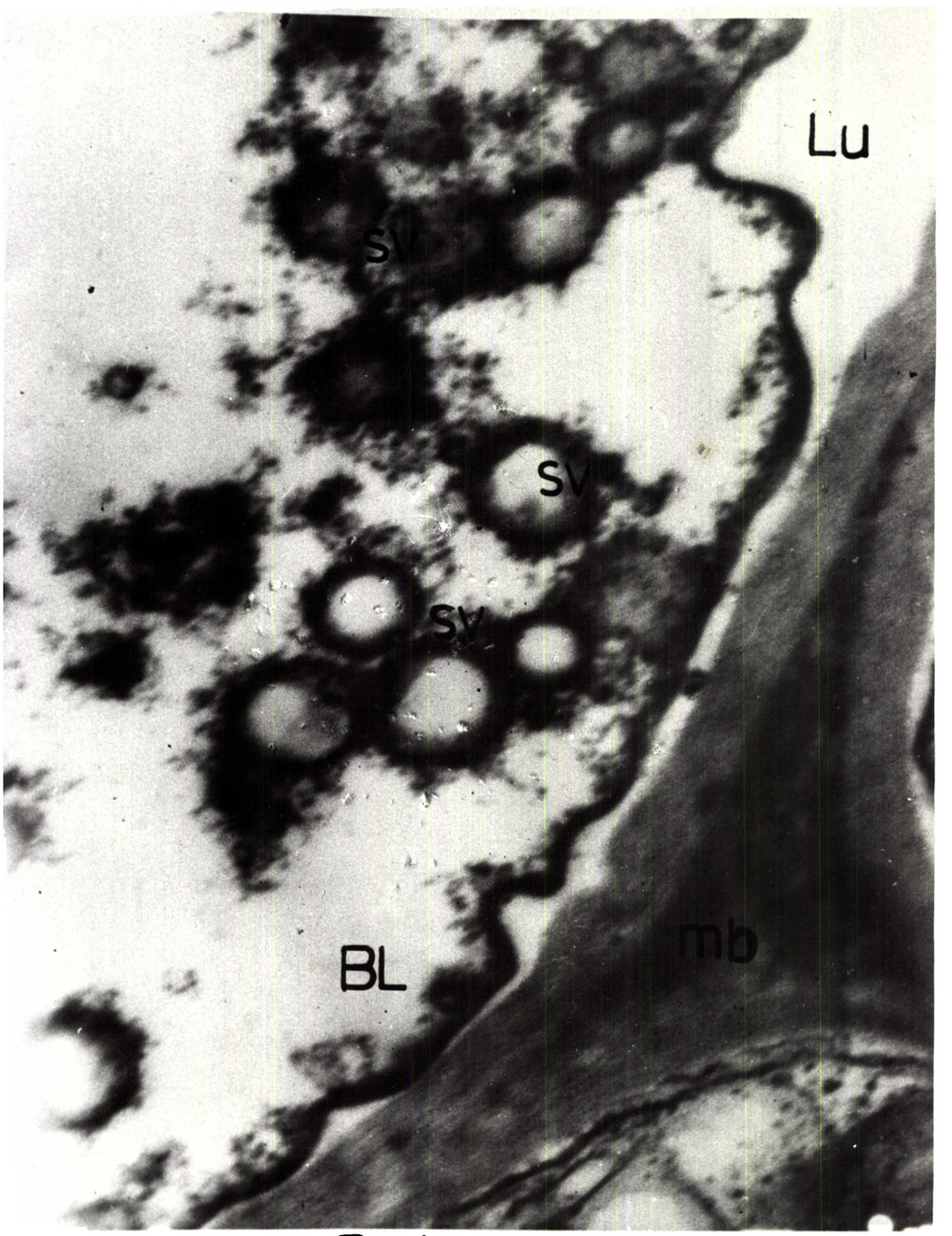


Plate 35

Plate 36: Electron-micrograph showing Secretory cell (B-Cell) with microvillar border (mi) and abundant smooth endoplasmic reticulum (rer), Note the numerous vesicles (V) some of which contain a dielectronic material and the small spherical mitochondria (m) X 12000.

ph - phagosome; db - dense bodies

Plate 36

Plate 37: Electron-micrograph showing Secretory (B-cell) and Fibrillar (F-cell). Note the vesicles (v) in the B cell and large vacuole, few mitochondria (m) in the F cell X 3,000.

Lu - lumen in between the cells.

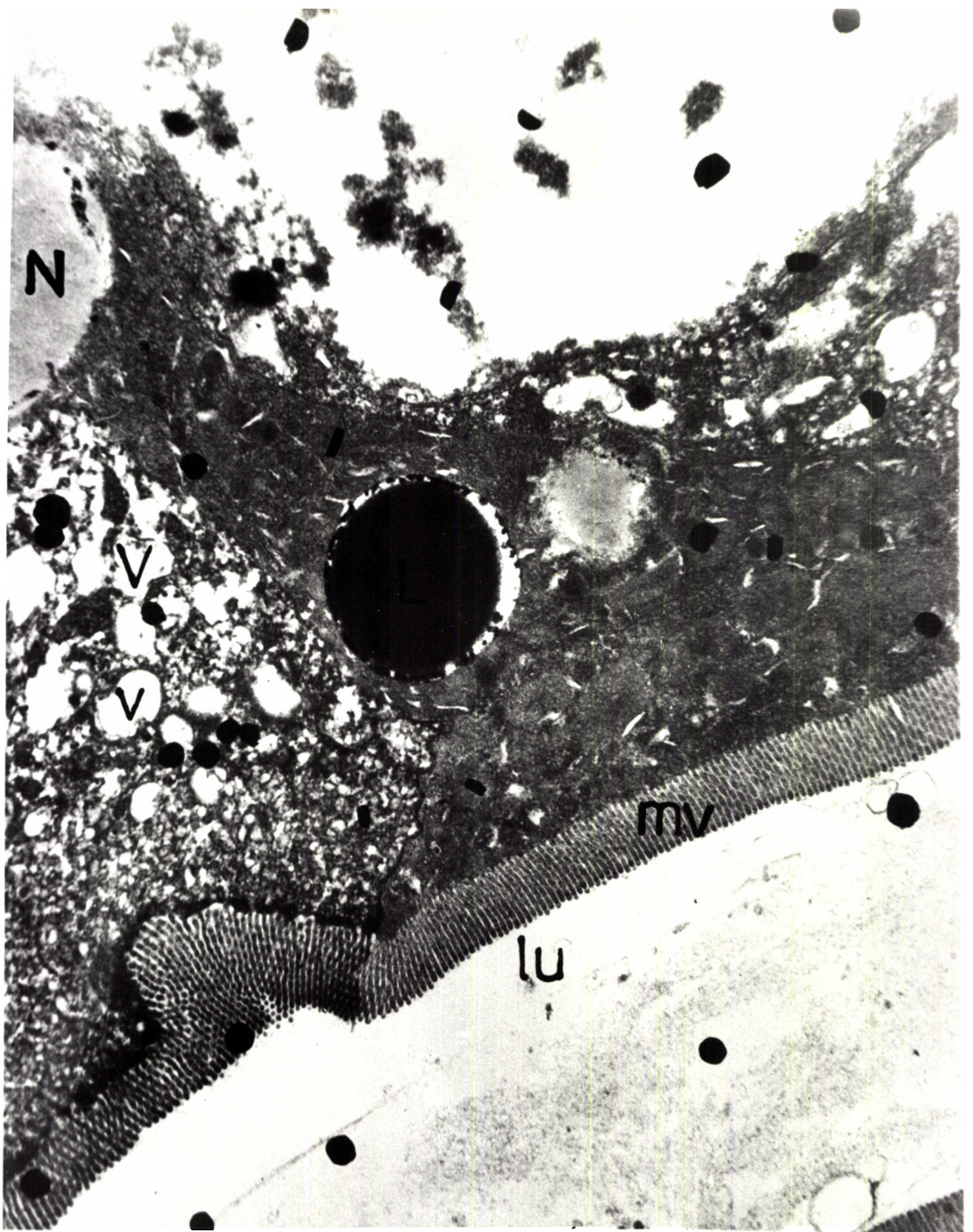


Plate 37

Plate 38: Electron-micrograph showing Absorptive cell (R-cell) and nucleus (N), nucleolus (n), lipid droplet (L) mitochondria (m) and cisternae of rough endoplasmic reticulum (rer). X 3000.

av - autophagic vesicle,

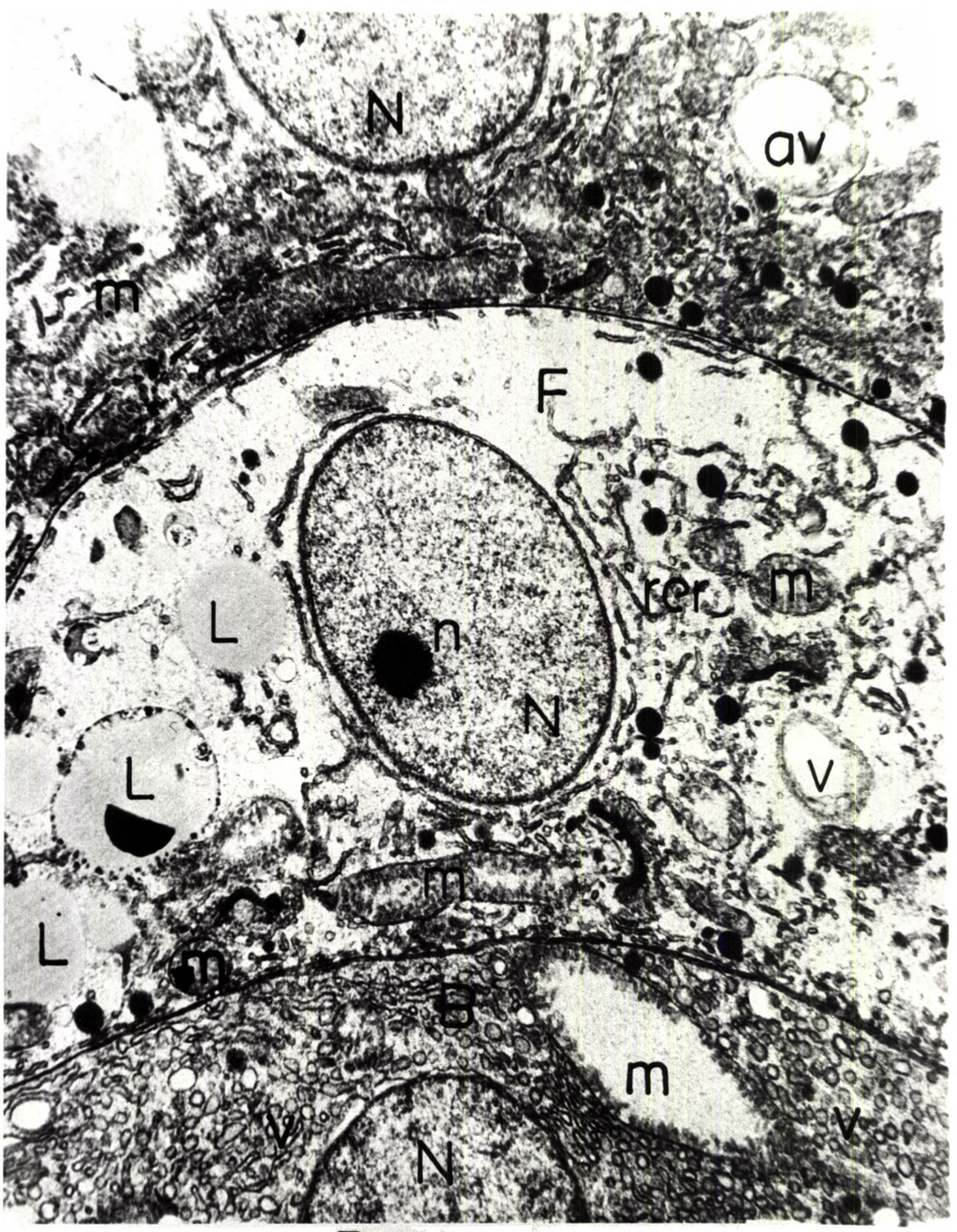


Plate 38

Plate 39: Electron-micrograph of a Absorptive cell (R) with a large lipid droplet (L) and mitochondria (m) and cisternae X 7000. db - dense bodies, rer - rough endoplasmic reticulum.

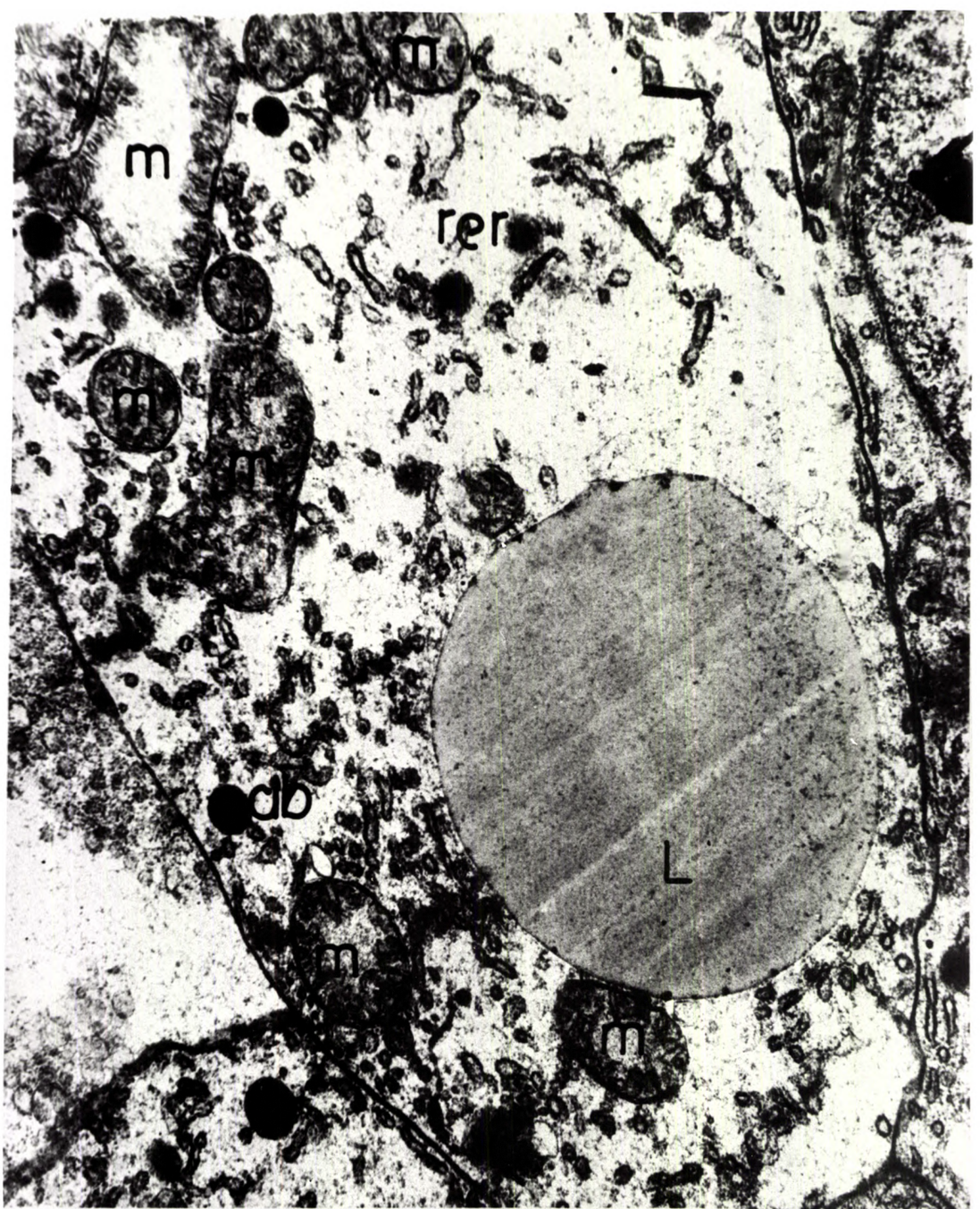


Plate 39

Plate 40: Electron-micrograph of a Absorptive cell (R) with autophagic vacuole (av) and lipid droplets (L) and numerous vesicles with dense inclusions X 7000
rer - rough endoplasmic reticulum.

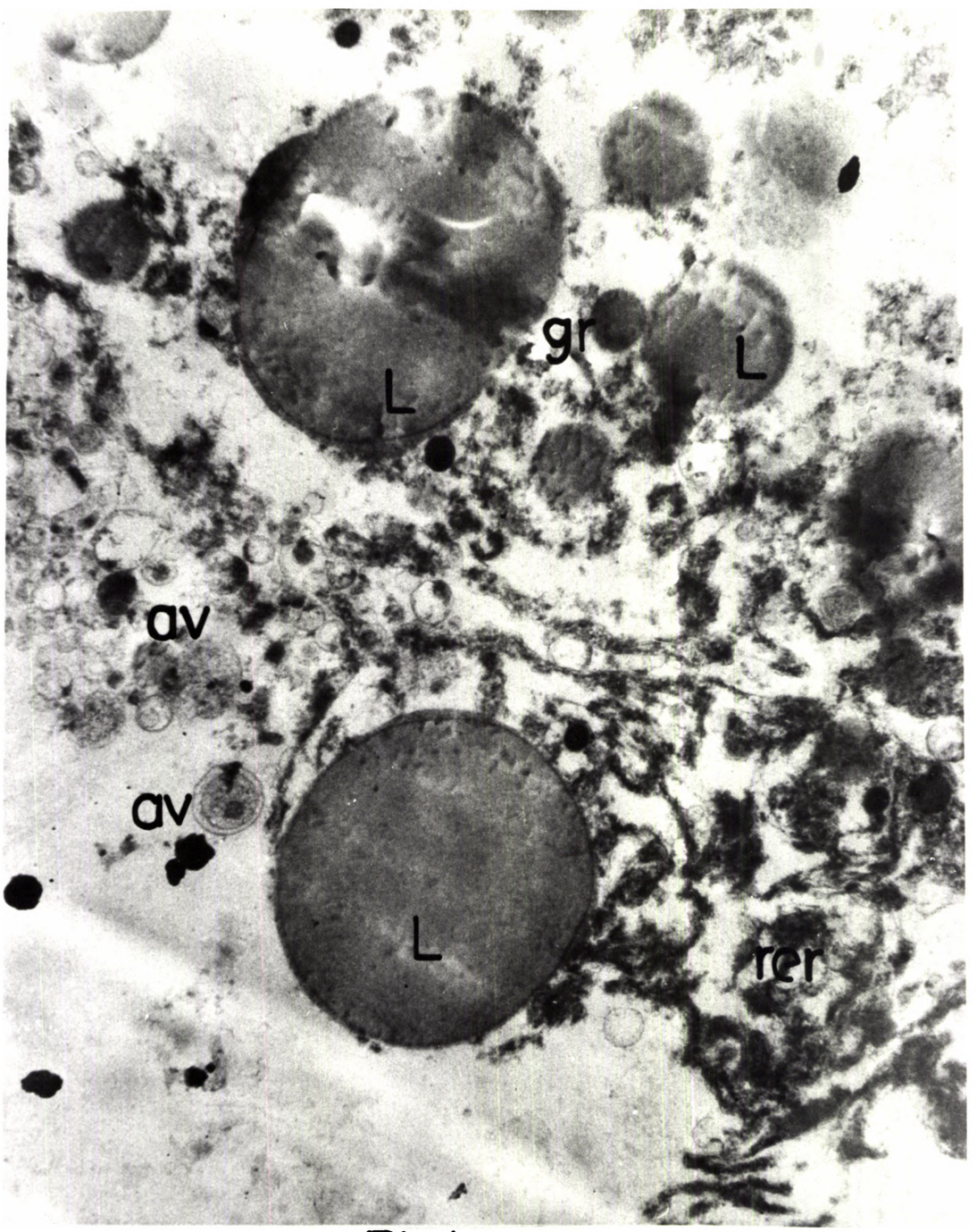


Plate 40

Plate 41: Electron-micrograph of a Absorptive cell with autophagic vacuoles at the proximal region of R-cell and a large lipid droplet (L) mitochondria (m), X 7000.
ph - phagosome, mv - microvilli

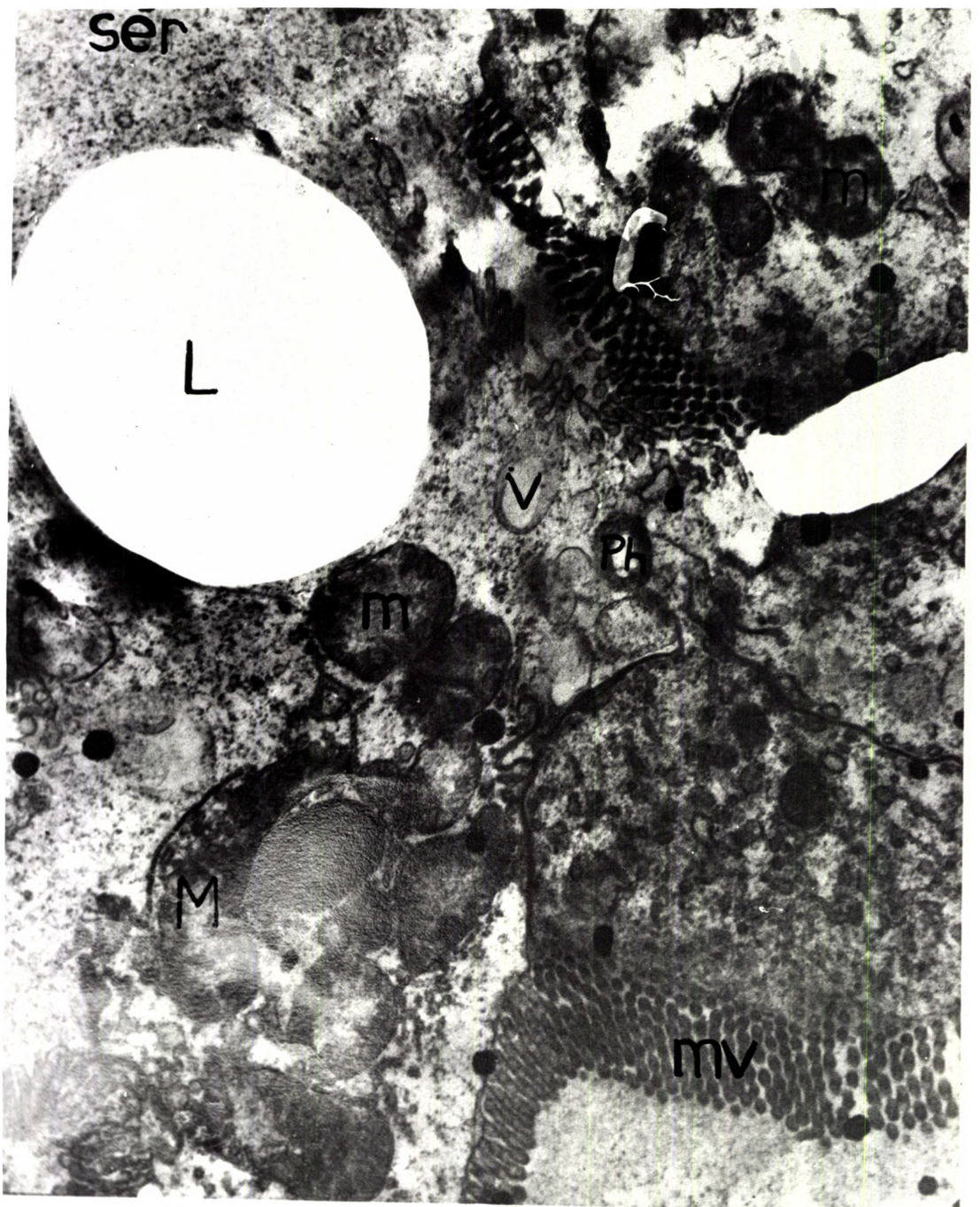


Plate 41

Plate 42: Electron-micrograph of the apical microvilli of the R and F cell, with an intracellular space filled with translucent material and vesicles. Note the microvilli on the surface of the cells, and the filaments in the core of each microvillus of the R-cell X 7000

Cy - cytoplasm, BL-basal lamina,

ph - phagosome filled with myelin figures and debris.

Plate 42

Plate 43. Electron-micrograph of a Absorptive cell (R) showing the apical portion; separated by debris material remaining after extrusion of a degenerated R-cell X 12,000

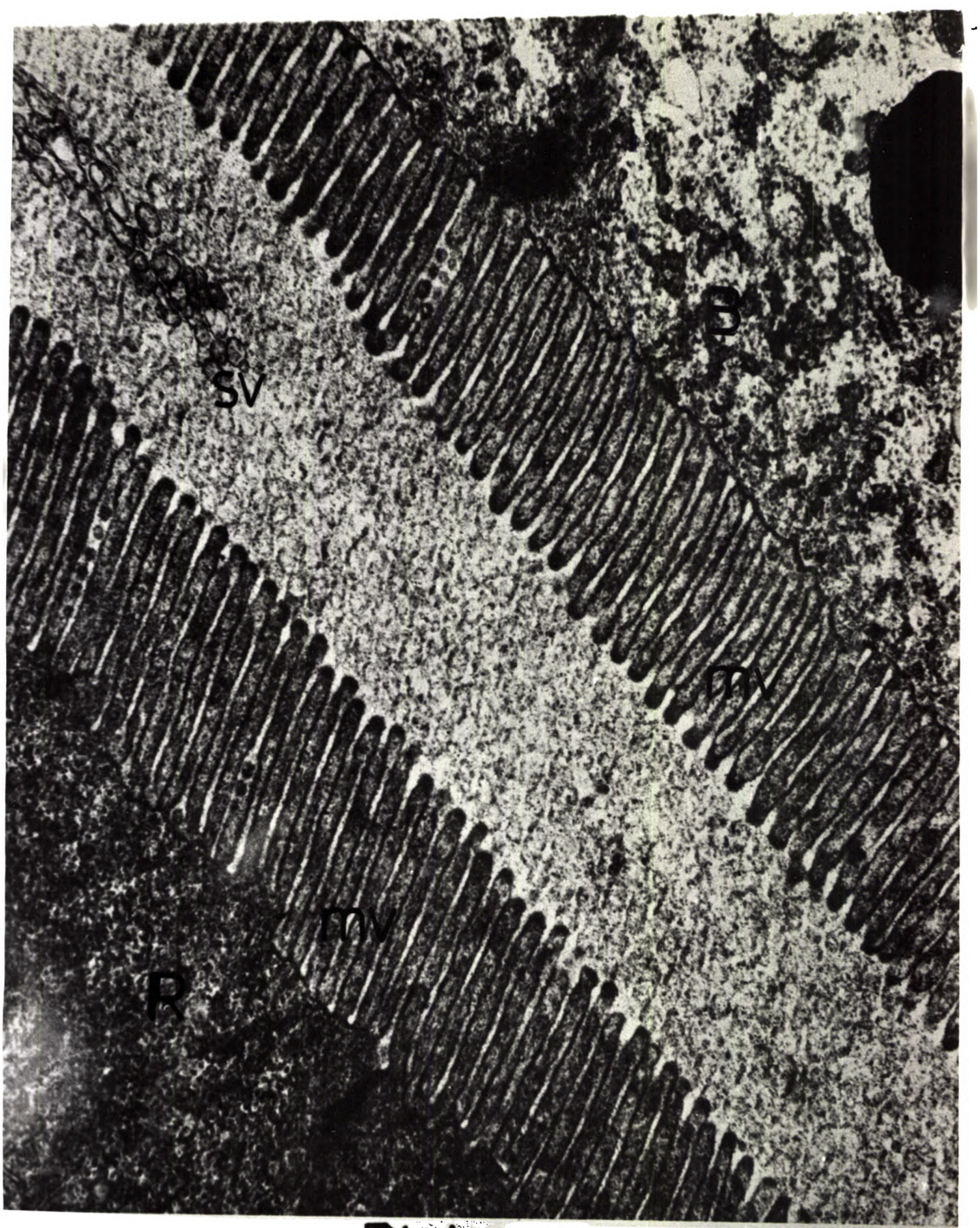


Plate 43

CHAPTER 2

QUALITATIVE ANALYSIS OF DIGESTIVE ENZYMES AND EXPERIMENTAL STUDIES ON ENZYME ACTIVITY

2.1. QUALITATIVE ANALYSIS OF ENZYMES FROM DIFFERENT REGIONS OF THE DIGESTIVE TRACT

INTRODUCTION

Enzymes are proteins specialized to catalyze biochemical reactions. They are among the most remarkable biomolecules known because of their extraordinary specificity and catalytic power, which are far greater than those of man-made catalysts.

The name 'enzyme' was not used until 1877; but much earlier it was suspected that biological catalysts are involved in the fermentation of sugar to form alcohol (hence the earlier name "ferments"). The first general theory of chemical catalysis published in 1835 by J.J. Berzelius included an example of what is now known as an enzyme, diastase of malt, and pointed out that hydrolysis of starch is more efficiently catalysed by diastase than by sulphuric acid (Waksman and Davison, 1926).

Although Louis Pasteur recognized that fermentation is catalyzed by enzymes, he postulated in 1860 that they are inextricably linked with the structure and life of the yeast cell. It was therefore a major land mark in the history of enzyme research when in 1897, Eduard Buchner succeeded in extracting from yeast cells the enzymes catalyzing alcoholic fermentation. This achievement clearly demonstrated that these important enzymes, which catalyze a major energy yielding metabolic pathway can function independently of cell structure. However, it was not until many years later that an enzyme was

first isolated in pure crystaline form. This was accomplished by J.B. Sumner in 1926. For the enzyme, urease, isolated from extracts of the jack bean, Sumner presented evidence that the crystals consists of protein and he concluded that enzymes are proteins. During the period between 1930 and 1936 J. Northrop crystalized the enzymes pepsin, trypsin and chymotrypsin. Today nearly 2000 different enzymes are known. Many have been isolated in pure homogeneous form and at least 200 have been crystallized. It was Kuhn in 1867, who suggested the name enzyme (Price and Lewis, 1982).

A considerable amount of kiterature has accumulated on the digestive enzymes of the decapod crustaceans. Mansour-Bek (1954) in "Tabulae Biologicae" made a full list of all the species investigated for the presence of digestive enzymes till that time. Later, studies of crustacean digestive enzymes have been reviewed by Vonk (1960); Hartenstein (1964), Huggins and Munday 91968); Van Weel (1970); Devillez (1975), Wickins (1976), Gibson and Barkin (1979) and Dall and Moriarty (1983).

Among the carbohydrases which have been identified from Decapoda, amylase is found in most species. Blandamer and Beechey(1964,1966) identified the enzyme from *Carcinus maenas* as an -amylase. Amylase has been reported from *(Penaeus indicus*(Gopalakrishnan,1957), *Homarus americanus*(Wojtowicz and

Brockerhoff, 1972), *Parapenaeopsis hardwickii* (Karunakaran and Dhage, 1977), *Parapeneopsis stylifera* (Kulkarni *et al.*,1979) and from *Macrobrachium lamarrei* (Saxena and Murthy, 1981). Amylolytic activity in the hepatopancreas of *Uca minax, Uca pugnax* and *Uca pugilator* has been reported by Jeffrey *et al.*, (1985).

Takatsuki (1939) reported the presence of "glycogenase" from hepatopancreas of *Panulirus japonicus* and glycogen digestion has been noted by Reddy (1938) in *Paratelphusa hydrodromus* and Nagabushanam and Sarojini(1968) in *Diogenes bicristimanus.*

β-fructofuranosidase (sucrase, invertase or saccharase) is also known from several species of decapods. Saxena and Murthy (1982) made detailed studies of this enzyme from *Macrobrachium lamarrei.*

Other glycoside hydrolases include α-glucosidase (maltase) with a pH optimum ranging between 4.43 and 6.0. Van Weel (1970) recorded an optimum pH of 6.97 for *Thalamita crenata.* Trellu and Ceccaldi (1977) recorded a single glucosidase from hepatopancreatic extracts of *Palaemon serratus* with an optimum of pH 5 and a molecular weight of 65,000, whereas Brun and Wojtowicz (1976) indicated molecular weights of 47,000 and 86,000 for glucosidase from *Cancer borealis* and *Cancer irroratus,* respectively.

β-glucosidases (cellobiase, gentiobiase or amygdalinase) have a slight to moderate level of activity in Decapoda (Telford, 1970; Brockerhoff *et al.*,1970). They have been found in a number of species, but Brun and Wojtowicz (1976) are the only authors to comment on the pH of activity; in both *C. borealis* and *C. irroratus* optimal hydrolysis is at pH 5.0; the respective molecular weights from the two species being 44,000 and 51,000.

α and β-galactosidases (melibiase and lactase respectively) have been found in a number of species, the latter being the most frequently recorded. Kooiman (1964) noted galactosidase in the gastric juice in *Astacus fluviatilis*. α-galactosidases in *C. borealis* and *C. irroratus* have high molecular weights with respective values of 121,000 and 115,000 being obtained by Brun and Wojtowicz (1976). Trellu and Ceccaldi (1976) found that the enzyme from the hepatopancreatic extract of *Palaemon serratus*, together with several other enzymes, exhibited a variable degree of activity which depended upon the moulting cycle.

Chitobiase (N - acetyl - β -glucosaminidase) has been found on only a few occasions in Decapoda. In *Homarus americanus* , it has a molecular weight of 100,000 and optimal pH 5 (Brockerhoff *et al.*,1970). Brun and Wojtowicz (1976) obtained comparable results from studies on *Cancer borealis* and *C. irroratus*. The trisaccharide raffinose is hydrolysed by enzymes of *Astacus*, *Cancer*, *Carcinus*, *Maia* and *Orconectes*, but since this sugar can be digested by either galactosidase or fructofuranosidase (the latter enzyme being present in all these species), the presence of a specific raffinase is doubtful (Gibson and Barker, 1979). Laminaranase (lichenase or β -1, 3 - glucanase) appears in several species, as also do α -mannosidase (mannanase) and β -glucuronidase; Trellu and Ceccaldi (1976) isolated three β -glucuronidases from hepatopancreatic extract of *Palaemon serratus*, all possessing an optimal activity at pH 6, with molecular weights of 235,000, 275,000 and 320,000.

Cellulases (β -1, 4 glucanases) are rare in the Animal Kingdom, despite the fact that the polysaccharide cellulose forms a major dietary constituent in herbivorous organisms. Yokoe and Yasumasu (1964) recorded cellulase activity from eighteen species of Crustacea, the optimum activity of all falling within the range pH 5.8-7.2. Kooiman (1964) also reported cellulases from *Astacus fluviatilis* and *Homarus vulgaris* with an optimum pH in the range 4.0-4.5. Telford (1970) has been unable to demonstrate cellulose digestion in *Orconectes virilis, Carcinus maenas, Cancer borealis* or *C. irroratus*.

Chitinases have been reported from the hepatopancreas of *Eriocheir sinensis* Jeuniaux, (1960, 1963) and from hepatopancreatic or gastic juice extracts of *Astacus fluviatilis* and *Homarus vulgaris* (Kooiman, 1964). Jeuniaux (1960) could not determine whether the enzyme was of endogenous or bacterial origin, but Kooiman (1964) in noting that no chitinase activity could be obtained from bacterial suspensions argued in favour of hepatopancreatic secretion.

β - glucosaminidase and α- fucosidase have been reported from *Palaemon serratus* (Trellu & Ceccaldi, 1976, 1977), and α -xylosidase and xylanase from *Astacus fluviatilis* and *Homarus vulgaris* (Kooiman, 1964).

Telford (1970) found a moderate to strong degree of amylopectin digestion in extracts from species of *Orconectes, Cancer* and *Carcinus*; he did not specify the enzyme responsible; but amylopectin-1, 6-glucosidase will hydrolyse the α -1, 6-glucan links in this substrate (Gibson and Barker, 1979). Telford (1970) also noted the ability of these extracts to split

trehalose, the activity was strong in *C. maenas* but very slight in the remaining species. This is due to the presence of trehalase. The presence of trehalase in the extract of *M. lamarrei* has been reported by Saxena and Murthy (1981). Gopalakrishnan (1957), however, could not detect trehalase in *Penaeus indicus*.

Proteolytic digestive enzymes (proteases or peptide hydrolases) are of two major types: endopetidases and exopeptidases. Among the endopeptidases trypsin, chymotrypsin, cathepsin and clostridiopeptidase A (collagenase) are peptidyl peptide hydrolases, whilst the exopeptidases include α-amino-acyl - peptide hydrolases (aminopeptidases), peptidyl - aminoacid hydrolases (carboxypeptidases) and dipeptide hydrolases (dipeptidases).

Proteolytic activity in the gastric juice or hepatopancreas of Decapoda was first demonstrated by Hoppe-Seyler (1876) using casein, albumin, peptone, gelatin or fibrin as substrates.

Tryptic activity was mentioned by Cattaneo (1887), Maloeuf (1938) and Degkwitz (1957) but it was not until Devillez (1965) working with gastric juice extracts of *Orconectes virilis*, used the specific substrate p-toluene Sulphonyl -L-arginine methyl ester (TAME) that a definite trypsin - like enzyme was identified. Devillez and Johnson (1968) subsequently reported that a trypsin component had been found in thirteen genera of Decapoda. Devillez and Johnson (1968) purified the trypsin - like enzyme in *Orconectes virilis* having a molecular weight of 24,700. This corresponds with the molecular weights of 24,000 for the trypsin from *Astacus fluviatilis* (Pfleiderer,

et al., 1967), 24,000 for *Penaeus setiferus* (Gates & Travis, 1969), 25,000 for *Homarus americanus* (Brockerhoff *et al.*, 1970) and 20,500 in *C. irroratus* (Burn and Wojtowicz, 1976). Eisen and Jeffrey (1969) reported that trypsin accounted for some 33% of the total hepatopancreatic protein contents in *Uca pugilator*.

Huggins and Munday (1968) concluded that crustaceans are generally accepted as not possessing peptic enzymes. Devillez and Lau (1970) reported another enzyme 'alkaline proteinase' in *O. virilis* which acted upon hippuryl - L - arginine and carbobenzoxyglycyl phenylalanine. Pfleiderer *et al.* (1967) have also extracted a protease from *Astacus fluviatilis* with a molecular weight of 11,000 and Sonneborn *et al.* (1969) obtained the same values for *Astacus leptodactylus*.

The occurrence of chymotrypsin - like enzymes appears to have been first noted by Kleine (1966). Using six specific substrates with gastric and hepatopancreatic extracts of *Astacus astacus* and *Cambarus affinis* Kleine (1967) found that chymotrypsin activity was extremely low compared with that of trypsin. Chymotryptic enzymes have also been identified by Brockerhoff *et al.* (1970). Van Wormhoudt *et al.* (1972) have reported protease in *Penaeus kerathurus*. Trypsin activity was also reported from *Macrobrachium lamarrei* by Murthy and Saxena (1979). Araujosaker *et al.* (1982) characterized the proteolytic enzymes in hepatopancreas of juveniles of lobster *Panulirus laevicauda*. Lee *et al.* (1984) detailed the proteases of *Penaeus vannamei* and the relationship between enzyme activity, size and diet. Galgani (1984) identified the digestive proteases like trypsin, carboxypeptidase A and B

and leucine amino peptidase in *Penaeus kerathurus* and *Penaeus japonicus*.

Collagenolytic enzymes with an optimal activity at pH 7.5 have been reported by Eisen and Jeffrey (1969) Eisen *et al.* (1973) Brockerhoff *et al.*(1970) reported this enzyme in *Homarus americanus* which hydrolyzed "Azocoll" (collagen coupled with an azo dye). Grant *et al.*(1983) have also reported a collagenolytic serine protease with trypsin like specificity in *Uca pugilator*.

A number of exopeptidases have been found in decapod crustaceans. Aminopeptidases have been reported as aminopolypeptidases from *Maia squinado* (Mansour-Bek, 1932), leucylpeptidases from *Homarus americanus* (Berger and Johnson, 1940; Brockerhoff *et al.*,1970); aminopeptidase from *Carcinus maenas* (Degkwitz, 1957) and *Astacus astacus* (Kleine, 1966), arylamidase from *Astacus astacus* and *Cambarus affinis* (Kleine, 1967 ; Kleine & Ponyi, 1967), and leucine and valine aminopeptidase from *Palaemon serratus* (Trellu and Ceccaldi, 1977).

Peptidyl-amino-acid hydrolases (carboxypeptidases) have been recorded from several species. Devillez (1965, 1968) and Kleine (1966) recorded the enzyme from *Astacus astacus* as carboxypeptidase A. Sonneborn (1969) isolated two carboxypeptidases A and B from *Astacus fluviatilis*, possessing a molecular weight of 27,000. Brockerhoff *et al.*(1970), on the other hand, were unable to detect carboxypeptidase B activity and found only moderate carboxypeptidase A hydrolysis at pH 7.5. Rodriguez *et al.* (1976) also recorded the A variety in the gastric juice of *Palaemon serratus*. Gates and Travis (1973)

however, have recorded carboxypeptidase A with a molecular weight of 30,000 and carboxypeptidase B with a weight of 34,200 from *Penaeus setiferus*.

Galgani and Benjamin (1985) in a radioimmunoassay of shrimp trypsin studied the specificity of *Penaeus japonicus* trypsin antibodies which interacts with trypsin from the digestive glands of *Penaeus stylirostris*, *Penaeus vannamei*, *Penaeus merguiensis* and *Penaeus kerathurus*. They also concluded that during the larval development of the *Penaeus japonicus* the variations in the enzyme activity depended on the various stages of the development.

Tsai *et al.* (1986) for the first time isolated two chymotrypsins from the hepatopancreas of *Penaeus monodon*. They were found to be single chained with molecular masses of 26 and 27 KDa respectively. Tsai *et al.*(1987) quantified trypsin, chymotrypsin carboxypeptidases A & B and leucine aminopeptidases in the midgut gland of Atlantic blue crab *Callinectes sapidus*.

Lipolytic enzymes (carboxylic ester hydrolases) are of wide spread occurrence in the Decapoda. Early reports of lipolytic activity include those of Hoppe-Seyler (1876), Yonge (1924) and Reddy (1938). True lipases which act only on an ester-water interface and preferentially hydrolyse the outer ester links have been demonstrated only from *Homarus americanus*. Brockerhoff *et al.* (1967) reported the digestion of the triglyceride triolein by *Homarus* gastric juice at pH 4.7. Brockerhoff *et al.*(1967), whilst acknowledging the fact that many esterases were capable of hydrolysing tributyrin, concluded that the breakdown of this substrate at pH 6.5 and of triolein at pH 7.5 by the gastric juice of the lobster is related to a true lipase with a molecular weight

of 43,000. On the basis of tributyrin digestion by hepatopancreatic extracts of *Cancer borealis* and *C. irroratus* at pH 6.5 the presence of a true lipase has been indicated (Brun and Wojtowicz 1976). Van weel (1960) also recorded a "tributyrine esterase" with an optimum pH of 6.97 from *Thalamita crenata.*

Gopalakrishnan (1957) working with hepatopancreatic extracts of *Penaeus indicus* detected the presence of lipase using fresh cow's milk. Nagabushanam and Sarojini (1968) recorded lipolytic activity from *Diogenes bicristimanus* with a pH optimum of 7.4. They also noted that the enzyme responsible was effective upon a wide range of substrates, a wide specificity is characteristic of carboxylic-ester hydrolases (carboxyl esterases). Hoyle (1973) has recorded lipase activity in the American lobster *Homarus americanus*. Trellu and Ceccaldi (1977) stated that there was no true lipase in *Palaemon serratus* although they did find two esterases. Lee *et al.*(1980) in the quantitative analysis of digestive enzymes of the freshwater prawn *Macrobrachium rosenbergii* could detect high esterase activity and only a trace of lipase activity.

The present study has been undertaken to survey the important digestive enzymes in the alimentary system of *Penaeus indicus* and to study changes in the hydrolytic activity with respect to size groups, eyestalk ablation, starvation as well as with varying levels of starch in the diet. In order to give a more logical and efficient approach to the study, α-amylase has been selected for detailed characterisation and purification and has been dealt separately in another Chapter 3 (Pages 79-108).

MATERIALS AND METHODS

Collection of animals: Adult *Penaeus indicus* measuring 120-130mm in length were obtained from Marine Prawn Hatchery, Narakkal and transported in oxygen filled polythene bags to the laboratory. They were immediately transferred to well aerated plastic pools and fed a fresh clam meat diet twice daily.

Preparation of tissue extract: Animals selected for preparation of crude enzyme extracts were starved for 48 hrs to avoid any undigested particles in the digestive system. All procedures in the preparation of the crude enzyme extracts were done in the cold at O° to 4°C. Prawns in the intermoult stages were weighed individually and then dissected over ice. The digestive tract was ligated at specific points. Stomach, midgut, hepatopancreas and hindgut were dissected-out; cleaned with cold distilled water; blotted dry on filter paper and weighed. Tissues were pooled and homogenised in ice-cold distilled water. The homogenate was centrifuged at 20,000 rpm for 20 minutes at 4°C in a Sorvall RC 5 B refrigerated centrifuge. The supernantant was subsequently used as the crude enzyme extract. Two or three drops of toluene were added as an antibacterial agent. All enzyme tests were run on the extracts within 2 days after separation from the tissues.

To find out the sites of secretion, enzyme extracts obtained from different parts of the gut were incubated separately with different substrates.

The substrates used for determining the presence of carbohydrases were 1% starch, raffinose, inulin, cellulose, 2% glycogen, maltose, lactose, and 5% sucrose. Extract (10%) was incubated with the above substrates upto 96 hrs at 37°C in a constant temperature water bath with a shaking device. Qualitative tests were carried out using Benedicts and Barfoed's reagents in the end product analysis after digestion. A few drops of toluene were added to the digestive mixture as an antibacterial agent (Hawk *et al.*, 1954).

Proteolytic enzymes were qualitatively demonstrated by incubating 10 ml of 5% gelatin with 1 ml of the different extracts for 48 hrs. at 37°C. The liquefaction of gelatin was considered for the presence of proteases while in control experiments it remained solid (Agrawal, 1963).

Extract (10%) was incubated with each of the substrates-1% fibrin, peptone and egg albumin for 48 hrs, the proteins were precipitated by 5% trichloroacetic acid and the products of digestion were determined by biuret reagent (Bodansky and Fay, 1947).

The presence of lipases was tested (Hawk *et al.*, 1954) by the use of condensed milk. Two drops of bromothymol blue were added to 25 ml of 10% milk solution. To this was added 1% sodium hydroxide solution until the colour changed to light blue. 1 ml of this blue milk solution was incubated with a few drops of extract. The change in colour of the milk to yellow was considered for the presence of lipase.

The results are summarised in Tables 3, 4, 5 & 6. The sign ++ denotes a vigorous reaction; sign + a definite positive reaction, ± only a trace of reaction. While the sign - indicates no reaction.

pH Measurements

pH of the different parts of the gut was also determined because activity of enzymes is dependent on the hydrogen ion - concentration. Fresh fed prawns were dissected to measure the pH in the different parts of the digestive tract. Stomach, hepatopancreas, midgut and hindgut regions were separately dissected out, washed with cold distilled water and homogenised. After centrifuging the extract at 15,000 r.p.m. in a refrigerated centrifuge at 4°C for 10 minutes the pH of the clear supernatant was measured both by indicator paper (BDH indicator paper) and pH meter with a microelectrode. pH measurements were also made in prawns starved for about 48 hrs for comparison.

Tables 7 & 8 shows the average ph of the different parts of the gut of fed and starved prawns.

RESULTS

Preliminary qualitative experiments with the enzyme extract of hepatopancreas revealed that most of the tested carbohydrates are digested by the enzymes secreted by the gland (Table 3). Inulinase, raffinase and cellulase are relatively less active and take more time for complete hydrolysis. Among the substrates for protein digestion fibrin, peptone and egg-albumin are readily acted upon by proteases, but gelatin is not hydrolysed by hepatopancreatic extract. The fat digesting enzymes also take a long time (more than 24 hours) to digest the substrate.

In the stomach, amylase, glycogenase, lactase, raffinase and cellulase are present indicating their role in the preliminary digestion of the ingested food. Maltase is not present. Fibrin, peptone and egg albumin are hydrolysed in the stomach but not gelatine. Fat digesting enzymes could not be detected in the stomach (Table 4).

In the midgut, starch, glycogen, maltose, lactose and sucrose are easily digested; whereas the other carbohydrate substrates are not hydrolysed. Slight digestion of fibrin and egg-albumin was observed. Whereas only weak lipid digestion was recorded (Table 5).

In the hindgut, very few enzymes like inulinase, raffinase, sucrase are present. Very weak protease and total absence of lipase was observed (Table 6).

The hydrogen -ion concentration measured (Table 7) indicate an acidic medium for stomach and hepatopancreas and alkaline medium for the midgut and hindgut regions in fed prawns. In the starved prawns the pH value indicates a highly acidic medium in the stomach and a highly alkaline medium in other regions of the digestive tract (Table 8).

DISCUSSION

The hepatopancreas of *Penaeus indicus* as in several other crustaceans contains carbohydrases, lipases and proteases as evident from the Table 3. It is also clear from the results that the hepatopancreas is the major site of digestion of food materials in *P. indicus*. Majority of the carbohydrases and to a certain extent proteases and lipases are very active in the hepatopancreas. Stomach and midgut showed slight activity for some of the enzymes. The hindgut exhibited very weak activity for carbohydrases, but no activity for proteases and lipases.

The existence of separate enzymes in the hepatopancreas has been shown in several crustaceans and the nature and strength of the digestive enzymes are correlated to the feeding habits of the animals concerned (Yonge, 1931; Vonk, 1937, 1960).

The hepatopancreatic extract could hydrolyse a variety of carbohydrates such as starch, glycogen, maltose, lactose, sucrose; but cellulase and raffinase are not present in the extract. Gopalakrishnan (1957) also could not

detect raffinase, cellulase and inulase in *P. indicus*. However, Kooiman (1964) observed the presence of maltase, cellulase, mannanase, chitinase, xylanase and saccharase in the digestive juices of *Homarus* and *Astacus*.

The presence of protease capable of digesting casein, fibrin and peptone is also evident from the present study. Gelatin is poorly digested. The over all study indicates that hepatopancreas is the major site of enzyme production. Since the hepatopancreas in crustaceans has the dual function of the liver and pancreas of the higher animals it is no wonder that it stores and synthesises almost all the digestive enzymes.

In *Penaeus indicus* the lipolytic activity is rather weak as evident from the results. However, the extract of hepatopancreas in *Penaeus indicus* contain a lipase as reported in *Nephrophs* (Yonge, 1924) and in *Paratelphusa* (Reddy ,.1937). A more active esterase rather than a lipase has been reported in *Macrobrachium* (Lee *et al.*, 1980).

Starvation also seems to have an effect on the constituents of the secretion material, other than enzymes. This becomes visible in a change in pH of the gastric juice. Starvation always resulted in a low pH value in the gut regions than in freshly caught or well-fed animals. Van Weel (1970) has listed a number of species wherein starvation caused a drop in the pH value of the gland extract or stomach juice. Since it is believed that the pH of the digestive juice is caused by NaH_2Po_4 and $CaH_2(Po_4)_2$, starvation might have its effect on the liberation of

of these inorganic compounds (Van Weel, 1970). The present observations in *P. indicus* also agrees with the above findings. All the regions showed a drop in the pH value.

TABLE 3: Digestion of various substrates by the crude enzyme extract from hepatopancreas of *Penaeus indicus*

Sl. No.	Substrate	Duration of reaction and extent of digestion			Control		
		After 24 hrs	After 48 hrs	After 96 hrs	After 24 hrs	After 48 hrs	After 96 hrs
1.	1% starch solution	++	+	+	-	-	-
2.	2% glycogen	-	++	+	-	-	-
3.	2% Maltose	+	+	-	-	-	-
4.	2% lactose	+	+	+	-	-	-
5.	1% inulin	-	+	-	-	-	-
6.	1% raffinose	-	+	+	-	-	-
7.	5% sucrose	+	+	-	-	-	-
8.	1% cellulose	-	+	-	-	-	-
9.	5% gelatine solution	Remains Solid			Remains Solid		
10.	1% fibrin	-	+	-	-	-	-
11.	1% peptone	-	+	-	-	-	-
12.	1% egg albumin	+	-	-	-	-	-
13.	Condensed milk	slight change in colour (Yellow)			No change		

TABLE 4: Digestion of various substrates by the crude enzyme extract from stomach of *Penaeus indicus*

Sl. No.	Substrates	Duration of reaction and extent of digestion			Controls		
		After 24 hrs	After 48 hrs	After 96 hrs	After 24 hrs	After 48 hrs	After 96 hrs
1.	1% starch solution	+	++	+	-	-	-
2.	2% glycogen	-	+	+	-	-	-
3.	2% maltose	-	-	-	-	-	-
4.	2% lactose	+	-	-	-	-	-
5.	1% inulin	-	-	-	-	-	-
6.	1% raffinose	±	±	-	-	-	-
7.	5% sucrose	+	+	-	-	-	-
8.	1% cellulose	+	-	-	-	-	-
9.	5% gelatine	Remains Solid			Remains Solid		
10.	1% fibrin	+	+	-	-	-	-
11.	1% peptone	±	+	+	-	-	-
12.	1% egg albumin	+	+	-	-	-	-
13.	Condensed milk	No change in colour			No change		

TABLE 5: Digestion of various substrates by the crude enzyme extract from midgut of *Penaeus indicus*

Sl. No.	Substrate	Duration of reaction and extent of digestion			Controls		
		After 24 hrs	After 48 hrs	After 96 hrs	After 24 hrs	After 48 hrs	After 96 hrs
1.	1% starch solution	+	+	-	-	-	-
2.	2% glycogen	±	+	-	-	-	-
3.	2% maltose	-	+	-	-	-	-
4.	2% lactose	-	+	-	-	-	-
5.	1% inulin	-	-	-	-	-	-
6.	1% raffinose	-	-	-	-	-	-
7.	5% sucrose	+	-	-	-	-	-
8.	1% cellulose	-	-	-	-	-	-
9.	5% gelatine	Remains Solid			Remains Solid		
10.	1% fibrin	+	-	-	-	-	-
11.	1% peptone	-	-	-	-	-	-
12.	1% egg albumin	-	+	-	-	-	-
13.	Condensed milk	Colour change to yellow			No change		

TABLE 6: Digestion of various substrates by the crude enzyme extract from hindgut of *Penaeus indicus*.

Sl. No.	Substrate	Duration of reaction and extent of digestion			Controls		
		After 24 hrs	After 48 hrs	After 96 hrs	After 24 hrs	After 48 hrs	After 96 hrs
1.	1% starch solution	-	-	-	-	-	-
2.	2% glycogen	-	-	-	-	-	-
3.	2% maltose	-	-	-	-	-	-
4.	2% lactose	-	-	-	-	-	-
5.	1% inulin	-	+	+	-	-	-
6.	1% raffinose	+	-	-	-	-	-
7.	5% sucrose	+	-	-	-	-	-
8.	1% cellulose	-	-	-	-	-	-
9.	5% gelatine	Remains	Solid		Remains	Solid	
10.	1% fibrin	-	-	-	-	-	-
11.	1% peptone	-	±	-	-	-	-
12.	1% egg albumin	-	±	-	-	-	-
13.	Condensed milk	No change in colour			No change		

TABLE 7: **pH** of the digestive tract in fed *Penaeus indicus*.

Sl. No.	Stomach	Midgut	Hepatopancreas	Hindgut
1.	5.13	7.52	5.98	8.51
2.	5.43	7.43	6.10	9.42
3.	4.98	7.35	5.82	8.54
4.	4.85	7.25	5.98	8.35
5.	6.01	7.41	6.02	8.20
Avg. pH.	5.28 ± 0.462	7.39 ± 0.100	5.98 ± 0.102	8.404 ± 0.136

TABLE 8: pH of the digestive tract in starved *Penaeus indicus*

Sl. No.	Stomach	Midgut	Hepatopancreas	Hindgut
1.	5.12	7.42	6.28	8.23
2.	4.80	7.32	6.10	8.15
3.	4.82	7.25	6.47	8.02
4.	4.59	7.03	6.36	8.14
5.	5.20	7.20	6.25	8.28
Avg pH	4.906 ± 0.250	7.24 ± 0.145	6.29 ± 0.137	8.16 ± 0.099

2.2 DIGESTIVE ENZYME ACTIVITY PATTERN IN VARIOUS SIZE GROUPS

INTRODUCTION

Several authors have suggested that crustaceans such as *Artemia* (Bellini, 1957), *Palaemon serratus* (Van Wormhoudt, 1973) and *Penaeus japonicus* (Laubier - Bonichon *et al.*, 1977) show wide variations in protease and amylase activities during the larval development. They described the influence of developmental stages on these variations, as have other authors for mammals (Corring and Aumaitre, 1970; Lebas *et al.*, 1971) and amphibians (Urbani, 1957). Experiments on prefeeding stages (developing embryos) in *Artemia* (Bellini, 1957) and chick (Kulka and Duskin, 1964) demonstrated the existence of periods for genetic expressions of amylase and protease synthesis. McGeachin *et al.*, (1972) have reported the variations in α-amylase during the life span of the mosquito. Tanaka *et al.* (1972) studied the activity changes of digestive enzymes in the fish, *Pecoglossus altivelis* during larval and juvenile stages.

Van Wormhoudt and Sellos (1980) based on biochemical studies of growth and digestive enzymes in *Palaemon serratus* reported that amylase/protease ratio did not increase regularly from the larvae to the adult stage. Van Wormhoudt (1981) compared the evolution and control of amylase and protease activity during larval development and the early juvenile stages in *Palaemon serratus*, *Macrobrachium rosenbergii* and *Penaeus japonicus* and attributed the changes in the enzyme activity to modification of nutrition and to the appearance of an hormonal control.

The present investigation was undertaken to study the amylase, protease and lipase activity in the postlarvae, juveniles and adults of *Penaeus indicus* and to understand variations, if any, during these growth stages.

MATERIALS AND METHODS

Prawn specimens for the study were collected regularly from Narakkal. Postlarvae (total length 15-30 mm) were collected from the plastic lined nursery pools of the Prawn Hatchery at Narakkal using hand-nets, transported alive in oxygen packed polythene bags and maintained in the laboratory in well aerated sea water of salinity 17 ± 3 ppt for 2 days. Juveniles (total length 50-90 mm) and adults (total length 130 mm) were collected from grow-out ponds in 50 l plastic bins containing fresh sea water of salinity 20 ± 2 ppt. After transportation to the experimental laboratory transferred to 500 l plastic pools having well-aerated sea water of salinity 20 ± 2 ppt. All the prawns were fed with a pelleted diet once daily during the acclimation period of two days. Animals in the intermoult stages were selected. Prior to dissection the animals were starved for 24 hrs.

Enzyme extract preparation was made following the methods given in Chapter 3 (Pages 86-88) Enzyme assays for the experimental studies were performed in the following way.

The quantitative estimation of the amylase activity was determined using Bernfeld (1955) method. The reaction mixture consisted of 0.5 ml of 1.0 percent soluble starch solution, 0.1 ml of 0.5 M NaCl, 0.5 ml of

Sorensen's phosphate buffer and 0.1 ml of the (1%) enzyme extract. This mixture was incubated for 50 minutes at 37°C in a constant temperature water bath. The reaction was stopped by adding 0.5 ml of 3,5- Dinitro salicylate reagent. Shook well. The tubes were covered with marbles and kept in a boiling water bath for 10 minutes, after which they were cooled and diluted to 6 ml with double distilled water. The orange-red colour formed was measured at 540 nm using a reagent blank containing boiled enzyme extract in a ECIL UV/VIS spectrophotometer.

A standard graph for maltose was calibrated. A standard solution of maltose was prepared at a concentration of 1 mg/ml.

The protein content of the crude extract was determined according to the method of Lowry *et al.* (1951) method using bovine serum albumin. Fraction V as the standard.

Total proteolytic activity was determined by the method of Kunitz (1947). Activity was calculated on the basis of tyrosine content of peptides produced by proteolysis in the trichloro acetic acid supernatant per minute.

The reaction mixture consisted of 0.5 ml of 1% casein, 0.4 ml of 0.1 M Tris-HCl buffer of pH (7.8) and 0.1 ml of enzyme extract (1%) and incubated in constant temperature water bath at 37°C for 30 minutes. Digestion was stopped by adding 2ml of 10% TCA(Trichloro acetic acid)solution. After standing for 10 minutes the precipitate was removed by contrifugation and to one portion of the supernatant was added 0.5 ml of diluted Folin-Ciocalteu

reagent and measured at 660 nm against boiled extract being used as control in a ECIL UV/VIS Spectrophotometer. For the evaluation of data the tyrosine calibration curve was used (Lowry *et al.*, 1951).

For lipolytic activity, titrimetric method of Cherry and Crandall (1932) with suitable modification was employed.

Olive oil emulsion prepared by homogenising equal volumes of pure olive oil and 1% polyvinyl alcohol served as the substrate.

The assay mixture consisted of 3.0 ml of distilled water, 1.0 ml of 5% enzyme extract, 0.5 ml of phosphate buffer and 2.0 ml of olive oil emulsion. Shook well using a shaker and incubated at 37°C for 24 hours. Then 3 ml of 95% ethyl alcohol and two drops of 2% phenolphthaline indicator were added to each tube. Boiled enzyme extract along with the reaction mixtures were used as the control. Titrated separately against 0.02 N NaOH in a microburette. The end point of titration was marked by the appearance of a permanent pink colour. The activity of lipase was determined from the amount of NaOH required minus the amount of NaOH required by the control. Amylase activity was represented as the relative or total activity (mg maltose formed per minute per ml of the enzyme extract) and specific activity (mg maltose formed per minute per mg protein in the extract).

Protease activity was represented as the relative or total activity (µg tyrosine formed per minute per ml of the enzyme extract) and specific activity (µg tyrosine formed per minute per mg protein in the extract).

Standard graphs of maltose and tyrosine are represented in (Fig. 6).

Preliminary standardisation for the enzyme assays were performed. Amylase , has been characterized in detail in (Chapter 3) and proteases and lipases assays were conducted to find out their optimum pH and incubation time, Fig. 7 indicates the optimum conditions for enzyme activity. Enzyme assays were conducted using the entire animal tissue in the postlarval group. Whereas, in the juveniles and adults hepatopancreas were dissected out and pooled for the preparation of crude enzyme extract. A series of three experiments were conducted with each size group and the values of enzyme assay were represented as mean of six different determinations from each of the experiments.

In this experiment, the amylase and protease activities were represented as the amount of product formed per ml of whole animal tissue extract per minute for postlarvae, whereas for juveniles and adults, represented as amount of product formed per ml of hepato-pancreatic extract per minute. Lipases were represented as titer value obtained per ml of whole animal extract for postlarvae and titer value per ml of hepatopancreatic extract for juveniles and adults.

RESULTS

Table 9 shows the activity patterns of enzymes in the three groups. In the postlarval group all the tested enzymes showed relatively lower activities than the other two groups.

Juveniles and adults had significantly higher values for amylase than the postlarvae. Amylase activity did not differ significantly between juveniles and adults. However, the specific activity of amylase showed a steady rise from postlarval to the adult stage, with the latter showing the highest value. Protease activity was markedly higher in the juveniles (three and a half fold) and adults (twenty two folds) than the postlarvae. The specific activity also recorded a consistent increase from postlarvae to adult stage.

Lipase activity was rather low in the postlarval and juvenile groups, but a two fold increase in activity was recorded in the adult group when compared to the juveniles.

Soluble proteins gave a different picture (Table 9). Postlarval group had the highest level being 15.10±0.98 mg/ml followed by juveniles and the adults. The high level recorded in the postlarval group is mainly because of the entire animal tissue homogenate being used for the assay.

DISCUSSION

Digestive enzyme activities vary during larval growth (Ceccaldi, 1982). According to him almost all the marine larvae usually begin to eat phytoplankton and later zooplankton. Van Wormhoudt (1973) and Van Wormhoudt and Sellos (1980) have observed marked changes in the enzyme activity during the metamorphosis of zoeal stages to mysis to postlarval stages in *Palaemon serratus* and *Penaeus japonicus*. However, there appears to be no previous information regarding the changes in digestive enzyme activity beyond the postlarval stages in the penaeid groups. It is clear from the present study that proteases are the dominant digestive enzymes in all the three size groups suggesting that protein is the predominant nutrient in the diet and being effectively utilized by all the three size-groups. However, the significant activity levels recorded for all the three enzymes in the three size-groups of prawns clearly reflect upon their omnivorous feeding habit. The significant increase in the amylase activity in juveniles when compared to postlarvae suggests that the juveniles have better capability to utilize carbohydrates as a source of energy nutrient.

The highly significant increase in the activity of proteases and lipases from the postlarval to the adult stage reflect upon the shift in the food preference of the animals. As the animal grows to become adult it seems to require nutritionally rich diet with substantial amounts of proteins and lipids when compared to postlarva. Observations of Hall(1962) show that in nature the smaller specimens feed mainly on plant tissue

while the large specimens fed predominantly on larger crustaceans. But no size - related differences in the diet was noticed by Kuttyamma (1974) in *P. indicus* but she noted larger quantity of debris in young prawns, and small molluscs, and plant matter in adults. Gopalakrishnan (1952) also observed that vegetable matter and crustaceans formed the bulk of the food consumed by *P. indicus*. Since the same pelleted feed, was fed to all the three groups, no diet induced variation in enzyme activity can be expected. The variations in activity of the three groups of enzymes recorded during the study indicates that the observed variations are mainly size related.

The ability of an organism to digest a given substance is predominantly dependent on the presence of appropriate enzymes (Smith, 1980). The adults of *Penaeus indicus* are known to be predominantly carnivores. In order to effectively utilise the protein and lipid rich animal matter in the diets, the prawns have to essentially secrete more of the digestive proteases and lipases and thus increased activity of the enzymes are recorded.

Thus it is clear that digestive enzyme activity changes with the stage of the animal. While amylase showed maximum activity in the juvenile stage; the adults, getting prepared for the maturation and propagation of the species showed greater protease and lipase activities demonstrating their preference for a carnivorous type of diet.

TABLE 9. Digestive enzyme activity in different size groups of *Penaeus indicus*.

	Postlarval group	Juvenile group	Adult group
Amylase - Total activity	7.49 ± 0.816	10.13 ± 0.351	10.04 ± 0.352
Specific activity	0.496 ± 0.054	0.92 ± 0.032	0.936 ± 0.33
Protease - Total activity	19.55 ± 0.84	74.10 ± 20.20	173.25 ± 33.80
Specific activity	1.302 ± 0.72	6.835± 1.84	16.16 ± 3.15
Lipase - Total activity	0.894 ± 0.72	1.058 ± 0.024	2.34 ± 0.178
Soluble proteins	15.10 ± 0.98	10.96 ± 2.51	10.64 ± 3.18

Total activity - mg/ µg products released/ml of whole animal tissue/ min in post-larval group and mg/ µg products released/ml of hepatopancreatic extract/min in juvenile and adult groups.

Specific activity - mg/ µg products released/mg protein/min.

Lipase - Milliliter of 0.02 N NaOH required for neutralisation of fatty acid liberated.

Fig. 6. Standard graph for quantitation of protein, maltose and tyrosine. Bovine serum albumin, maltose and L. tyrosine were used as standards.

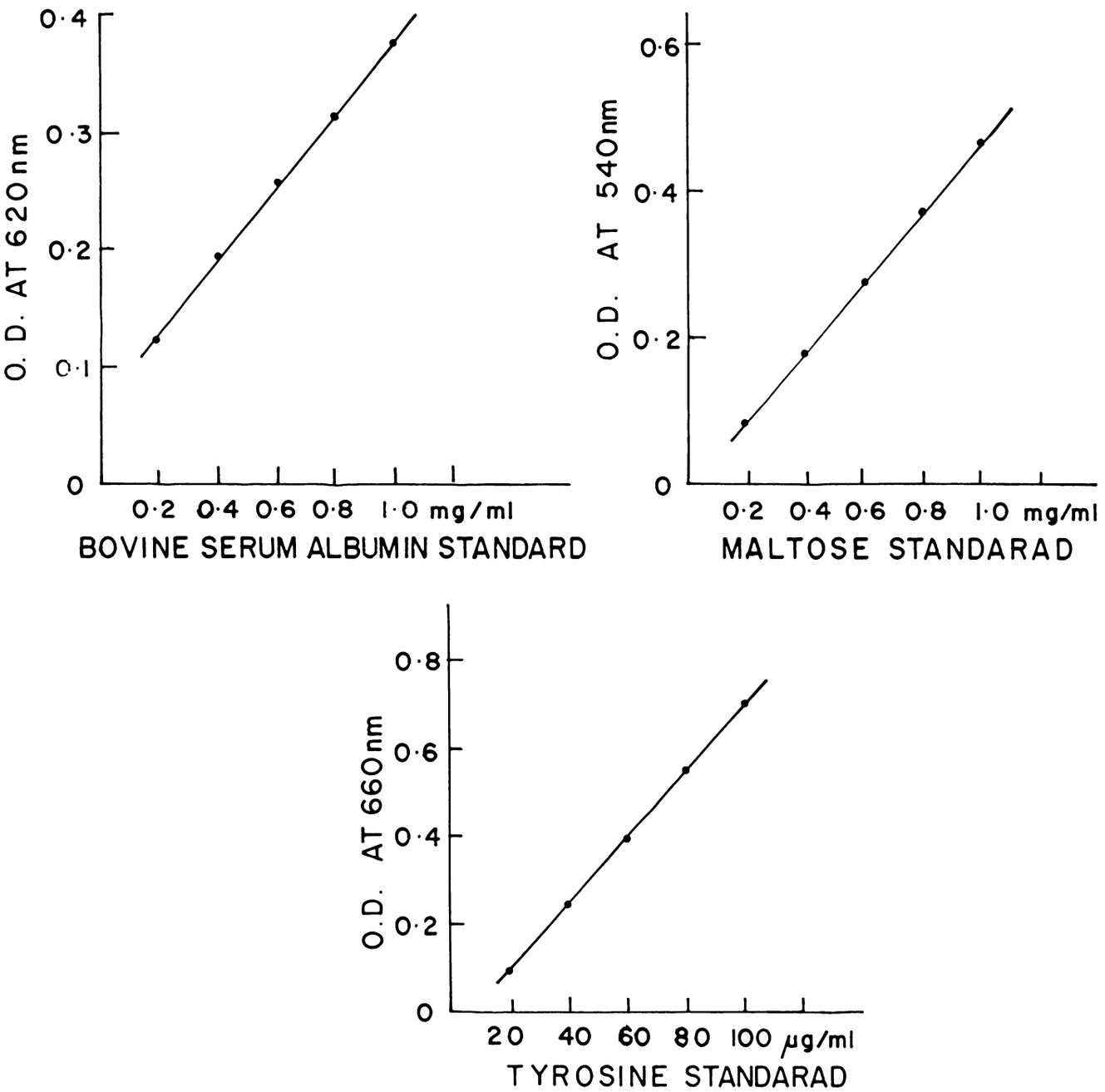
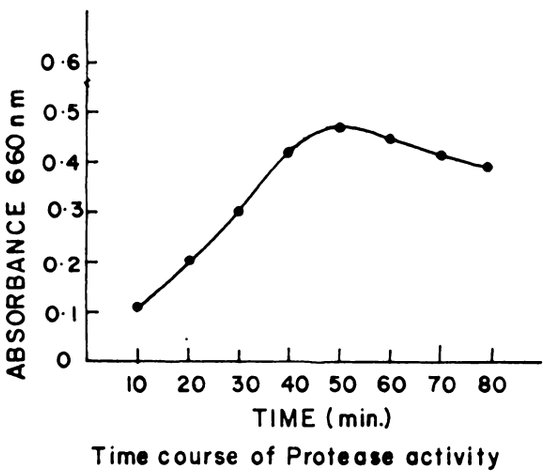
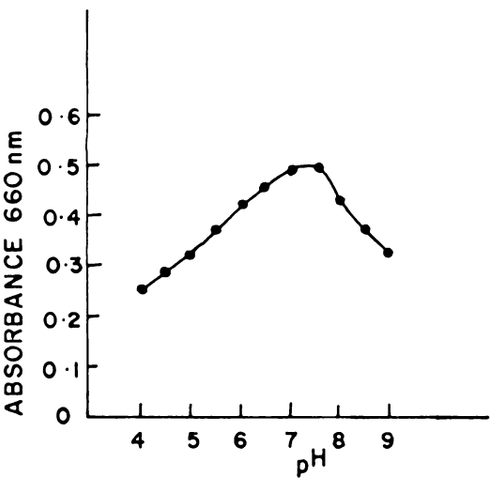

O. D. AT 620nm
0·4
0·3
0·2
0·1
0
0·2 0·4 0·6 0·8 1·0 mg/ml
BOVINE SERUM ALBUMIN STANDARD
O.D. AT 540nm
0·6
0·4
0·2
0
0·2 0·4 0·6 0·8 1·0 mg/ml
MALTOSE STANDARAD
O.D. AT 660nm
0·8
0·6
0·4
0·2
0
20 40 60 80 100 µg/ml
TYROSINE STANDARAD

FIG. 6

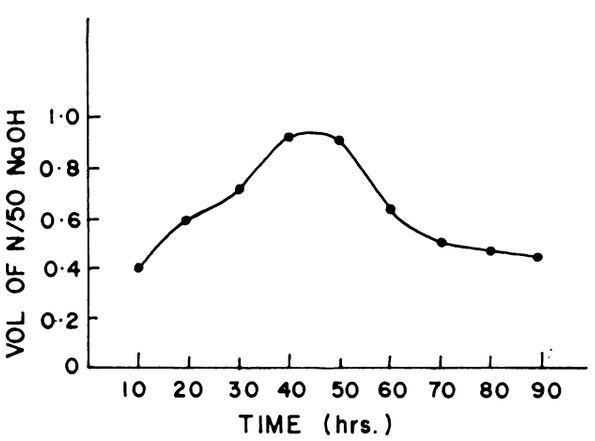
Fig. 7. Time course and effect of pH on the activity of protease and lipase in Penaeus indicus



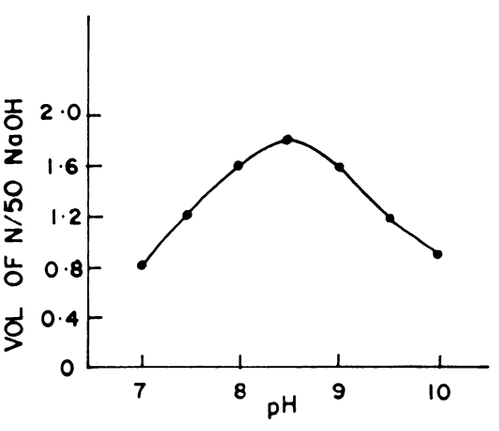
Time course of Protease activity



pH Optimum for Protease activity



Time course of lipase activity



pH Optimum for lipase activity

FIG. 7

Fig. 8. Activity of amylase and protease in the post-larvae(PL) juvenile (J) and adult(A) of Penaeus indicus. Each point represents a mean of six independent determinations.

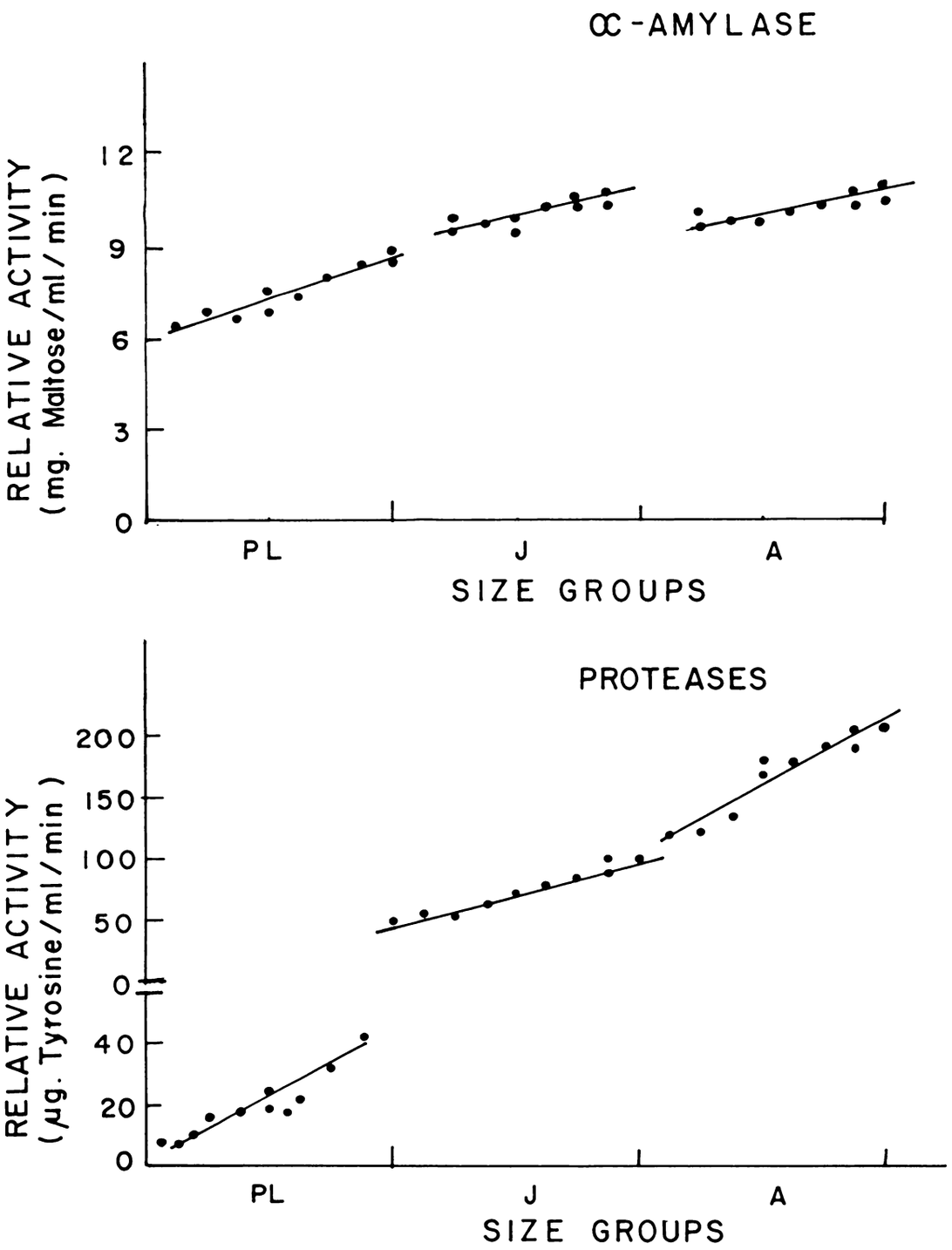

α-AMYLASE
RELATIVE ACTIVITY
(mg. Maltose/ml/min)
12
9
6
3
0
PL
J
A
SIZE GROUPS
PROTEASES
RELATIVE ACTIVITY
(μg. Tyrosine/ml/min)
200
150
100
50
0
40
20
0
PL
J
A
SIZE GROUPS

FIG. 8

Fig. 9. Lipase activity and soluble protein level in postlarvae (PL) Juvenile(J) and Adult (A) groups of Penaeus indicus. Each point is a mean value of six independent determinations.

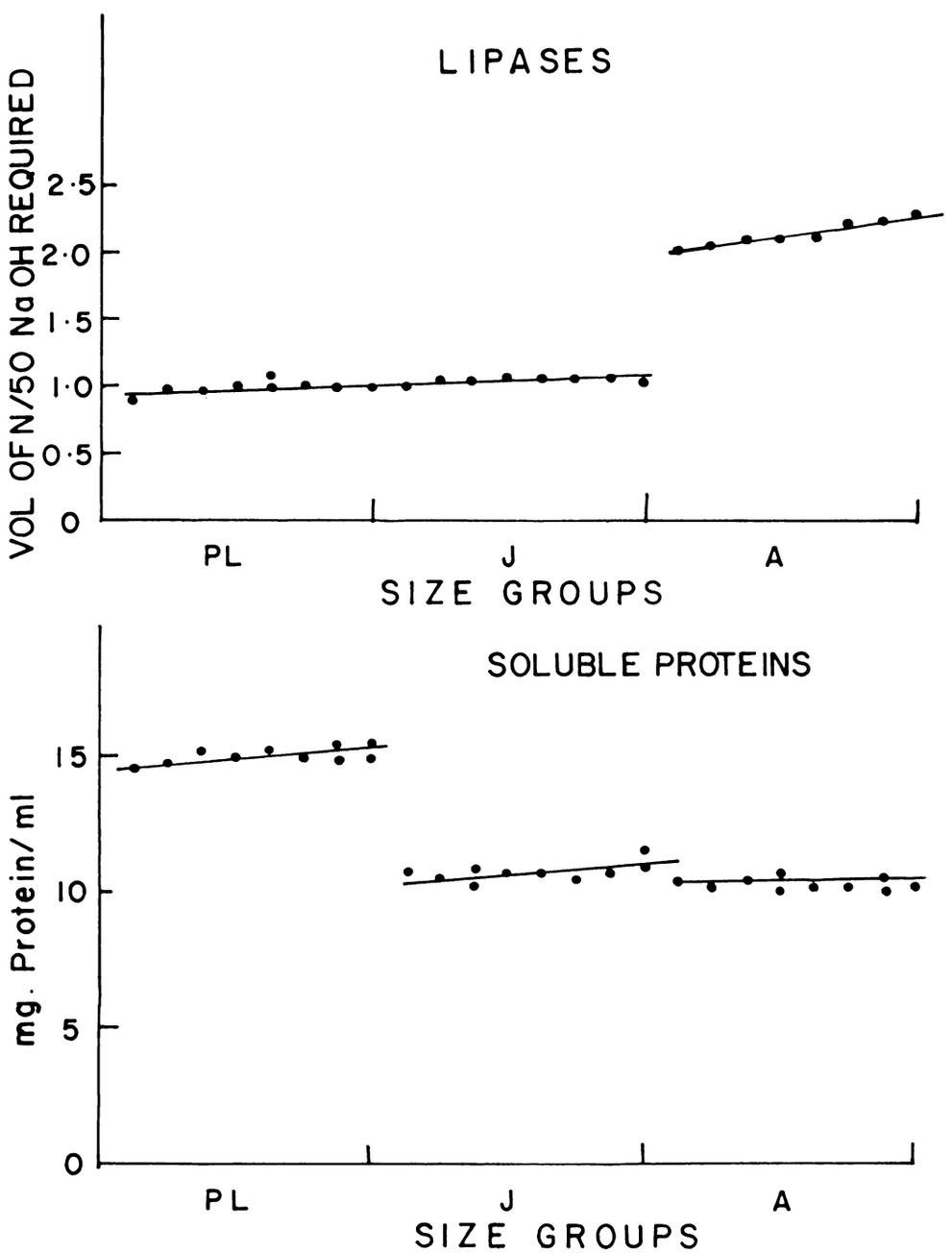

LIPASES
VOL OF N/50 NaOH REQUIRED
2·5
2·0
1·5
1·0
0·5
0
PL
J
A
SIZE GROUPS
SOLUBLE PROTEINS
mg. Protein/ ml
15
10
5
0
PL
J
A
SIZE GROUPS

FIG. 9

2.3 EFFECT OF STARVATION AND FEEDING ON DIGESTIVE ENZYMES

INTRO DUCTION

Many of the starvation studies in crustaceans dealt with the changes in the biochemical compositionn of blood, muscle and hepatopancreas. Djangmah (1970) found that starvation reduced blood proteins in *Crangon*, particularly haemocyanin and glycoproteins, and that these changes were reversed by feeding. Busselin (1970) reported that a glycoprotein fraction in *Carcinus* disappeared entirely during starvation. Alikhan and Lysenko (1973) described the disappearance of a glycoprotein and four other minor protein fractions from the blood of *Porcellio laevis* after 15 days starvation, while haemocyanins were lowered by about 10%. Cuzon and Ceccaldi (1972) found qualitative and quantitative blood protein changes during starvation of *Penaeus kerathurus* and stated that haemocyanin played the role of a nutritional reserve. Dall (1974) reported that starvation caused a greatest decrease in the protein and non-protein amino acid concentration. Dall (1975) in another set of starvation experiments on *Panulirus longipes* concluded that the concentrations of gastric fluid protein (GFPC) and non-protein amino acids are both considerably reduced. Clifford and Brick (1983) while studying the substrate metabolism in fasting juvenile freshwater shrimp *Macrobrachium rosenbergii* concluded that energy metabolism in fasting shrimps was initially dominated by carbohydrate substrates, followed by a shift towards lipid and protein oxidation respectively, as starvation progressed.

There are relatively few studies combining starvation and digestive enzymes. Hoyle (1973) found that absolute amounts of proteinase and lipase in 'stomach juice' rose after a period of feeding following starvation in lobsters. Cuzon *et al.* (1980) while studying starvation effect on metabolism of *Penaeus japonicus* made passing reference to enzyme activity. They concluded that digestive enzyme activity increased in the fed animal and declined considerably in the starved animal.

Studies in insects by Teo and Woodring (1985) showed a decreased amylolytic activity in the starved house cricket *Achaeta domesticus*. Starvation of crickets upto 10 days had the general effect of reducing all activity levels of enzymes measured in mid and hind guts (Thomas and Nation, 1984). A 60% decrease in amylase level was observed in the beetle, *Alphitobius laevigatuos* after one day starvation (Sio and Teo, 1974/75). Khan and Ford (1962) also found a loss of 50% amylase activity in *Dysdercus fasciatus*. Starvation stress also led to slight decline of digestive enzyme activity in adults of the insect, *Schizodactylus monstrosus* (Sanjay *et al.*, 1981).

In the present investigation, starvation induced changes in the major hydrolytic enzymes of the hepatopancreas has been studied in *P. indicus* after starving the animals for a period of 15 days and the results are compared with normal fed animals.

MATERIALS AND METHODS

Juvenile *Penaeus indicus* (4g average weight) obtained from culture ponds were maintained in 500 liter capacity plastic lined pools and fed fresh clam meat once daily during the acclimation period of 3 days. For the experiment, two 500-liter sea water pools with a salinity of 20±2 ppt were maintained at a temperature of 28±1°C. One hundred and forty prawns were distributed between the two pools, each pool with 70 animals. No food was given to the prawns in pool I (starved) while prawns in the pool II(fed) were fed once daily *ad libitum* with live clam meat. The experiment was carried out for 15 days.

Prawns were sampled from both pools initially (t_0) 5th day (t_5) 10th day (t_{10}) and on the 15th day (t_{15}) at 0800 Hrs and 10 individuals at intermoult (Stage c) (Drach and Techernigovtzeff, 1967) were kept for analysis. The hepatopancreas from the selected animals were dissected over ice; dried between filter paper folds and weighed. Preparation of enzyme extract, the concentration of the extract used and the methods of analysis of amylase, protease, lipase and general proteins are as in Chapter 2 (Pages 41-43 and 49-51) Each value represented is a mean value of 6 replications. The experiment was repeated three times.

RESULTS

The results of the experiment are summarised in Table 10. The soluble proteins in the hepatopancreatic extract increased gradually from 10.56 ± 0.01 to 18.17 ± 0.2 mg/ml in fed animals; but starvation induced a gradual decrease in the soluble protein levels from 10.56 ± 0.01 to 5.82 ± 0.03 mg/ml.

Amylase activity in fed animals increased from 2.04 ± 0.06 to 3.59 mg maltose/ml of extract/min, during the experiment; in the starved animals the total amylase activity showed a slight drop in the value from 2.04 ± 0.06 to 1.73 ± 0.003 mg maltose/ml extract/min in the 5th day followed by a slight increase in activity reaching 2.18 ± 0.01 mg maltose/ml of extract/min. on the 15th day. The increase was only 6.42% when compared to the Ist day starvation. Specific activity (Table 11) in the fed group showed a slight drop on the 5th day and then increased considerably on the 10th and 15th days. In the starved ones, however, there was slight increase in the activity from 1st day to 15th day.

Total proteases activity in the fed animals showed an increase with time, from 208.41 ± 1.22 to 258.46 ± 5.33 µg tyrosine/ml of extract/min; whereas the specific activity showed a decline during this period, from 19.74 ± 0.089 to 14.22 ± 0.292 µg tyrosine/mg of protein/min. In the starved prawns the total activity showed a decline from 208.46 ± 1.26 to 114.09 ± 2.11 µg tyrosine/ml of extract/min; but the specific activity (Table 11) showed a slight increase on the 5th day followed by a sharp drop in activity by the 15th day.

Lipase showed very slight increase in activity during the 15 days time in the fed group, whereas the starved ones showed a slight increase in the beginning dropping significantly after one week.

DISCUSSION

The effect of a natural diet and starvation stress on amylase, protease and lipase enzymes of juveniles of *Penaeus indicus* was studied at selected time intervals. The results indicate that the levels of soluble proteins in the hepatopancreas and digestive enzyme activities increase steadily in fed animals reflecting an adjustment in the production levels of amylase, protease and lipase according to the ingested food quality and growth requirements. During inanition, however, the amount of digestive enzymes like proteases and amylases decline in parallel with hepatopancreatic protein content, reflecting a decline in protein synthesis due to the absence of food. Dall (1975) also noted a similar reduction in concentration of the gastric fluid protein (GFPC) in *Panulirus longipes*. According to him the protein content of the secretion was a function of the number of cells in the digestive gland as well as quality of secretion from the cells. In *Penaeus indicus* prolonged starvation probably has resulted in degeneration of a large number of digestive gland cells and thereby affecting the amount of protein produced. Ultrastructural studies on hepatopancreas by Papathanassiou and King (1984) in *Palaemon serratus* emphasised that the fine structure of storage lipid cell (R-cell) and of protein producing cell (F-cell) are mainly affected and the absorption and protein synthesis are disturbed. The general depression in activity of the enzymes observed in *P. indicus* may be due to alterations in the functioning of cell machinery as a result of starvation induced stress. Total amylase activity in the fed prawn-group showed a constant increase, whereas in the starved group a slight drop

occurred in the initial days followed by a slight increase in the activity, which is however, not significant when compared to the fed ones. Cuzon *et al.* (1980), however, recorded a sharp decline in amylase activity during a prolonged starvation period of 48 days. The present experimental period seems to be inadequate for expressing the full effects of starvation.

The specific activity of amylase in the fed group showed a drop in the first two days followed by an increase upto 15 days; whereas, the starved ones recorded a steady increase from the first day onwards. In *Penaeus japonicus*, Cuzon *et al.* (1980) did not observe much fluctuations in the specific activity of amylase, in the fed group but observed a sharp decline in the specific amylase activity in the starved group, quite contradictory to the observation in *Penaeus indicus*. In *Penaeus indicus*, the amount of soluble proteins in the hepato-pancreas seems to drop during the initial phase of starvation, while the concentration of amylase drops slightly or remains relatively unchanged thereby producing apparent increase in specific activity. Probably, the duration of experiment was not adequate enough to bring about significant impact of stress on the metabolic activity of starved prawns.

Proteases showed a steady increase in the total activity in the fed group as against a constant drop in the starved group. At the same time the specific activity of both the fed and the starved groups declined steadily. Cuzon *et al.* (1980), in *Penaeus japonicus*, also recorded a sharp decline in the specific activity of proteases by the end 14 days starvation period.

Thus, it may be noted that varying degree of fluctuation occurs in the specific activity, and there was no proper correlation between the total and specific activity of the enzymes, though protease activity was affected slightly by the total soluble proteins. The steady increase in total activity of the fed group was associated with the steady increase in the amount of soluble proteins in the hepatopancreas. At the same time in the starved groups, the amount of soluble proteins showed a reduction from 5th day to 15th day, thus influencing the specific activity of the proteases in the starved prawns.

Lipases showed an increase in the fed animals; whereas, the starved ones did not show any significant fluctuations. Thus it may be concluded that the short term starvation is not able to bring about any significant changes in the lipases activity. This might probably due to the effective utilisation of the stored products in the hepatopancreas which provide necessary energy during the initial stages of starvation.

Cuzon *et al.* (1980) reported tryptic activity for *Penaeus japonicus* in which one group was fed live *Mytilus* and another group was starved for 4 weeks. Although the tryptic activity in the digestive gland of starved shrimp dropped by 50% to a rather constant level in 14 days compared with that of fed shrimp, the specific activity in starved shrimp remained higher than that of the fed group until the middle of the third week. At that time, the specific activity of starved shrimp declined to levels significantly lower than in fed shrimp. The authors suggested that bacterial flora associated with sand in the tank was ingested, thereby maintaining tryptic activity. In the present investigation too, the inconsistent trend observed in the enzyme activities may be attributed to the bacterial flora ingested by the prawns from the water.

TABLE 10: Digestive enzyme activity in the fed and starved groups of *Penaeus indicus*

	Fed				Starved			
	Ist day t_0	5th day t_5	10th day t_{10}	15th day t_{15}	1st day t_0	5th day t_5	10th day t_{10}	15th day t_{15}
Soluble proteins (mg/ml)	10.56± 0.01	13.71± 0.25	15.15± 0.05	18.17± 0.02	10.56± 0.01	9.73± 9.25	7.42± 0.09	5.82± 0.03
Amylase activity (Total) mg maltose/ml of extract/min	2.04± 0.06	2.20± 0.06	2.76± 0.03	3.59± 0.08	2.04± 0.06	1.73± 0.03	1.83± 0.03	2.18± 0.01
Protease (Total) (µg tyrosine/ml of extract/min)	208.46± 1.22	221.84± 10.5	228.88± 6.09	258.46± 5.32	208.46± 1.22	176.06± 3.23	161.37± 3.22	114.09± 2.11
Lipase (ml of .05 N NaOH required for neutralisation)	2.09± 0.01	2.13± 0.02	2.13 0.01	2.14± 0.01	2.09± 0.01	2.11± 0.01	2.10± 0.02	2.08± 0.02

TABLE 11. Specific activity of α-amylase and protease in fed and starved groups of *Penaeus inducs*.

	Fed		Starved	
	Amylase (mg maltose/ mg protein/ min)	Protease (μg tyrosine/ mg protein/ min)	Amylase	Protease
1st day	0.193±0.004	19.74±0.089	0.149±0.004	15.20±0.089
5th day	0.161±0.005	16.18±0.770	0.164±0.003	16.67±0.306
10th day	0.182±0.002	14.75±0.393	0.188±0.002	16.57±0.331
15th day	0.198±0.004	15.22±0.292	0.224±0.001	11.726±0.21

Fig. 10. Soluble proteins, amylase, protease and lipase activity in the starved and fed groups of Penaeus indicus at t_0, t_5, t_{10}, t_{15} time interval. Data are given as mean ± S.D.

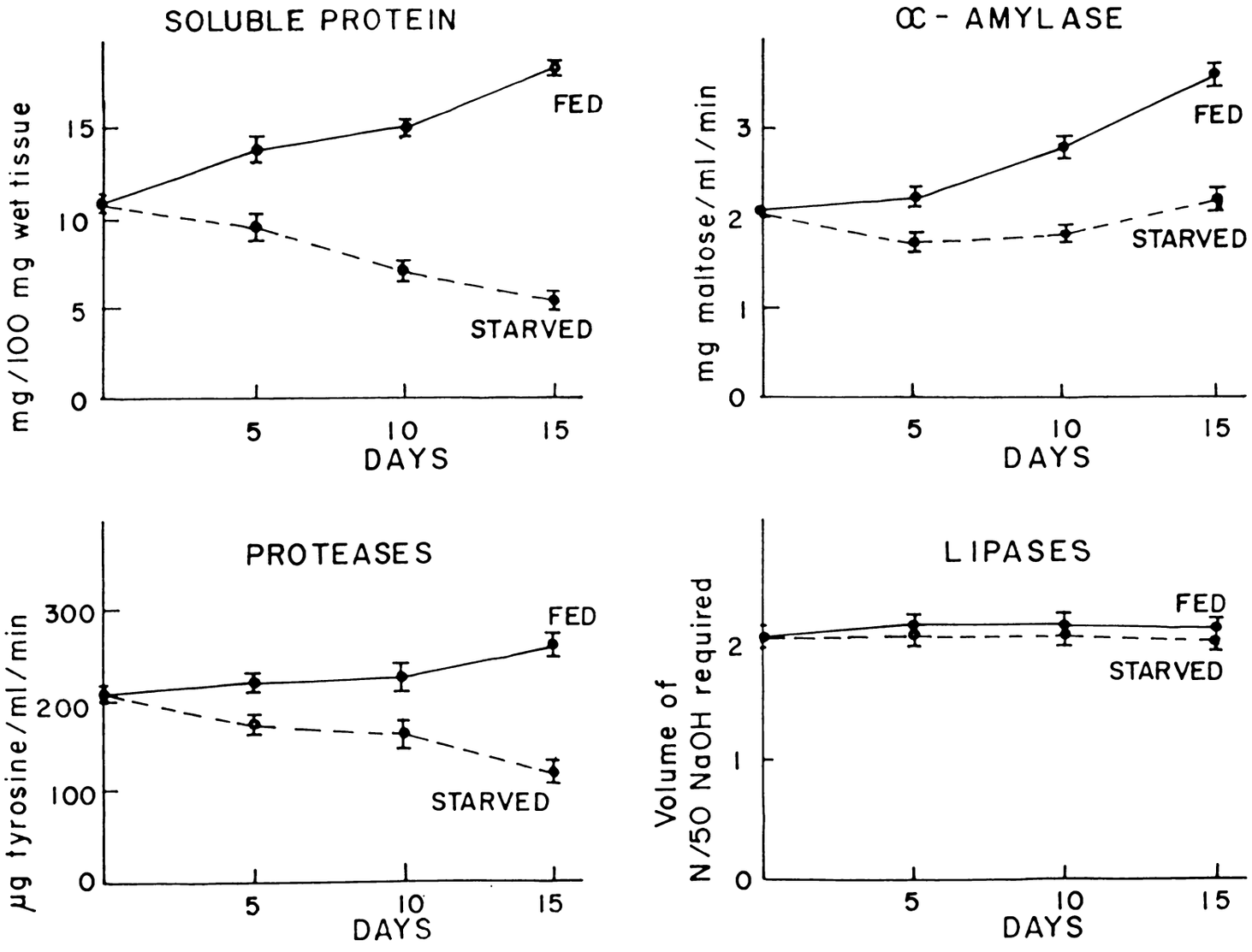

SOLUBLE PROTEIN
mg/100 mg wet tissue
0
5
10
15
FED
STARVED
5
10
15
DAYS
α - AMYLASE
mg maltose/ml/min
0
1
2
3
FED
STARVED
5
10
15
DAYS
PROTEASES
µg tyrosine/ml/min
0
100
200
300
FED
STARVED
5
10
15
DAYS
LIPASES
Volume of N/50 NaOH required
0
1
2
FED
STARVED
5
10
15
DAYS

FIG. 10

2.4 EFFECTS OF STARCH LEVELS IN THE DIET ON ACTIVITY OF DIGESTIVE ENZYMES

INTRODUCTION

Nutritional studies conducted with shrimps and prawns have classically been confined to empirically designed dietary trials, while aspects such as bioenergetics and digestive physiology of these organisms received relatively less emphasis. Digestibility of feeds, nutrients balance and bioenergetics of marine and freshwater prawns are currently receiving more attention (New, 1976, 1980). All of these studies have proven useful inspite of the inherent limitations of each. One of the major limitations is the inability to assess the specific digestive capabilities of an organisms. If the specific digestive potential of an organism can be assessed by utilising digestive enzyme analysis, a nutritionally balanced diet can be designed.

Several reviews (Van Weel, 1970; Gibson and Barker, 1979; Dall and Moriarty, 1983) dealing with the comparative physiology of digestion in Crustacea have been published, but have contained very little quantitative information. Since crustaceans, particularly the prawns, are now being widely used for commercial culture, changes in the digestive enzyme activities during the life cycle and adaptation to diets are being examined quantitatively (Hood and Meyers, 1977; Laubier-Bonichon *et al.*, 1977; Trellu and Ceccaldi, 1977; Van Wormhoudt *et al.*, 1982a,b). Hoyle (1973) showed that prolonged feeding of lobsters with diets of

varying starch levels, composed of 60% protein demonstrated a preferential production of protease, which he ascribed to a possible adaptation to dietary substrate. In another investigation, Van Wormhoudt *et al.* (1972) linked variations in growth rate of *Palaemon serratus* to changes in the wet weight of the hepatopancreas and digestive enzyme levels, demonstrating an adaptation to various levels of dietary carbohydrate and protein.

The phenomenon of adaptation of the digestive mechanism to major dietary constituents has been discussed since Pavlov's time. This type of response, namely, a 'purposeful adaptation' has ample evidence in both vertebrates (Grossman *et al.*, 1943; Howard and Yudkin, 1977) and invertebrates (Fisk and Shambaugh, 1952; Ishaaya *et al.*, 1971). Grossman *et al.* (1943) found a pronounced increase in the amylase activity in rats after feeding a high carbohydrate diet for 3 weeks. Similarly, a high protein diet resulted in an elevated trypsin level. Howard and Yudkin (1963) obtained essentially the same result, but also noted changes in enzyme content after only one day's change in diet. Vonk (1927) was the first to record that an adaptation of digestive enzymes to feeding habits exists; carnivorous fish have more protease activity than herbivorous, while herbivorous fish have more amylase. Nagase (1964) showed that trypsin activity can be increased with a protein rich diet, but there is no adaptation of lipase and pepsin to food in *Tilapia mossambica*. Other studies in these fields were made by Agrawal *et al.* (1975), Goel (1975), Sinha (1975), Hofer (1979a,b) and Hofer and Scheimer (1981).

The objective of the present study was to obtain information regarding the activity of certain digestive enzymes in *Penaeus indicus* with respect to graded levels of starch in purified diets, so that the information if relevant can be used for the formulation of practical diets for prawn culture.

MATERIALS AND METHODS

Formulation and preparation of experimental feeds:

Nine different diets (Table 12) were prepared using purified ingredients purchased from Nutritional Biochemical Corporation, Ohio, U.S.A. and Sigma Biochemicals, U.S.A. The feeds were prepared as follows: Weighed out casein, mineral mixture and essential aminoacids, and mixed thoroughly. To this mixture, fat-soluble vitamins in oil and water soluble vitamins were added and the whole mixture was mixed thoroughly. Dissolved gelatin and agar in about 50 ml of boiling distilled water (for 100 g diet) in a beaker. After removing the gelatin and agar mix from the heater added starch, mixed thoroughly and again heated. After adequate cooking of the starch, the container was removed from the heater and the mixture containing all other ingredients were added as the temperature dropped to about 40°C. The above dough-like mass with a pH of 6.8 ± 0.4 was extruded into pellets using a laboratory extruder. After extrusion these diets were dried in an oven for 48 hours at 50°C and stored in air-tight plastic bags at 4°C. Gelatin (2%), agar (3%) and cooked starch served as binders. Gelatin also is a good source of the essential amino acid arginine. The proportion of starch and α-cellulose was adjusted to maintain the total

carbohydrate content in the diet. Chromic oxide (0.5%) was added to the diet as an inert marker to study the digestibility coefficient of starch, the variable component in the diet.

Feeding Experiments

Feeding experiments were conducted with the diets on prawn juveniles of mean length 30 ± 5 mm and mean live-weight 100 ± 10 mg. They were randomly selected and stocked in 50 litre capacity circular plastic troughs, containing filtered sea water with a salinity of 17 ± 1‰. In each through 20 animals were kept with three replicates for each treatment. The animals were fed a restricted ration of five percent of body weight once daily in the afternoon with dry pellets. The left-over food and faecal matter were collected daily; water was changed once in two days. The duration of the feeding experiment was four weeks.

After the experiment, 10 animals were picked up at random from each trough and dissected alive over ice after recording their length and weight. Hepatopancreas were removed, pooled and the total weight noted. The glands were homogenized and the tissue extract centrifuged at 15,000 r.p.m. in a refrigerated centrifuge for 10 minutes and the clear supernatant was used as a crude enzyme extract. A 1% concentration of the crude enzyme extract was prepared to conduct the assay. The assay methods and unit of expression are same as described earlier (vide pages 49-51). Growth, survival and weight gain of the animals were also recorded during the experiment. The feeding experiment was repeated two times and the biochemical analysis and enzyme assays reported as mean of six determinations.

Collection of faecal matter

Once daily in the evening, the water from the trough was siphoned out and the faecal matter was collected over a bolting silk cloth 35 μ . The faecal matter was collected in a aluminium foil and dried in an oven. After drying the samples were kept in desiccator until analysis. The samples collected from each treatment were pooled in order to carry out the chemical analysis.

Digestibility coefficient:

The digestibility of each of the diet was determined by an indicator method using chromic oxide (Cr_2O_3) as the marker. The digestibility coefficient was calculated using the formula:

$$\text{Digestibility (\%)} = 100 - 100 \left(\frac{\%Cr_2O_3 \text{ in food}}{\%Cr_2O_3 \text{ in faeces}} \times \frac{\% \text{ nutrient in faeces}}{\% \text{ nutrient in food}} \right)$$

Chromic oxide in the feed and faecal matter was determined by a spectrophotometric method as outlined in CMFRI special publication No. 8. (1982)

Proximate analysis of feed and faecal matter:

Weighed samples of experimental diets and faecal matter were oven dried for 48 hours at 60 ± 2°C, cooled in a desiccator and the dry weights were taken. The moisture content was determined using the formula:

$$\text{Moisture (\%)} = \frac{\text{wet weight of samples - dry weight of sample}}{\text{wet weight of sample}} \times 100$$

Total nitrogen was determined by micro-kjeldahl procedure and conversion factor of 6.25 was used to get the crude protein content (AOAC, 1965).

Modified Bligh and Dyer (1959) method was followed for determining the lipid content in both feed and faecal matter.

Total carbohydrates were determined spectrophotometrically using phenol-sulphuric acid method with D-glucose as standard (Dubois *et al.*, 1956)

The samples in powdered form were ignited at 550°C in a muffle furnace in silica crucibles for 6 hours to obtain ash and the ash percentage was determined.

RESULTS

Hepatopancreatic amylase, protease and lipase activities in prawns fed the experimental diets are given in Figs. 12&13. The activity pattern of amylase showed an increase as the level of starch increased in the diet. In prawns fed the control diet (starch-free), the activity of amylase was highly insignificant. From the Table 14 it is evident that, as the percentage of starch in the diet increased, the amylase activity showed a corresponding increase with the maximum rate at 20% starch. Beyond this level the activity steadily declined attaining significantly low levels at 40% level.

Proteases, however, did not show any significant variation in the activity pattern with reference to starch level in the diet. The values remained more or less steady without wide fluctuations (Table 14). The maximum activity was noted between 15% and 30% starch in the diet, and optimum between 20% and 25%. Lipase activity was also found to be relatively steady

without any significant variations with respect to the percentage of starch in the diet.

The results of feeding the purified diets on mean individual weight increase and survival of prawns are presented in Table 13. Prawns with an initial mean weight of 1.11 ± 0.05 g and fed on the 20% starch diet had a significantly increased growth rate over those receiving the other levels of starch. The increase was very significant. Almost 55% increase was noticed in this group. The other groups, however, did not show any significant growth rate when compared to the 20% starch diet. Diets with starch levels between 10% and 30% provided better growth rate, survival and enzyme turn over than the other diets.

The digestibility coefficient data (Table 15) as determined by chromic oxide indicator method gave the highest coefficient value of 81% for carbohydrates 73.34% for proteins and 84.23% for lipids for the animals fed with 20% starch diet. Besides, digestibility coefficient for the diets was also relatively higher when starch level was in the range 10% - 30%.

DISCUSSION

The results of the experiment clearly indicate that dietary starch level has a highly significant influence on the secretion of -amylase. However, starch level had no significant effect on proteases or lipases. This may be expected as the diets were iso-proteic and iso-lipidic, with starch being the variable. The variations in the availability of starch substrate affected the amylase enzyme system alone. Besides, the study clearly shows that amylase secretion increases with the substrate availability upto 20% starch in the diet. Higher starch levels seems to have inhibitory effect on the enzyme.

The digestibility coefficient data (Table 15) also support the above findings. There was a steady increase in the digestibility of carbohydrates upto 20% starch in the diet. Digestibility of the carbohydrates also was significantly reduced when starch was included at higher levels. These results suggests that for optimum amylase turn-over and digestion of starch the diet should contain about 20% of starch; although digestion of starch occurs upto the 40% level tested in the study, but with reduced enzyme turn-over efficiency.

Hoyle (1973) who studied the digestive enzyme secretion associated with dietary variation in the American lobster (*Homarus americanus*) reported that on feeding 0, 5 and 20% starch diet for periods upto 37 days, the specific activity as well as the absolute amounts of proteinase

lipase and amylases increased, when compared to the base-line, or true fasting enzyme level which was considered to be those values found after feeding for two days on a 5% starch diet. Hoyle (1973) attributed the increase in enzyme activity for two reasons: 1) supply of the necessary amino acids, 2) induction of enzyme production of specific components, or digestion products of the diet. The present study further substantiates the observation, of the enhanced activity profile of the amylase system, influenced by varying level of starch in the diet.

Reimer (1982) observed that activity of amylase, trypsin and lipase could be increased with a diet rich in carbohydrates, protein or fat in Amazon fish *Brycon melanopterus*. Nagase (1964) confirmed this type of adaptation with amylase and proteases in an experiment on *Tilapia mossambica (Sarotheroden mossambicus)* with change in the diet. However, Nagase (1964) and Agarwal *et al.* (1975) did not observe any correlation between the fat content of the diet and the lipase activity in the Teleost fishes. Though there are only few studies in invertebrate group Van Wormhoudt *et al.* (1980) and Maugle *et al.* (1982b) in *Palaemon serratus* and *Penaeus japonicus* noted that some nutritional variables affected the enzyme activity. Since the above studies were conducted feeding the animal using natural diet, the component in the diet responsible for stimulation of the enzyme activity could not be identified.

TABLE 12: Composition of experimental diets with varying levels of starch.

Ingredients	Diets Nos.								
	1	2	3	4	5	6	7	8	9
1. Casein (fat-free)	35	35	35	35	35	35	35	35	35
2. ∝ -Starch	0	0	10	15	20	25	30	35	40
3. Glucosamine HCl	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
4. Sodium citrate	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
5. Sodium succinate	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
6. Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
7. Cod liver oil	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
8. Vitamins[a]	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5
9. Minerals[b]	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
10. Cellulose powder	41.4	36.4	31.4	26.4	21.4	16.4	11.4	6.4	1.4

[a]Vitamins	mg.	[a]Vitamins	mg.	[b]Minerals	g
Thiamine HCl (B1)	4.9	Menadione	4.0	K_2HPO_4	2.0
Riboflavin (B_2)	8.0	B-Carotene	9.6	$Ca_3(PO4)_2$	2.72
P. aminobenzoic acid	10.0	-Tocopherol (Vitamin-B)	20.0	$NaH_2PO_4 2H_2O$	0.76
Biotin	0.4	Calciferol	1.2	$MgSO_4.7H_2O$	3.02
Inositol	400.0	Cyanocobalamine (B_{12})	0.08	$MnSO_4.5H_2O$	0.004
Niacin	40.0	Na-ascorbate (Vitamin C)	2000.0	$FeSO_4.7H_2O$	0.015
Ca-pantothenate	60.0	Folic acid	0.30		
Pyridoxine HCl	12.0	Choline Chloride	600.00		

TABLE 13: Weight gain and survival of prawns fed on the experimental diets (duration of feeding experiment 4 weeks)

Diet No.	Starch level (%)	Weight gain (%)	Survival (%)
1	0	6.08	86.6
2	5	4.35	83.3
3	10	5.22	76.6
4	15	2.61	90.0
5	20	25.22	93.5
6	25	20.87	83.5
7	30	14.78	90.0
8	35	11.30	86.6
9	40	18.26	90.0

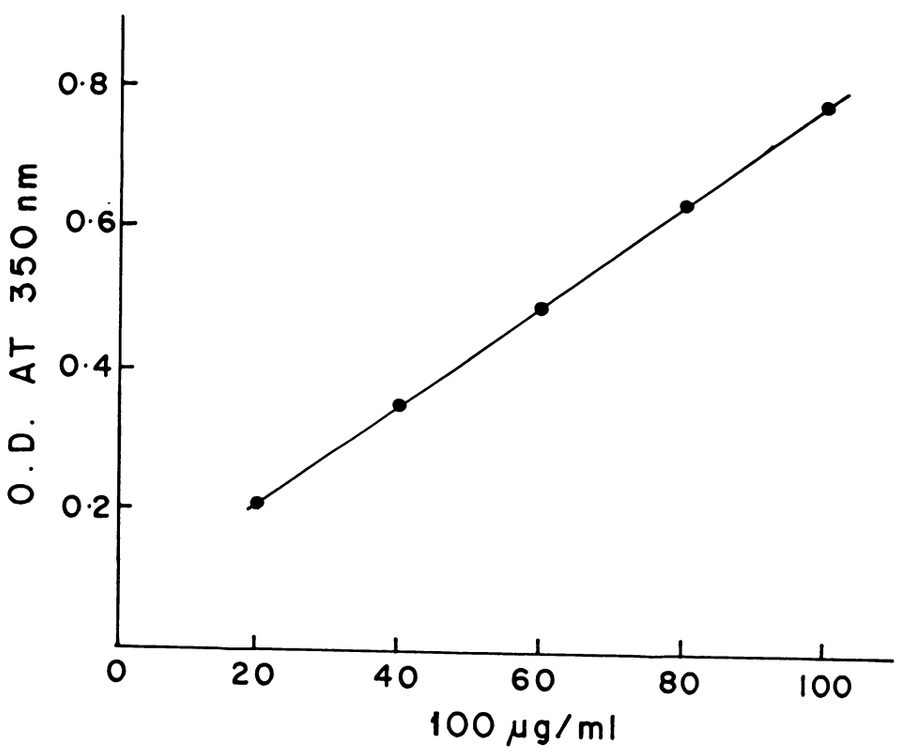
TABLE 14: Influence of starch level in the diets on digestive enzyme activity of *Penaeus indicus*.

Diet Nos.	Amylase Total activity (mg maltose/ml/min)	Protease Total activity (μg tyrosine/ml/ min)	Lipase Total activity (ml of N/50 NaOH required for fatty acid neutralisation)
1	5.13 ± 5.82	201.41 ± 1.22	2.30 ± 0.072
2	25.18 ± 2.68	205.27 ± 2.19	2.33 ± 0.05
3	40.42 ± 5.41	204.58 ± 2.20	2.35 ± 0.01
4	38.00 ± 12.96	208.1 ± 1.05	2.38 ± 0.01
5	43.97 ± 4.08	210.57 ± 1.22	2.42 ± 0.03
6	24.40 ± 2.60	210.22 ± 1.06	2.39 ± 0.01
7	25.26 ± 2.97	208.11 ± 1.05	2.44 ± 0.01
8	21.01 ± 4.60	205.97 ± 1.05	2.39 ± 0.01
9	18.36 ± 4.13	200.57 ± 1.22	2.42 ± 0.03

TABLE 15: Digestibility of dietary carbohydrate fed to *Penaeus indicus*.

Diets	Carbohydrate content in the diet (%)	Digestibility (%) (Carbohydrate)
1	12.24	24.12
2	16.76	35.90
3	15.42	65.29
4	15.18	81.00
5	16.0	87.73
6	19.5	68.36
7	18.8	50.45
8	15.0	52.49
9	19.30	38.57

Fig. 11. Experimental calibration curve for quantitative estimation of chromic oxide.



STANDARD GRAPH FOR
CHROMIC OXIDE ($Cr_2 O_3$)

FIG. 11

Fig.12. Relative and specific amylase activities with varying levels of starch in the diet. Data represented as Mean ± S.D.

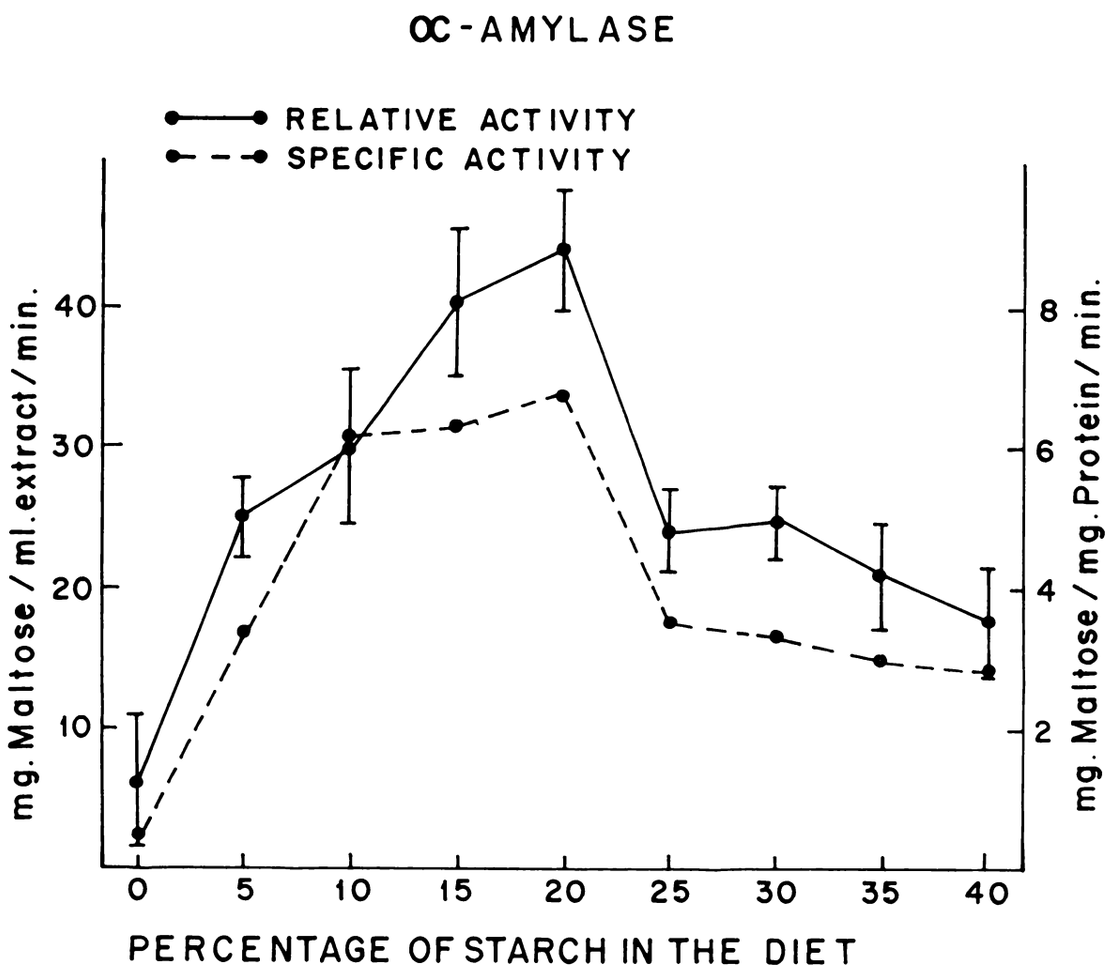

α-AMYLASE
RELATIVE ACTIVITY
SPECIFIC ACTIVITY
mg. Maltose / ml. extract / min.
mg. Maltose / mg. Protein / min.
10
20
30
40
2
4
6
8
0
5
10
15
20
25
30
35
40
PERCENTAGE OF STARCH IN THE DIET

FIG. 12

Fig. 13. Protease & lipase activities with varying levels of starch in the diet. Data are given as Mean ± S.D.

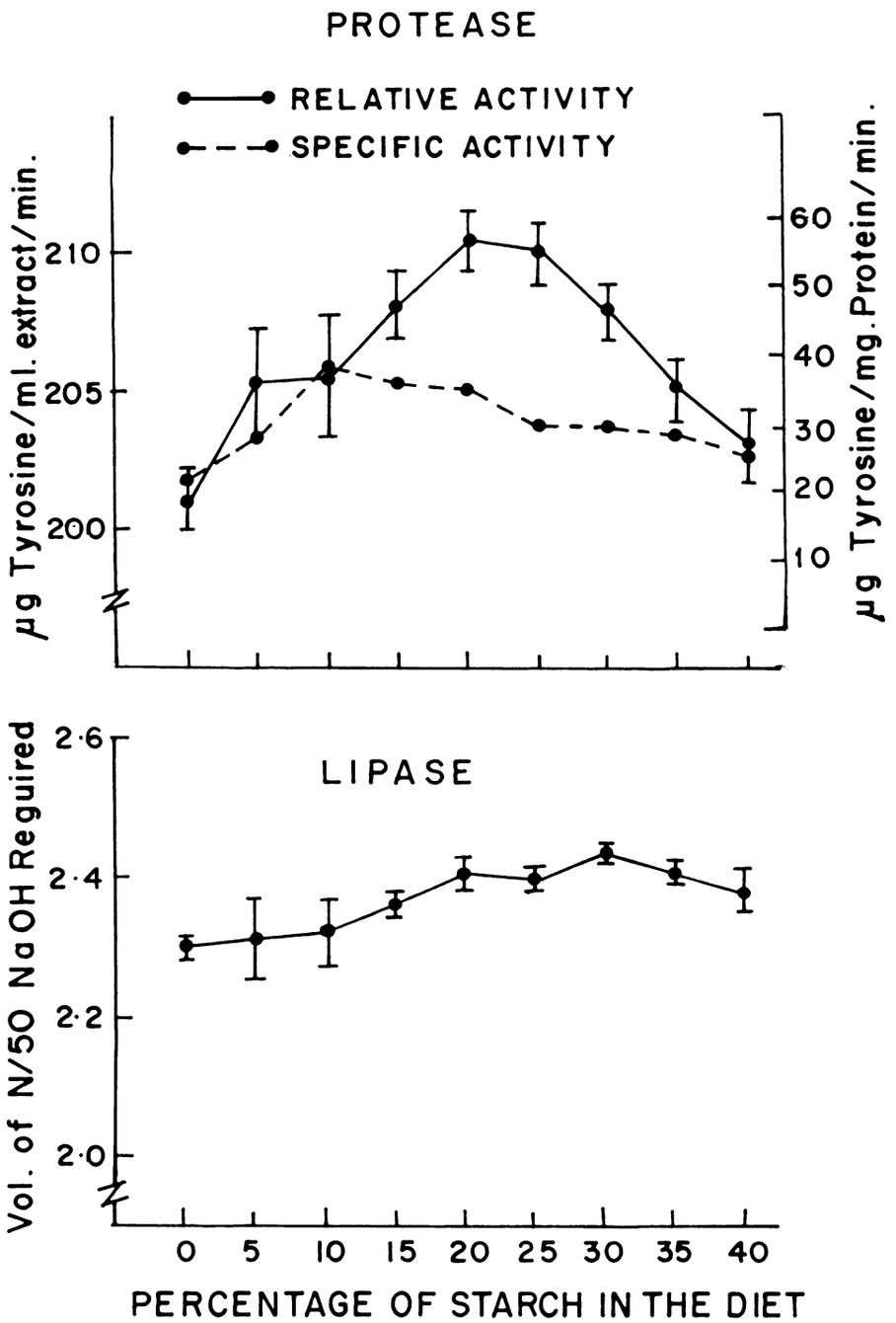

PROTEASE
RELATIVE ACTIVITY
SPECIFIC ACTIVITY
µg Tyrosine/ml. extract/min.
210
205
200
µg Tyrosine/mg. Protein/min.
60
50
40
30
20
10
LIPASE
Vol. of N/50 NaOH Required
2·6
2·4
2·2
2·0
0 5 10 15 20 25 30 35 40
PERCENTAGE OF STARCH IN THE DIET

FIG. 13

2.5. EFFECT OF EYESTALK ABLATION ON ENZYME ACTIVITY

INTRODUCTION

In crustaceans, many of the metabolic functions such as oxygen consumption (Scudamore, 1947), frequency of molting (Carlisle, 1954), heart rate (Hara, 1952), chromatophore activity (Fingerman, 1965) and the maturation of gonads (Takewaki and Yamamoto, 1950) are known to be under the control of eyestalk hormones. However, very few contributions exist on the relationship between eyestalk hormones and the hepatopancreas of decapod crustaceans. Bilateral eyestalk ablation of the crayfish, *Procambarus clarkii*, resulted in histological changes in the hepatopancreas and the implantation of sinus glands into the destalked animals retarded the degeneration of the tissue (Yamamoto, 1960). Miyawaki and Tanoue (1962) observed some changes in mitochondria and endoplasmic reticulum after eyestalk ablation in the hepatopancreas of *P. clarkii*. Miyawaki *et al.* (1966) described the relationship between eyestalk hormones and the ultrastructure of the hepatopancreas cells in *P. clarkii*. Fingerman *et al.* (1967) for the first time stated that the hormones in the eyestalk of *P. clarkii* are involved in the regulation of enzymes of the hepatopancreas.

There is considerable evidence that the synthesis of digestive enzymes within the hepatopancreas as well as the uptake of material from the gut lumen is under the control of neuroendocrine factors, produced by the sinus gland - X

organ complex. Fingerman *et al.* (1967) showed that the maintenance of ribonucleic acid synthesis in the hepatopancreas of *Orconectes ozarkae* and *Procambarus blandingii* depended upon a neurosecretory substance emanating from the eyestalks. Fingerman and Yamamoto (1965) and Fingerman *et al.* (1967) demonstrated that the effects of eyestalk ablation in *P. clarkii* included a decrease in gastric juice amylolytic activity as well as a drastic increase in pH. They also linked ribonucleic acid activity in the hepatopancreas with amylase synthesis. Similar results have been obtained by Thornborough (1968), Gorell and Gilbert (1971), Van Wormhoudt *et al.* (1973), Van Wormhoudt (1974), Momin and Rangneker (1974, 1975a), Nagabushanam and Diwan (1974) and Jyothi and Nagabushanam (1977). Relatively few studies on these lines have been conducted in penaeid species. Although eyestalk ablation technique has been practised to induce maturation of penaeid prawns information on the influence of eyestalk ablation on digestion and enzyme activity is very limited. Hence experiments were designed to study the role of eyestalk factors in the regulation and synthesis of digestive enzymes in the hepatopancreas of *P. indicus*.

MATERIALS AND METHODS

Three groups, each with 20 number of *Penaeus indicus* measuring 130 ± 15 mm and weighing 22 ± 3.5 g were maintained in plastic pools of 1000 L capacity. One of the groups was kept as a control (without eyestalk ablation); another group was subjected to unilateral ablation and the third group was subjected to bilateral ablation.

Eyestalk ablation was performed using an electrocautery apparatus. The wound inflicted by this operation was not harmful and ensured 100% survival. All the three groups of prawns were maintained in well aerated seawater of salinity 28 ± 2 ppt. All the animals were kept at the room temperature 29 ± 1°C fed once in the evening and water was changed once in two days. The prawns were fed with a restricted ration (5% body weight) of fresh clam meat once everyday. To avoid changes brought about during moulting, only prawns in the intermoult stage were selected. All the adult specimens tested were immature. The animals were sacrificed after one week and prior to dissection they were starved for 24 hours. Methods of crude enzyme extract preparation and analysis of digestive enzymes are as that described in Chapter 2 (Pages 41-43 and 49-51).

RESULTS

The removal of eyestalk from the experimental animals resulted in a highly significant (ANOVA 1% significant) increase in the amylolytic activity. (Table 16). The unilaterally ablated prawns recorded an increase of 19.92% of activity over the control animals. Bilateral ablation induced a highly significant increase in activity (25.11%) over the control prawns.

Proteolytic activity was however, not significantly affected by the removal of eyestalk. The unilaterally ablated prawns recorded a slightly lesser proteolytic activity(5.83%) in the hepatopancreatic extract than the control ones. Bilateral ablation did not alter the proteolytic activity significantly (Table 16).

Lipolytic activity showed a highly significant (1%) increase (Table 16) after ablation. The unilateral animals recorded an increase of 15.2% over the control animals and bilateral ones showed an increase of 24.8% over the control animals and almost an increase of 8.5% over the unilaterally ablated prawns.

Soluble protein levels were relatively higher in both the ablated groups. While the hepatopancreatic extracts of control animals had 14.15 mg protein/ml, the unilaterally and the bilaterally ablated prawns had 20.62 and 19.66 mg protein/ml respectively. Therefore, the specific activity of amylase did not differ significantly between the three groups, whereas proteases showed a lower specific activity in unilaterally and bilaterally ablated prawns than the control ones. Specific activity of protease was also comparatively much lower than the specific activity of amylase. The amylase to protease ratio was relatively higher for the unilaterally ablated prawns (Table 17).

DISCUSSION

The present findings indicate that the removal of enzyme inhibiting hormonal factors from the eyestalk of *P. indicus* accelerates the activity of amylolytic and lipolytic enzymes. This is quite contradictory to the findings of Fingerman (1967) in ablated starved crayfish *Procambarus clarkii*, Thornborough (1968) in *Palaemonetes vulgaris* and Jyothi and Nagabushanam (1977) in *Caridina weberi*, who have recorded a considerable

decrease in amylase activity, following eyestalk ablation. The accelerated activity of amylase and lipase in *Penaeus indicus* is probably due to the significant increase in the soluble protein levels or due to the metabolic state of the animal during which the operation was conducted. As the prawns were fed they had to continuously release the enzymes to digest the ingested food.

The present finding agrees with the results obtained by other workers who combined the eyestalk ablation with the availability of nutritionally enriched food. Mauoit and Castell (1976) have suggested that diet may be a key factor in survival of ablated juvenile lobsters. Their studies suggested that the growth rate and survival of ablated lobsters were made much more sensitive to diet composition (protein content) than the control. No mortality was encountered among the ablated prawns during the present study. The prawns were very active and quite normal. Since, the animals were properly fed with fresh clam meat they were not exposed to any kind of major stress.

The total activity of hydrolytic enzymes were significantly higher than their specific activity. The protein content of the hepatopancreatic extract did not differ significantly between the control and the ablated prawns. The amylase to protease ratio (A/P) in ablated prawns recorded significantly higher values than the intact ones (Table 17); whereas protein to amylase ratio(P/A) showed a significant drop in the eyestalkless animals when compared to the intact ones. Carbohydrases are the enzymes subjected to much changes under the eyeless condition and the proteases activity dropped very much during this period.

It has been well established that the X-organ sinus gland complex in the eyestalk plays a major role in the control of moulting and growth in crustanceans. Radhakrishnan and Vijayakumaran (1982) obtained tremendous growth rate and weight gain for bilaterally ablated lobsters, *Panulirus homarus* within six months. Their experiments showed that at *ad libitum* level of feeding food consumption of ablated lobsters was twice that of the control animals, recording 2 to 3 fold increase in food conversion efficiency. The increased efficiency in food conversion observed by the above authors in eye-ablated lobsters clearly indicate that the ingested food nutrients are better digested and metabolised than the control lobsters. This increased efficiency in digestion and conversion of food could only be brought about by an increase in the secretion of the hydrolytic enzymes. The present study clearly substantiates this fact atleast in respect of α-amylase, which showed a tremendous increase in eyestalk ablated prawns even under restricted feeding.

The triggering action of the digestive enzymes in ablated prawns may indicate that the animal is under a metabolic stress and it needs extra energy to cope up with the stress. In the presence of adequate level of food the animal responds by increased secretion of digestive enzymes to digest the food nutrients with a view to meeting its additional energy demand. The increased activity of amylase and lipase in eye-ablated prawns further indicate that carbohydrates and lipids are perhaps the nutrients utilised first in times of stress. Further the accelerated amylase and lipase activity induced by eyestalk ablation indicates that hormonal factors in the eyestalk are somehow associated with the regulation of enzyme secretion. However, proteases do not seem to be significantly affected by eyestalk removal.

Thus it may be concluded that the eyestalk ablation, has a very significant influence on the hydrolytic activity in the hepatopancreas. The removal of the enzyme regulatory factors from the eyestalk causes an overall increase in the activity of amylase and lipase. Further studies will help in ascertaining the factors controlling proteolytic activity since in the present experiments no significant response was observed.

One of the important observations that could be of immense use to prawn hatcheries is that when prawns are ablated for induction of maturation, it is very essential to feed them adequately to meet their increasing nutritional demand so as to achieve maximum success. The observations of Mauoit and Castell (1976) and Radhakrishnan and Vijayakumaran (1982) further substantiate that eyeablated crustaceans require additional nutrients to cope with the increased metabolic rate when compared to eyestalk intact individuals.

TABLE 16: Influence of eyestalk ablation on digestive enzymes in *Penaeus indicus*.

Treatments	Enzyme activity		
	Amylase (mg maltose/ml/min)	Protease (µg tyrosine/ ml/min)	Lipase (ml. of N/50 NaOH required for neutralisation of fatty acids.
Control	39.54 (2.79)	185.05 (1.306)	1.97
Unilateral	47.42 (2.30)	174.26 (0.845)	2.27
Bilateral	49.47 (2.5)	184.02 (0.936)	2.46

Values in the brackets denotes specific activity - mg/products/mg protein/min. formed.

TABLE 17: Specific activity & ratio of the digestive enzymes in *Penaeus indicus*

Treatments	Specific activity			
	Amylase (mg maltose/mg protein/min)	Protease (mg tyrosine/mg protein/min)	A/P ratio	P/A ratio
Control	2.79	1.307	2.13	0.47
Unilateral ablation	2.30	0.845	2.72	0.37
Bilateral ablation	2.50	0.936	2.67	0.37

Fig. 14. Amylase, protease and lipase activities in the control (eyestalk intact), and unilateral and bilateral eyestalk ablated prawns. Values are represented as Mean ± S.D.

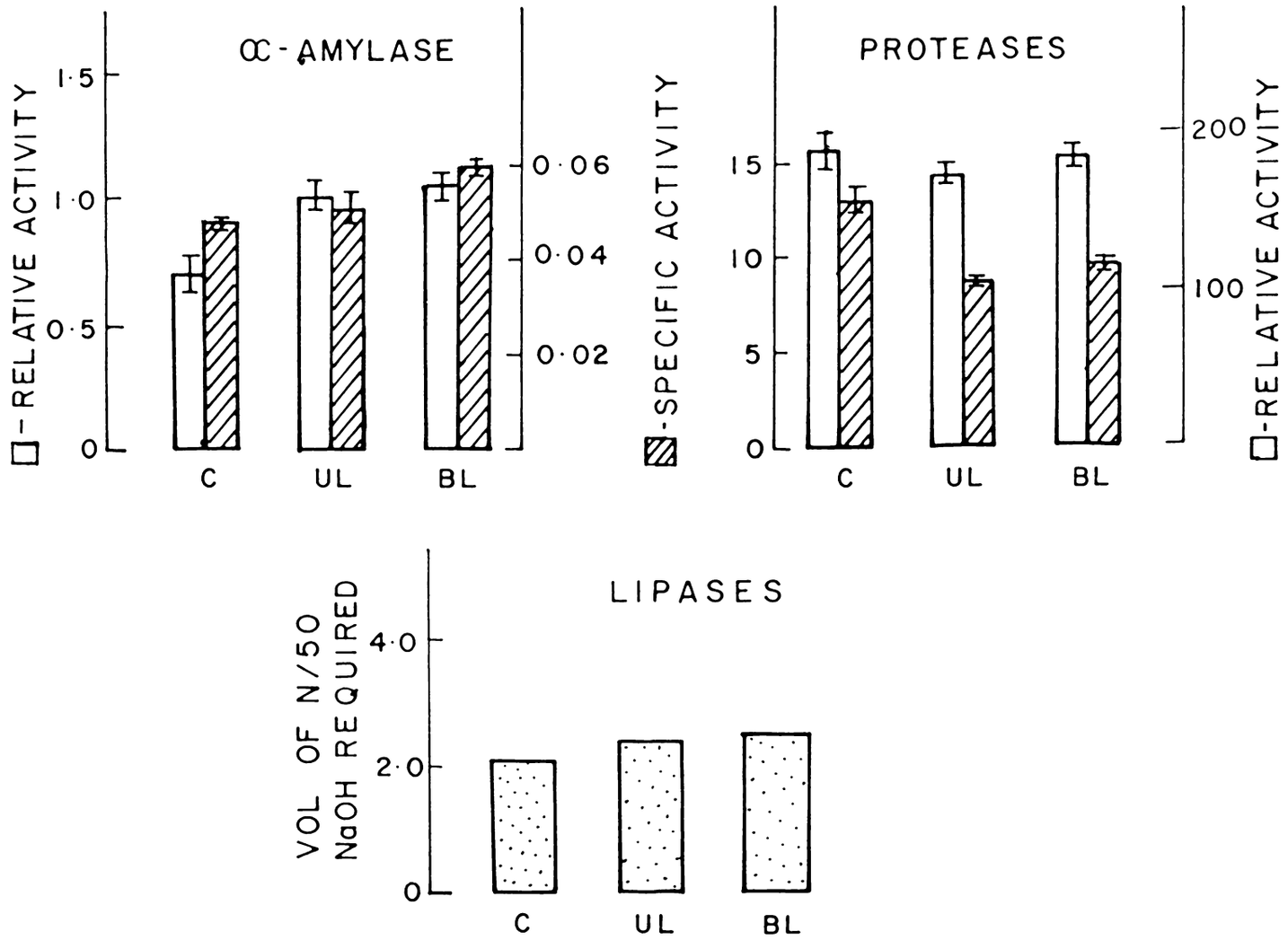

α-AMYLASE
□-RELATIVE ACTIVITY
1·5
1·0
0·5
0
0·06
0·04
0·02
C
UL
BL
PROTEASES
▨-SPECIFIC ACTIVITY
15
10
5
0
200
100
□-RELATIVE ACTIVITY
C
UL
BL
LIPASES
VOL OF N/50 NaOH REQUIRED
4·0
2·0
0
C
UL
BL

FIG. 14

CHAPTER 3

BIOCHEMICAL CHARACTERIZATION OF ALPHA -AMYLASE

3. BIOCHEMICAL CHARACTERISATION OF ALPHA AMYLASE

INTRODUCTION

α - Amylase (1, 4 -α-D - Glucan glucanohydrolase, EC 3.2.1.1.) hydrolyses α-1 $\rightarrow$ 4-glucan bonds in polysaccharides such as starch, amylopectin and glycogen, and their degradation products with a chain length of at least 3-d-glucose residues. The hydrolysis of the polysaccharide chain occurs via the principle of "multiple attack". Once the enzyme-substrate complex is formed, the enzyme can hydrolyze several bonds of the polymer successively before the enzyme is again liberated in the free form.

Starch is hydrolysed by α-amylase to fragments whose reducing hemiacetal groups can be determined with 3, 5-dinitrosalicylic acid. The concentration of the nitroaminosalicylic acid formed is measured colorimetrically; it corresponds to the concentration of newly formed terminal groups and therefore directly to the enzyme activity.

The enzyme is found in micro-organisms, in plants and in particular high activity in germinating cereals. In animals it is found in the secretory granules of the cells of the salivary glands, the pancreas, and also in the secretions of these organs; slight activity is also reported in blood, serum and urine of normal individuals. The enzyme from pig pancreas, human saliva, human pancreas and rat pancreas have been crystallized.

α-amylase is the most widely investigated carbohydrase among the crustaceans. Qualitative studies have been conducted by several workers in the early nineteen hundreds. In subsequent years, the enzyme was subjected to detailed investigations elucidating the properties like pH, temperature, stability, incubation period, substrate and enzyme concentrations (Gopalakrishnan, 1957; Takahashi *et al.*, 1964; Blandamer and Beechey, 1963, 1964, 1966, Wojtowicz and Brockerhoff, 1972; Karunakaran and Dhage, 1977; Kulkarni *et al.*, 1979, Robson, 1979; Saxena and Murthy, 1981, Van Wormhoudt *et al.*, 1982; Azzalina and Trainer, 1985). Earlier investigation of amylase in decapods dates back to 1876, when Hoppe-seyler, detected amylolytic activity in the hepatopancreatic secretion in crab. Jordan (1904) detected amylolytic ferments in *Astacus fluviatilis*. This was again supported by Wiersma and Van der Veen (1928). Roaf (1906) working on the digestive gland in different Crustacea revealed that extract of the gland could digest starch along with glycogen, maltose, sucrose and lactose. The investigations of Yonge (1924) indicated that the hepatopancreatic extract of Norway lobster *Nephrops norvegicus* contains various carbohydrases and that their optimum action is in a neutral medium. Verwey (1930) working with *Uca* indicated the presence of amylase activity in gastric secretion. Reddy (1937) while studying the physiology of digestion in the Indian carb *Paratelphusa hydrodromus* showed the existence of amylase enzyme in the digestive gland. Gopalakrishnan (1957) while working on the biology of penaeid prawns, detected amylase activity in *Penaeus indicus*, and studied its optimum activity. Blandamer and Beechey (1964, 1966) identified the enzyme from *Carcinus maenas* as an α-amylase, noting that its activity

was enhanced in the presence of halions; and its optimal activity was in the pH range 5.0-7.9. High level of amylase activity was reported from the hepatopancreatic extracts of *Metapograpsus messor* and *Procambarus clarkii* (Sather, 1969). Takahashi *et al.* (1964) reported starch hydrolysis by the extracts of liver and intestine in the spiny lobster, *Panulirus japonicus* . The optimum pH and temperature requirements were 5.0-5.6 and 45°C respectively.

Wojtowicz and Brockerhoff (1972) isolated and studied the properties of digestive amylase of the American lobster *Homarus americanus*. Isolation and purification studies were undertaken by affinity chromatography followed by electrophoresis on polyacrylamide gel. Its molecular weight was determined as 41,000 and its optimum activity at pH 5.2. The purified enzyme was activated by sodium chloride.

The years which followed, saw a great revolution in the enzyme studies. Primary hydrolytic enzymes were investigated with reference to different moult stages, size-groups, seasons, larval development, effects of eyestalk ablation and dietary variations.

Van Wormhoudt *et al.* (1972) reported that during moult cycle, the enzyme activity varies drastically and variations are synchronised. The same authors (1974) showed a circadian rhythm for amylase activity in *Penaeus kerathurus*. A seasonal variation in amylase activity, with maximum in winter and autumn and minimum in spring was also reported (Van Wormhoudt *et al.*, 1973). The removal of eyestalk induced a lowering of the enzyme

only in May and September, and hence the existence of a double hormonal mechanism controlling the activities was suggested (Van Wormhoudt *et al., 1973)* in *Palaemon serratus.* Ceccaldi (1973) studied the amylase activity during vitellogenesis, and came across a marked similarity in enzyme activity during vitellogenesis as during molting in *Palaemon serratus.*

Hoyle (1973) fed *Homarus americanus* with diets containing 0, 5, 20% starch for periods upto 37 days and compared the enzyme activities in the stomach juices of fed and starved lobsters. Amylase activity was found to rise on prolonged feeding and alteration of starch content of the diet had no consistent effect. Van Wormhoudt *et al.* (1980) studied the effect of the carbohydrate and protein composition of compounded diets on growth rate and amylase activity of the hepatopancreas of *Palaemon serratus* and found that the maximal specific amylase activity is reached when assimilable carbohydrate amounts to 2.8%. In another study Maugle *et al.* (1982a)compared the growth, survival and digestive enzyme activities in *Penaeus japonicus* , which were fed with live short-necked clam (L-SNC), freeze dried short-necked clam (F-SNC) and a compound dry diet (CDD). According to them exogenous digestive enzymes from the live-food source affect the shrimp's amylase activities. The live short-necked clam group showed an increase in amylase activity, whereas F-SNC group exhibited decreased amylase activity. These changes seemed to be an adaptation of endogenous enzyme activity to the diet's enzyme content. Besides, the amylase activity in the F-SNC group increased greatly when L-ascorbic acid was added to the extract solution.

Van Wormhoudt (1981) while studying the digestive enzymes during larval development and early juvenile stages in *Palaemon serratus*, *M. rosenbergii*, and *Penaeus japonicus* came across a significant increase in the activity of amylase than protease. These changes appeared mainly at the end of the nauplius and mysis stages in *Penaeus* and during mysis stages in *Palaemon* and *Macrobrachium*. They are related to modification of nutrition and to the appearance of an hormonal control. Earlier to this, another study conducted by Laubier-Bonichon *et al.* (1977) revealed the development of a new stock of digestive enzymes during the zoeal stages. The variations of amylase and protease as well as their ratio showed that the diet was predominantly based on glucids at the end of zoea and at the beginning of mysis stage.

Fingerman *et al.* (1967) demonstrated that the secretion of digestive enzymes in *Procambarus clarkii* was controlled by neuroendocrine factors. They noted that eyestalk removal caused a decrease in the amylase activity. Similar observations have been made by Nagabushanam and Diwan (1974) on freshwater crab, *Barytelphusa cunicularis* and Jyothi and Nagabushanam on freshwater prawn *Caridina weberi*. These studies revealed that amylase synthesis is regulated by a neuroendocrine mechanism mediated via ribonucleic acids.

Karunakaran and Dhage (1977) analysed the amylase activity in the digestive tracts of *Penaeus indicus* and *Metapenaeus monoceros*. They found out that the activity was maximum in the hepatopancreas and decreased along the posterior parts of the alimentary canal. The optimum activity was at slightly acidic medium for foregut, pyloric and hepatopancreas, but the intestinal

extract showed optimum activity at neutral medium. The temperature optimum was between 35 and 40°C and the optimum substrate concentration between 3.0 and 3.5% starch.

Kulkarni *et al.* (1979) studied amylase activity of the digestive system in marine penaeid prawns, *Parapeneopsis hardwickii* and *Parapeneopsis stylifera*. Maximum amylase activity was found in the midgut gland, whereas it decreased in the foregut and hindgut parts. In both the species the optimum pH for enzyme activity was little less than 7 while optimum temperature was between 32°C and 35°C. Maximum activity of this enzyme was found when the substrate concentration was between 3 and 3.5% in *P. hardwickii*, and 2.5 and 3.0% in *P. stylifera*.

While studying the enzyme profile of *Macrobrachium rosenbergii*. Lee *et al.* (1980) reported strong amylase activity in the hepatopancreatic extract. Saxena and Murthy (1981) characterised hepatopancreatic amylase activity in *Macrobrachium lamarrei*. Effect of factors like pH, temperature, incubation period, enzyme and substrate concentration, dialysis, metallic chlorides, amino-acids was studied in detail by the authors. Maugle *et al.* (1982b)identified significant amylase activity in *Penaeus japonicus* with a distinct optimum at pH 6.8 and temperature 40°C. Chinnayya (1972) reported the optimum pH and temperature for amylase activity to be 6.8 and 65°C respectively in *Caridina Weberi*.

Amylase exists in its isoenzyme pattern. This was first reported by Van Wormhoudt (1982) in *Palaemon serratus*. During the moult cycle, in summer, the

amount of dominant α2-amylase isoenzyme increases 2 fold between inter and premoult stage. It's maximum activity is reached at stage D_1' while the maximum specific activity of α-amylase is attained at stage D_1'''. In winter, however no significant variations were measured. Van Wormhoudt and Favrel (1988) while studying the amylase polymorphism during the intermoult cycle by electrophoretic characterization detected seven forms in *Palaemon elegans*. . Their molecular weights ranged from 29,000-78,000.

Purification of α -amylases by gel-filtration chromatography was performed for the first time on *Carcinus maenas* by Blandamer and Beechey (1966). Robson (1979) isolated and purified α-amylase in *Asellus aquaticus*. Van Wormhoudt *et al.* (1982) purified the enzyme from *Euphausia superba*.

The present investigation, has been carried out to characterize the α-amylase of *Penaeus indicus*. The study has been designed to determine the optimum pH, temperature, incubation period, substrate and enzyme concentration levels. The action of varying concentrations of metallic chloride solutions, amino acids and vitamins have also been studied. This enzyme has been partially purified by gel filtration chromatography.

MATERIALS AND METHODS

Substrates and Chemicals

Potato starch (soluble), bovine serum albumin (protein standard, fraction V), maltose standard, amino acids, vitamins, all chemicals and reagents for poly-

acrylamide gel electrophoresis and buffer preparations were purchased from BDH, London or Sigma Chemicals USA, Sephadex gels grade G-25, G-100, and Blue Dextran were procured from Pharmacia Fine Chemicals AB, Uppsala, Sweden. All other chemicals used were of analytical grade.

Experimental Animals

Penaeus indicus adults ranging in lengths 100-120 mm (116 ± 2.5 mm) and weights 13-14.5g (13.43±0.15g) were used in the study.

Animals in the intermoult stages were selected. Moult stage identification was made by microscopic examination of the dorsal apical surface of the endopodite of the uropod (Drach, and Tchernigovtzeff, 1967). The prawns were fed with fresh clam meat *ad libitum* during the acclimation period. Prior to enzyme assays, prawns sacrificed for the study had been starved for two days.

Preparation of crude enzyme extract

All the operations were carried out in the cold (0°-4°C). Fresh specimens were dissected alive over ice and the hepatopancreas were carefully taken out. Utmost care was taken as not to cause any damage to the tissue membranes which would otherwise result in oozing of the glandular contents. Several glands were pooled together, dried between filter-paper sheets and weighed. Then transferred to precooled Potter-Elvehjem type tissue homogenizer, and homogenised in little amount of cold double distilled water. The homogenates were spun in a Sorvall Model RC5B - refrigerated centrifuge at 20,000 r.p.m. for 20 minutes at 0°-4°C and the supernatant diluted to a concentration of 10 mg wet weight/ml

(0.1 ml = 1mg of hepatopancreas). All enzyme tests were run on the extract within three days after separation. A few drops of toluene was added as preservative to the extract. Preliminary standardisation was carried out to determine the concentration of crude enzyme extract necessary for action on appropriate substrate concentration within the optimum incubation period of 60 minutes.

Crude enzyme extracts ranging from 5mg% to 10mg% were tested taking varying substrate (starch) concentrations from 0.5%-3%.

The results of the preliminary standardisation showed that 1% starch solution produce the best turnover of the product with the 10 mg % enzyme extract.

The assay system consisted of appropriate buffer 0.3ml, 1% starch 0.2 ml, and enzyme extract 0.1 ml. In controls a heat denatured enzyme was added. After incubating the mixtures at 37°C in a constant water bath for 60 minutes, following Bernfeld(1955) colorimetric methods for estimating hexose sugars, the reaction was stopped by adding 0.5 ml of 3,5 dinitrosalicylic reagent; thereafter the mixtures were warmed for 15 minutes and their volume raised to 6 ml with double distilled water. Readings were taken at 540 nm using a ECIL UV-VIS spectrophotometer.

Maltose was used to construct a calibration curve and absorbance values were converted into mg of maltose equivalents liberated per ml of extract per minute and as expressed relative activity. Specific activity was represented

as mg maltose liberated per mg. of protein per minute (Bernfeld, 1955). Protein was determined according to the method of Lowry *et al.* (1951) using Bovine serum albumin (fraction V) as a standard.

The crude enzyme extract was used for elucidating the effects of pH, stability, incubation period, enzyme and substrate concentrations, metallic chlorides, vitamins and amino acids. pH was the first factor to be studied in order to ascertain its optimum value at which the effect of remaining factors were investigated. For three factors (metallic chloride solutions, amino acids and vitamins) 0.1 ml of their solution was added to the assay mixture and then incubated.

Data analysis

All the experiments were replicated six times. Each assay for each experiment was done in triplicate and read on the spectrophotometer against their blank controls. Mean plus standard errors were calculated for most of the experiments.

Purification of α-amylase

Fresh hepatopancreas (30 g) were collected from prawns weighing 16-18 g and 120 - 130 mm in length. The glands were homogenized in ice-cold distilled water using a glass mortar and pestle, the crude enzyme extract was prepared by allowing the solution to stand overnight at 0°-5°C for complete extraction of the enzyme and then centrifuged at 20,000 rpm for 20 minutes at 4°C and the supernatant collected. The volume was made up to 100 ml using cold double distilled water.

The supernatant was brought to 30% saturation of ammonium sulphate, centrifuged, the precipitate collected and the supernatant was brought to 80% saturation of ammonium sulphate, stirred and then centrifuged at 20,000 rpm for 20 minutes at 4°C to collect the precipitate. The precipitates were combined and then dissolved in 50 ml of 0.02 M cold phosphate buffer with pH 7.5, centrifuged at 20,000 rpm for ten minutes at 4°C and the residue discarded.

A column with 40 cm length and 1.5 cm internal diameter was packed with 20 gm of sephadex G-25 equilibrated with the phosphate buffer pH 7.5. The internal volume Vi of the column was calculated from the mass and water regain of the dry sephadex. The void volume had been previously determined with Blue Dextran (Pharmacia-Gel Filtration Techniques). Twenty five ml of the extract collected as above was poured on top of a column packed with sephadex G-25. The extract was applied to the column very carefully and eluted with the phosphate buffer pH 7.5. A flow rate to 17 ml/hr was maintained and the fractions collected at one hour interval. Active fraction numbers 30-40 were pooled and dialyzed using Cellophane membrane in .02 M phosphate buffer pH 7.0. The remaining portions of the extract were treated in the same manner and the active fractions were combined and concentrated by dialysis.

The concentrated solution (100 mg protein in 12 ml) was applied to the sephadex G-100 (90 x 2.5 cm) column equilibrated with 0.02 M phosphate buffer. A flow rate of 12 ml/hr was maintained and the active fractions 10-15 were collected at one hour interval.

A summary of the specific activity and recovery of the enzyme activity at each purification process is shown in Table 22.

All steps of the purification process were carried out at 0°-4°C.

Homogeneity of active fraction obtained as above was examined by polyacrylamide gel disc electrophoresis. The details are as follows: About 36 µg of protein was applied and the migration observed.

Polyacrylamide disc gel electrophoresis

Electrophoretic separation of partially purified enzyme was performed in accordance with the technique of Ornstein and Davies (1962) and Hedrick and Smith (1968) with some modification using 7.0% polyacrylamide gel.

Stock solutions for the preparation of standard gel

This system stacks at pH 8.9 and runs at pH 9.5. The final concentration of the gel was 7.0 percent acrylamide.

STOCK A			STOCK B		
1N HCl	-	24 ml	1N HCl	-	48 ml
Tris	-	18.1 g	Tris	-	5.98 g
TEMED	-	0.12 ml. pH 8.9	TEMED	-	0.24 ml pH 6.7
Dist. water to make - 100 ml			Dist. water to make - 100 ml.		

STOCK C

Acrylamide - 28.0 g

NN'-methylene- 0.735 g

Bisacrylamide

Dist water to make - 100 ml

STOCK D

Acrylamide - 10 g

NN'-Bisacrylamide- 2.5 g

STOCK E

Riboflavin - 4.0 mg

Dist.water to make - 100 ml

STOCK F

Sucrose - 40.0 g

Dist.water to make - 10 ml

STOCK G-Catalyst

Ammonium persulphate - 200 mg

Dist.water to make - 100 ml

STOCK H-Buffer

Tris - 3.0 gm

Glycine - 14.4 gm pH 8.4

Dist. water to make - 100 ml.

Tracking Dye: 5 mg Bromophenol blue was dissolved in distilled water to make final volume to 100 ml.

Working solution

Separating Gel (Small pore gel, Running gel)

1 part - STOCK A

1 part - Distilled water

2 parts - Stock C

4 parts - Stock G.

Stacking Gel (Large pore gel, spacer gel)

1 part	-	Stock B
4 parts	-	Water
2 parts	-	Stock D
1 part	-	Stock E

Preparation of Gels

Separating Gel - 12 open ended glass tubes (0.5 cm inner diameter and 9.0 cm length) were sealed at one end with a rubber stopper. The running gel solution was carefully pipetted into the tubes taking necessary care to avoid the occurrence of any air bubble. A drop of distilled water was then gently layered at the top to ensure a flat surface. After 20-25 minutes, gel polymerization was detected as an appearance of a sharp refractive boundary separating gel from the water drop. After gel formation, the water drop was removed by firm shaking of the tube.

Stacking gel

A large pore solution was delivered on the running gel and was layered on top with a drop of water.

The tubes were then kept adjacent to a fluorescent tubelight for photopolymerization for about 30 minutes.

Application of sample:

The gel tubes ready for electrophoresis were inserted, spacer gel end up, into the upper bath tube adapters. 40 µl of the enzyme fraction was pipetted over

the stacking gel, and over the enzyme an equal amount of sucrose (40%) was added to provide density to the sample which prevented it from washing up into or mixing with the buffer solution. A drop or two of bromophenol blue solution was added to ensure the completion of the run. About 300 ml of electrolytic buffer was added in each of the bath chambers. In case of upper bath chamber buffer was added slowly to avoid washing-off of the dye.

Electrophoresis:

Electrophoresis was conducted at low temperature (0°-5°C) in a refrigerator. For first 20 minutes, a current of 10 mA was given and later increased to 40 mA. When the bromophenol blue comes to the lower edge of the gel tubes, the current supply was switched off.

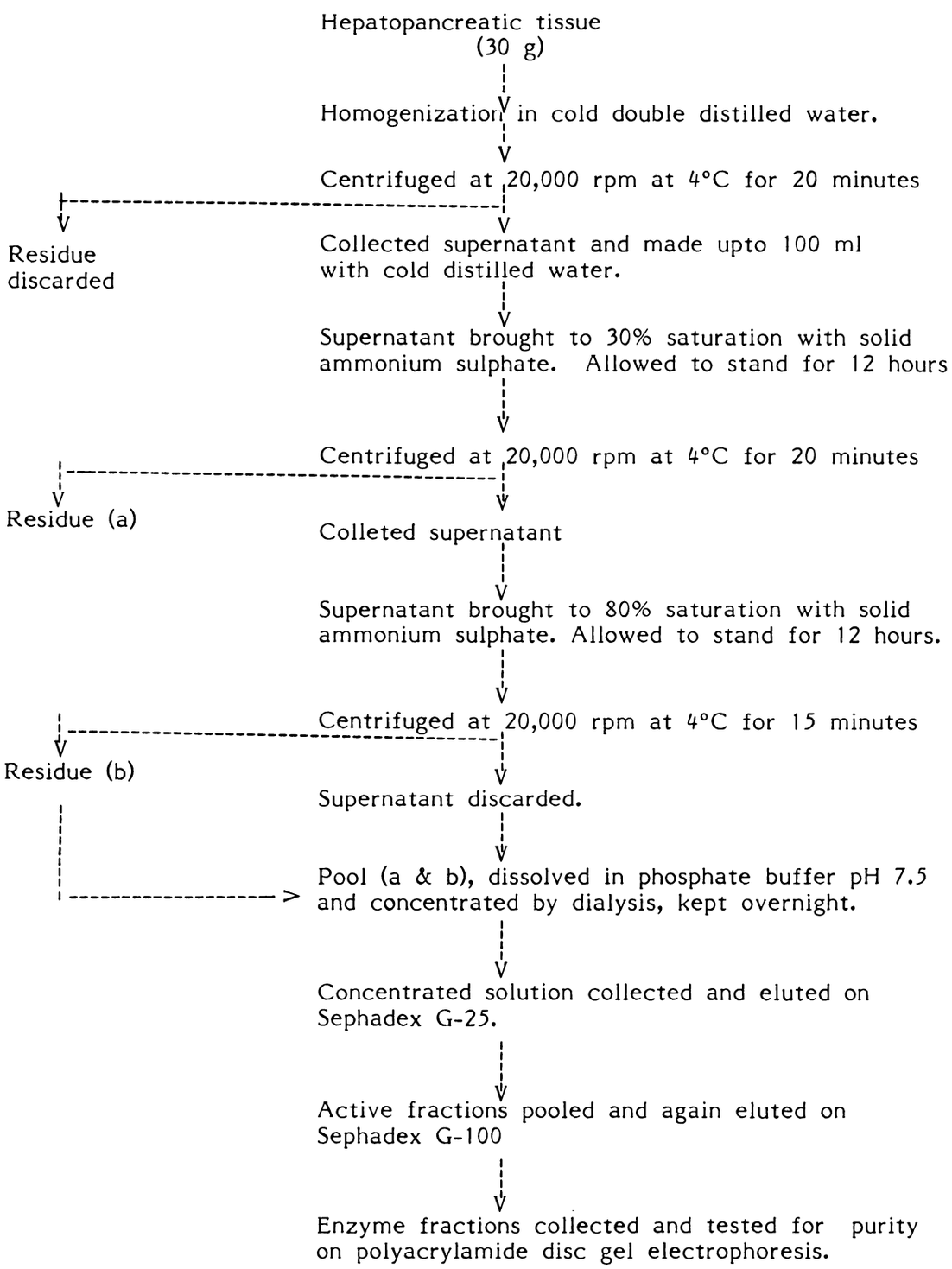
Staining:

After the completion of electrophoresis the gels were removed from the gel tubes carefully using a syringe filled with the used buffer. The gels were incubated in 10% T.C.A. for 30 minutes till the bromophenol blue mark becomes yellow. Then transferred to 0.25% Kenacid blue (BDH) in methanol-water-acetic acid mixture (5:5:1) in dark for 30 minutes. Destained the gels in methanol-water-acetic acid mixture (5:5:1) for 30 minutes in dark and then stored the gels in 7% acetic acid. This was done for the detection of general protein fractions.

Enzyme localisation:

Amylase activity was located by incubating gels for 12 hours in fresh 2% starch solution at 37°C. They were then transferred to a solution of 0.2%

FLOW CHART OF PURIFICATION OF ∝-AMYLASE



iodine in 2% potassium iodide and kept for 10-15 minutes. Gels were then washed and preserved in 7% acetic acid. At the loci of amylase activity, the starch in the gel gets hydrolysed. These loci appear as white bands and the rest of the gel remains blue coloured due to reaction of starch with iodine,

The iosenzyme bands were traced on a graph sheet.

RESULTS

The following experiments were designed to obtain information on the nature of the amylolytic factor in the enzymatic extracts and to determine optimal conditions for its activity.

pH optima determination

Four buffer sets were used

a) Glycine-HCl buffer (0.2M)
   pH range 2.0-4.0

b) McIlvaine buffer - (0.2 M)
   (citrate-phosphate)
   pH range - 4.0-6.0

c) Sorensen phosphate buffer (0.2M)
   pH range 6.0-8.0

d) Carbonate-bicarbonate buffer (0.2 M)
   pH range - 8.0-10.0.

Buffer solutions were prepared according to Gomori (Methods in Enzymology, 1954 volume I 138-141). The solutions were stored in bottles and kept in the refrigerator until used. The results obtained showed that the amylase remains quite active in the pH range 6.0-8.0, with optimum at pH 7.5 (Fig.15).

Effect of temperature

Amylase activity was tested at eight temperatures ranging from 10°C-80°C in a constant water bath. Although the enzyme remained fairly active from 30°C-50°C optimum activity was observed at 40°C. At higher temperatures there was negligible activity (Fig. 15).

Stability of the enzyme

The effects of freezing (-10°C), cold storage (0°-4°C) and room temperature (28±2°C) on stability of the enzyme has been studied using dilute enzyme extract.

Amylase, in general, was quite stable at the experimental temperatures. Virtually there was no change in activity of the enzyme extract under cold or frozen conditions upto 7 days (Fig. 16). At room temperature, however, the activity was reduced by 10-15% by the end of the week (Fig. 16).

Effect of incubation period

Assay mixtures were incubated for progressively increasing duration. In the first 15-20 minutes there was a sharp increase in the activity reaching the maximum at 50 minutes; thereafter it remained steady, more or less, without showing any drop in the activity (Fig. 17).

Effect of substrate concentration

Soluble starch solutions of 18 concentrations, from 0.1 mg/ml to 5.0 mg/ml were tested on enzyme activity.

Starch hydrolysis increased with increasing substrate concentration upto 0.8 mg/ml, thereafter it slowed down, becoming constant at 1.5 mg/ml (Fig.18). It thus seems that at 1 mg/ml, virtually all available enzyme is converted into E.S. complex (Km value 1 mg/ml) (Fig. 19).

Effect of enzyme concentration

Enzyme extracts of varying strengths, from 0.1 mg to 1 mg protein/ml hepato-pancreatic proteins were tested. The starch hydrolysis increased in a linear fashion upto 0.7 mg/ml and beyond that became constant at 1 mg/ml followed by a sharp decline in the activity (Fig. 17).

Effect of metallic chloride solutions

Fifteen metallic chloride solutions, each in six molar concentrations of 0.0001; 0.0005, 0.001, 0.005, 0.01, and 0.05 were tested on the enzyme activity. Aluminium chloride showed maximum activation at the lowest concentration of 0.0001 M. Inactivation occurred at 0.03 M solution (Fig. 20).

Ammonium chloride: Ammonium chloride activated the enzyme at all the testes concentrations. The activity increased from 0.0001 M to 0.05 M linearly. Maximum activation of 308% was obtained at 0.05 M concentration (Fig. 20). The optimum concentration obtained from Polynomial regression of the

data was 0.031 M. Antimony chloride inhibited the enzyme activity at all the tested concentrations (Fig. 20).

Barium chloride: Activated the enzyme at all concentrations. There was a steady increase in activity with the concentration of chloride solution. Maximum activation (421%) occurred at 0.05 M (Fig. 20). and optimum was derived at 0.026 M.

Calcium chloride: Induced a linear increase in the activity of the enzyme, in relation to the concentration. The maximum activation (313%) was at 0.05 M (Fig. 20) and the optimum was at 0.029 M.

Cobalt
Chloride: Activated at all the concentrations. The activity trend showed an increase from 0.0001 M to 0.01 M, followed by a slight decrease at higher concentrations. The maximum activation was 200% when compared to the control and occurred at 0.1 M concentration (Fig. 20); optimum was seen at 0.028 M.

Cupric chloride: Maximum activation of 60% occurred at 0.0001 M concentration; the remaining strengths were inhibitive (Fig. 21).

Ferric chloride: Showed slight activation at 0.001 M. Other concentrations were ineffective; optimum activation of 43% was observed at 0.0001 M (Fig. 21). Inhibition occurred at 0.288 M.

Magnesium chloride: All the six concentrations were very effective. Linear activation pattern was obtained. The maximum activity (252%) was at 0.05 M (Fig.21).

Managanese chloride: Effective at all strengths, maximum activation was seen at 0.05 M (454%). Manganese chloride solution showed the strongest activating effect from the lowest to the highest concentrations, when compared to the other metallic chlorides (Fig. 21) with an optimum at 0.0386 M

Mercuric chloride: Inhibited the enzyme at all concentrations, resulting in no activity at higher strengths (Fig. 21).

Nickel chloride: Showed maximum activation at 0.005 M. At higher and lower concentrations activity was relatively less. Optimum activation was obtained at 0.04 M (Fig. 21).

Potassium chloride: All the six concentrations were effective with maximum activation at 0.05 M (299%). A linear increase in the activation of the enzyme was observed with increasing concentration of the chloride solution. Optimum activation was at 0.034 M.

Sodium chloride: Progressive activation of the enzyme at various strengths were observed. Maximum activation was at 0.05 M. Strontium chloride also activated the enzyme at all concentrations. The activation increased from 0.0005 M concentration, reaching the maximum at 0.05 M concentration.

Effect of amino acids on enzyme activity:

Twenty one amino acids, each in six concentrations (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 M) were tested. Results are represented in Figs. 22,23,24 & 25 and Table. 19.

Alanine activated the enzyme at lower concentrations (0.0001 M-0.005 M). At 0.01 and 0.05 M concentrations, it inactivated;the rates of inhibition being 28% and 40% respectively. (Fig. 22)

Aspartic acid amino-butyric acid and arginine inhibited the enzyme at all the tested concentrations. (Figs. 22 & 23)

Cysteine showed slight activation at lower concentrations (0.0001, 0.0005, 0.001 M) but inhibited at other concentrations. About 57% inhibition was observed at 0.05 M.(Fig. 23)

DOPA (Dihydroxy-phenyl-alanine) was effective at higher concentrations. Maximum activation was found to be at 0.05 M concentration (Table. 19).

Lower concentrations (0.0001,0.005M)of glutamic acid activiated the enzyme. Maximum activation was observed at 0.0001M,0.005 M concentration. (Fig. 23)

Glycine did not activate the enzyme at any of the tested concentrations. (Fig. 23).

Histidine activated the enzyme with a maximum at 0.0005 M (about 12% when compared to the control). Inhibition was observed at higher concentrations. Inactivation of about 53% was seen at 0.05 M concentration.

Isoleucine induced only slight increase in the activity at 0.0005 and 0.001 M concentrations. Other concentrations did not have any significant effect. (Fig. 24)

Leucine showed maximum activation at 0.005 M. Higher concentrations showed inhibiting effect. (Fig. 24)

Lysine showed slight activation at 0.0005 M concentration. The rest of the concentrations showed inactivation.(Fig. 24)

Methionine showed inhibition at all strengths. At 0.05 M concentration there was no activity at all. (Fig. 24)

At lower concentrations nor-leucine activated the enzyme to a slight degree. Maximum activation of 26% occurred at 0.0005 M concentration. At other strengths the enzyme reactions were very negligible when compared to the control. (Fig. 24)

Ornithine activated the enzyme at lowest concentration. About 26% increase in activity was detected at 0.0005 M concentration. The enzyme activity was greatly reduced at higher concentrations. (Fig. 24)

Phenylalanine induced more activity at lower concentrations. Maximum degree of activation occurred at 0.0001 M. The activity got reduced to almost 50% at the 0.05 M level. (Fig. 25)

Proline inhibited the enzyme activity within the concentrations tested (Fig. 25). At all levels, the enzyme activity was low.

Serine exhibited inactivation at all concentration with the maximum inactivation at 0.05 M (about 38%). Threonine also showed inhibitory effect at all the strengths, with greater degree of inhibition (about 50%) at higher concentrations (0.05 M). (Fig. 25)

Tryptophan. Slight activation of 7% was observed at 0.01 M concentration, when compared to the control. All other concentrations were inhibitive, maximum inhibition being recorded at 0.0001 M concentration. (Fig. 25)

Valine activated the enzyme at a lower concentration of 0.0005 M 0.001 M, with 0.0005 M providing maximum activation of 347. At higher concentrations inhibition was noticed. (Fig. 25)

Effect of selected vitamins

Enzyme activity increased steadily with the increasing concentration of ascorbic acid reaching optimum levels at 0.001 M, thereafter the enzyme activity slowed down rapidly. At the highest concentration of the vitamin, maximum inactivation was observed. (Fig. 26)

Choline induced greatest activity at the lowest vitamin concentration (0.0001 M). Activity reduced with increasing concentration and maximum inactivation occurred at 0.05 M. (Fig. 26).

At all the tested concentrations inositol showed inhibitory effect with maximum inhibition at higher concentrations. (Fig. 26)

Calciferol induced a sharp increase in activity of the enzyme with the increase in concentration upto 0.001 M. At higher concentrations, the activity declined.

Nicotinic acid induced maximum activation at 0.0001 M. At higher concentrations, inhibition was noted. (Fig. 26)

Riboflavin activated the enzyme at 0.0001, 0.0005, 0.001, 0.005 M concentrations with an optimum at 0.0005 M. Inhibition was noted at 0.05 M concentration (about 98.36%.) (Fig. 26)

DISCUSSION

All crustaceans usually secrete a very strongly acting amylase with a pH optimum ranging from 5.0 to 7.8. In *Penaeus indicus* amylase showed maximum activity within the pH range 6.5 and 7.5, and optimum activity at pH 7.0 (Fig. 15). In an earlier study also the optimum pH for amylase activity in *P. indicus* was found to be in the range 7.0-7.2 (Gopalakrishnan, 1957). However, Karunakaran and Dhage (1977) reported the pH optimum for amylase as 6.6 in *P. indicus*. While Maugle *et al.* (1982) showed an optimum pH of 6.8 for the marine prawn *Penaeus japonicus*. Murthy and Saxena (1981) found the optimum at pH 6.5. for the freshwater prawn *Macrobrachium lamarrei* In the lobster *Panulirus japonicus*, the optimum was between 5 and 5.6 (Takahashi *et al.*, 1964) and in other decapods slightly higher values have been reported - for *Paratelphusa hydrodromus* 6.75-7.25 (Ammal, 1966), *Cancer borealis* and *C. irroratus* pH 7.0 (Brun and Wojtowicz, 1976).

Based on their pH optima the amylases of the Crustacea appear to fall into two disparate groups: amylases with pH optima below 6.3 have been reported in Isopoda Amphipoda and Reptantia-Astacura: amylases with pH optima above 6.8 have been reported in Natantia and Reptantia Brachyura (Robson, 1970). As it is clear from the study the amylase of *Penaeus indicus* fall into the latter category with an optimum pH 7.0.

The optimum temperature of hepatopancreatic amylase (40°C) recorded for *P.indicus* is comparable to the results of Maugle *et al* (1982)

for *Penaeus japonicus* but lower than that of *Cardina weberi* - 52°C (Chinnayya, 1972), *D. bicristimanus* - 49°C (Nagabhushanam and Sarojini, 1968), *Penaeus japonicus* - 45°C (Takahashi *et al.*, 1964) and *M.l amarrei* - 50°C (Murthy and Saxena, 1981). Kulkarni *et al.* (1979) reported an optimum temperature between 32°C and 38°C for *Parapeneopsis hardwickii* and *Parapeneopsis stylifera*. Karunakaran and Dhage (1977), however, reported an optimum temperature of 35°C while Gopalakrishnan (1957) reported the optimum temperature as 52°C for *Penaeus indicus*. The observed differences in the temperature optima for *Penaeus indicus* could be attributed to the variations in the ecological conditions in which the prawns were maintained. As the prawns are poikilothermic they have a tendency to adjust the body temperature to that of their environment. Probably amylase of *Penaeus indicus* is influenced by fluctuations in environmental temperatures to a certain degree.

There are only few reports available in the effect of starch concentration on amylase activity in crustaceans. Substrate concentration studies in *Macrobrachium lamarrei* showed an optimum amylase activity at 1.75% of starch (Murthy and Saxena, 1981). *Penaeus indicus* and *Metapenaeus monoceros* showed maximum activity at 3.0 and 3.5% of starch (Karunakaran and Dhage, 1977). In the present study optimum substrate concentration for amylase activity for *Penaeus indicus* was at 1 mg starch/ml of hepatopancreatic extract. It had a Km value of 1 mg starch/ml. The low value is an indication of the high affinity between the enzyme and substrate.

This observation is similar to the value of Michaelis constant reported for *Palaemon serratus* by Van wormhoudt (1977).

Experiments to determine the effect of incubation time, substrate concentration and enzyme protein levels on the enzyme activity showed an identical effect of an initial acceleration followed by retardation on the activity of hepatopancreatic amylase (Fig. 17 & 18). There was always an initial acceleration, reaching the optimum and thereafter a steady phase is being maintained. Retardation of amylase activity caused by prolonged incubation and stronger enzyme concentration may be attributed to the complete utilization of the starch substrate. That caused by stronger strengths of substrate is probably due to the conversion of the total enzyme into enzyme-substrate complex (Karlson, 1969).

As evident from the Fig. 14 metallic chlorides activated the enzyme at one or more strengths of the solution when compared to the control. Mercuric chloride was found to be a strong inhibitor of the enzyme even at the lowest tested concentration. In *Penaeus indicus* optimum activation of the amylase occurred at a chloride concentration of 0.05 M for ammonium, barium, calcium, magnesium, manganese, potassium, sodium and strontium, 0.01 M for cobalt chloride, 0.005 M for nickel chloride and 0.0001 M for aluminium, antimony, cupric and ferric chlorides. The activation of hepatopancreatic amylase by a halide particularly chloride suggests that it is related to the mammalian alpha-amylase (Bernfeld,1950). In the lobster *Homarus americanus* maximum activation of amylase occurs

at 0.05-0.1 M NaCl (Wojtowicz and Brockerhoff, 1972). An optimum of 0.1 M NaCl was reported for the amylase of the shore crab *Carcinus maenas* (Blandamer and Becchey, 1964) and the cray fish *Orconectes virilis* (Telford, 1970). However, surprisingly, the amylase of the freshwater isopod *Asellus aquaticus* was reported to be most active at 1.0 M NaCl, a very high concentration (Robson, 1979). Maugle *et al.* (1982) recorded increased amylase activity in *Penaeus japonicus* when two ions, Na_2SO_4 and $CaCl_2$ (particularly at a concentration of 10^{-2}M) were added to the assay solution. Saxena and Murthy (1981) found chlorides of Li, Mg, K, Mn, Ba, Cd to be activatory at six concentrations ranging from 5×10^{-5}M to 1×10^{-2}M. Chlorides of Na, Ca, Rb, Sr, Pt, Hg, Co, Zn and Sn exerted no effect at 1×10^{-4}M and/or 5×10^{-5}M; but at remaining strengths amylase was activated.

In view of the above facts, it may be concluded that with the exception of mercuric chloride the presence of chloride ions at optimal levels significantly enhances amylase activity in *P. indicus*. Being common to all, chloride may be inferred to be responsible for the activation. Ions have generalised effects on stereochemistry of the enzyme. Certain ions seem to have more specific functions in the activation of enzyme systems. An ion which activates an enzyme may (a) form an integral part of the enzyme molecule (b) serve to link enzyme and substrate (c) cause shift in the equilibrium position of the reaction (d) act indirectly by releasing ions which inactivate the enzyme system (Lockwood, 1971)

Although the effect of amino acids on crustacean amylase remains largely unknown, Hori (1969) seems to be the first worker to have studied the effect of 16 aminoacids on amylase from the salivary gland of the bug *Lygus disponsi*. In another study the effect of 19 amino acids on amylase has been worked out by Saxena and Murthy (1981) in *Macrobrachium lamarrei*. Three amino acids (asparagine, β- alanine, glycine) were ineffective, whereas glutamine, norleucine, phenylalanine, and leucine produced both effects depending upon the concentration and the remaining 12 were activatory at all six strengths ranging from 5×10^{-5}M to 1×10^{-2}M. In the present investigation, of the 21 amino acids tested, 16 amino acids were found to be effective activators at one or more molar concentrations. Aspartic acid, arginine, methionine, proline and threonine did not activate the enzyme at any of the tested concentrations. While the presence of -COOH and $-NH_2$ groups, being common to all in general, is responsible for the activation, the third acyl (R-) group which is specific for each amino acid could be held responsible for the degree of activation or inhibition (Lehninger, 1975).

Among the tested vitamins ascorbic acid, choline, calciferol, nicotinic acid, riboflavin, were found to activate amylase at 0.0001, 0.001, 0.0001, 0.0001, 0.0001, 0.0005M respectively. Inositol was found to be a strong inhibitor. All the vitamins were active at very low concentrations of the solution. Vitamins generally act as cofactors of the different metabolic enzymes. However, the mode of activation of digestive amylase is not clear perhaps they act as catalysts to trigger the amylase reaction.

From the present study it is evident that certain metallic chlorides, amino acids and vitamins have tremendous influence on the amylase activity. The activity pattern showed an optimum concentration level for few chlorides, amino acids and vitamins, which may suggest that the hydrolysis of the food component in the digestive system is being influenced by these factors. There is an increase in the activity pattern reaching an optimum and then a decline, indicating that supra-optimal concentrations do not have any positive influence on the activity. It is important to note that in culture operations, the diets given to the animals should contain the appropriate amounts of these factors which in turn would result in increased digestibility and optimum utilization of food.

The presence of isoenzymes has been reported by Van Wormhoudt (1980, 1982) in *Palaemon serratus* after purification of the amylase fraction by affinity chromatography and preparative electrophoresis. In *Penaeus indicus*, the purified fractions obtained after gel filtration chromatography when subjected to polyacrylamide gel electrophoresis to test its homogeneity exhibited two isoenzyme bands. May be these bands represent subunits of a single molecule (i.e. multiple groups on the same amylase species). This observation is consistent with the observation on *Palaemon serratus* by Van Wormhoudt (1980 and 1982). The presence of isoenzyme bands in *Uca pugnax* and *Uca pugilator* were attributed to the different NaCl binding sites (Azzalina *et al.*, 1985). Again, the heterogeneity of amylases from the hepatopancreas was studied by electrophoresis (Van Wormhoubt, 1988). Seven forms were detected. Their molecular weights were between 29,000 and 78,000. In Crustacea, only few reports

exist concerning α-amylase polymorphism. Two allelic forms were measured in *Asellus* (Lomholt and Christensen, 1970) and four allelic forms in *Palaemonetes species* (Christensen and Lomholt, 1972).

More studies are needed on the purified amylase enzyme in *Penaeus indicus* and other penaeid species of the Indian waters to further characterise the physio-chemical features of the enzyme molecule.

TABLE 18: Concentrations of metallic chloride solutions causing maximum activation or inhibition on hepatopancreatic amylase of *Penaeus indicus*

Metallic chlorides	Final molar concentration	Relative amylase activity	% Activation	% Inhibition
Control	-	100	-	-
Aluminium	0.0001	198	98	-
Ammonium	0.05	408.5	308.5	-
Antimony	0.0001	71	-	29
Barium	0.05	521	421	-
Calcium	0.05	413	313	-
Cobalt	0.01	300	200	-
Cupric	0.0001	160	60	97
	0.05	3.0	-	97
Ferric	0.0001	143	43	-
	0.0005	57	-	43
Magnesium	0.05	352	252	-
Manganese	0.05	554	454	-
Mercuric	Inhibition at all strengths			
Nickel	0.005	254	154	-
Potassium	0.05	399	299	-
Sodium	0.05	305	205	-
Strontium	0.05	325	225	-

TABLE 19: Concentrations of amino acid solutions causing maximum activation or inhibition on hepatopancreatic amylase of *Penaeus indicus*

Amino acid	Final molar concentration	Relative Activity	% Activation	% Inhibition
Control	Nil	100	Nil	Nil
Alanine	0.0001M	133	33	-
	0.005	59	-	41
Aspartic acid	0.0001	57	-	43
Aminobutyric acid	0.0005	94	-	6
	0.05	50	-	50
Arginine	0.0001	92	-	8
	0.05	60	-	40
L-cysteine	0.0001	131	31	-
	0.001	111	11	-
	0.05	43	-	57
DOPA	0.005	132	32	-
	0.01	126	26	-
	0.05	303	203	-
	0.0001	75	-	25
Glutamic acid	0.0001	106	6	-
	0.005	118	18	-
	0.05	68	-	32
Glycine	0.0005	103	3	-
	0.001	98	-	2
	0.05	65	-	35
Histidine	0.005	112	12	-
	0.05	47	-	53
Isoleucine	0.0005	105	5	-
	0.001	104	4	-
	0.001	55	-	45

Contd...

TABLE 19 (Contd.....)

Amino acid	Final molar concentration	Relative Activity	% Activation	% Inhibition
Leucine	0.005	121	21	-
	0.05	31	-	69
Lysine	0.0005	102	2	-
	0.05	62	-	38
Methionine	0.01	55	-	45
	0.05	0	-	100
Nor-leucine	0.0005	126	26	-
	0.05	47	-	53
Ornithine	0.0001	126	26	-
	0.05	58	-	42
Phenylalanine	0.0001	127	27	-
	0.0005	126	26	-
	0.05	53	-	47
Proline	0.05	42	-	58
Serine	0.001	104	4	-
	0.05	62	-	38
Threonine	0.05	54	-	46
Tryptophan	0.01	107	7	-
	0.0001	47	-	53
Valine	0.0005	134	34	-
	0.001	117	17	-
	0.05	51	-	49

TABLE 20: Concentrations of selected vitamin solutions causing maximum activatory or inhibitory effect on hepatopancreatic amylase of *Penaeus indicus*.

Vitamins	Final molar concentration(s)	Relative Activity	% activation	% inhibition
Control	-	100	-	-
Ascorbic acid	0.001	346.97	246.90	-
	0.05	52	-	48
Choline	0.0001	346.97	246.9	-
	0.05	52	-	48
Inositol	0.0001	57	-	43
Calciferol	0.001	356	256	-
	0.05	94	-	6
Nicotinic acid	0.0001	233	133	-
Riboflavin	0.0005	247	147	-
	0.05	64	-	36

TABLE 21: Data to find out Line weaver - Burk plot of α-amylase for the determination of Michaelis constant.

Sr.No.	Starch (mg/ml) (S)	1/(S)	Maltose formed (mg/mg protein/ min) (V)	1/V	Km (mg/ml)
1	0.25	4.00	0.273	3.660	
2	0.50	2.00	0.433	2.301	
3	0.75	1.33	0.658	1.520	
4	1.0	1.00	0.743	1.346	
5	1.5	0.667	0.743	1.346	
6	2.0	0.500	0.698	1.433	
7	2.5	0.400	0.699	1.431	
8	3.0	0.333	0.700	1.429	
9	3.5	0.286	0.700	1.429	
10	4.0	0.250	0.693	1.433	
11	4.5	0.222	0.695	1.439	
12	5.0	0.200	0.697	1.435	1.00 mg/ml

TABLE 22: Summary of Results of purification of ∝ -amylase of *Penaeus indicus*

	Fraction	Volume (ml)	Total activity (mg/ml)	Total protein (mg/ml)	Specific activity (mg/mg protein/ min)	Purification fold	Recovery (%)
1.	Crude extract	100	935	1670	0.56	1.00	100
2.	0.3% $(NH_4)_2SO_4$	150	792	1471	0.53	0.94	84.7
3.	0.8% $(NH_4)_2SO_4$	100	648	692	0.67	1.264	82.0
4.	Sephadex G-25	50	526	702	0.75	1.12	81.5
5.	Sephadex G-100	20	97.8	18.42	5.30	7.07	18.6

Fig. 15. The effect of pH and incubation temperature on the hepatopancreatic amylase of Penaeus indicus. Each value represents the mean of 6 independent determinations.

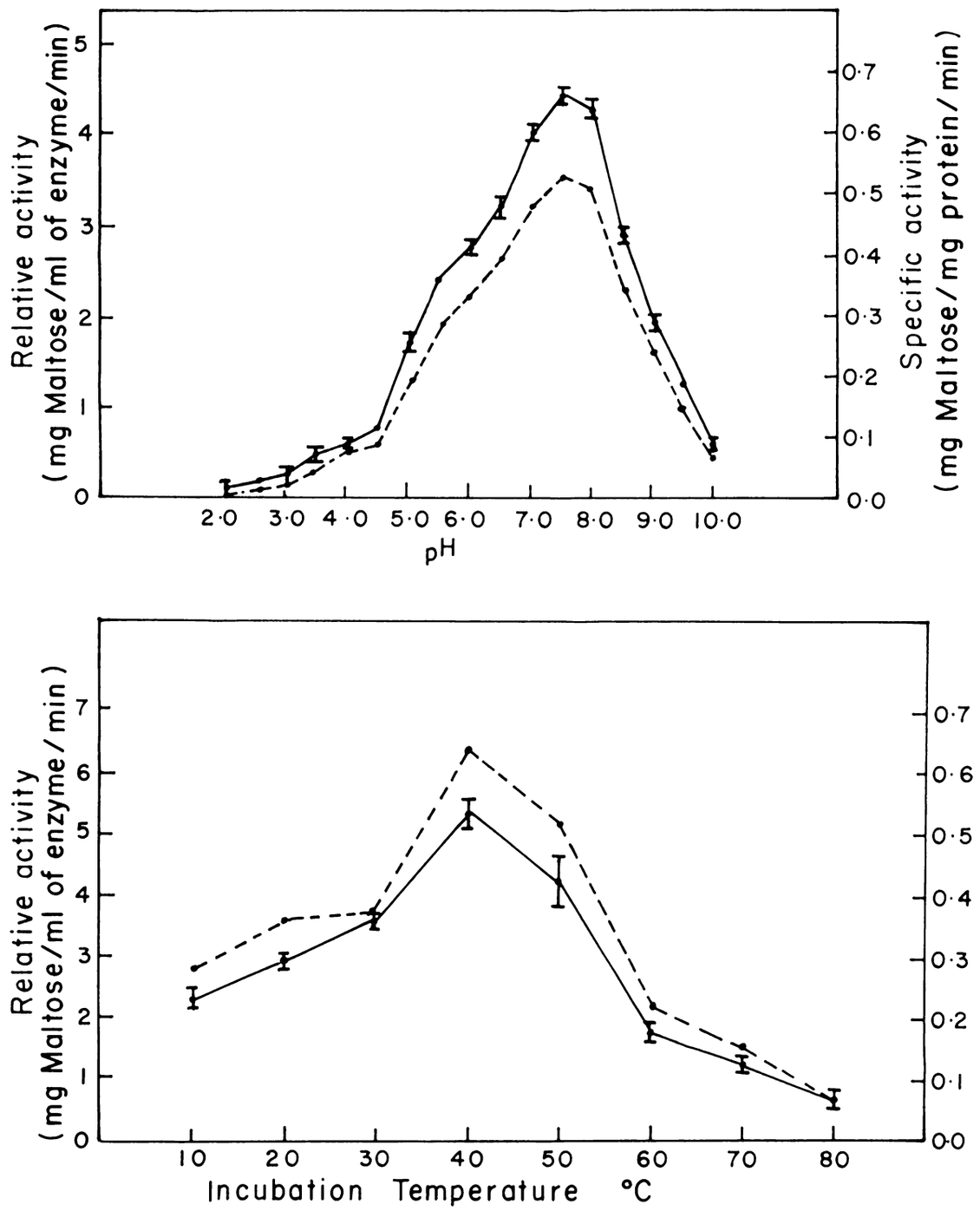

Relative activity
(mg Maltose/ml of enzyme/min)
Specific activity
(mg Maltose/mg protein/min)
0
1
2
3
4
5
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
2·0 3·0 4·0 5·0 6·0 7·0 8·0 9·0 10·0
pH
Relative activity
(mg Maltose/ml of enzyme/min)
Specific activity
(mg Maltose/mg protein/min)
0
1
2
3
4
5
6
7
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
10 20 30 40 50 60 70 80
Incubation Temperature °C

FIG. 15

Fig. 16. Stability of amylase of Penaeus indicus under cold, frozen and room temperature conditions for a period of 7 days Data given as mean of six replicates. Cold storage was at 0-4°c; frozen storage at -10°C; room temperature at 28 ± 2°C.

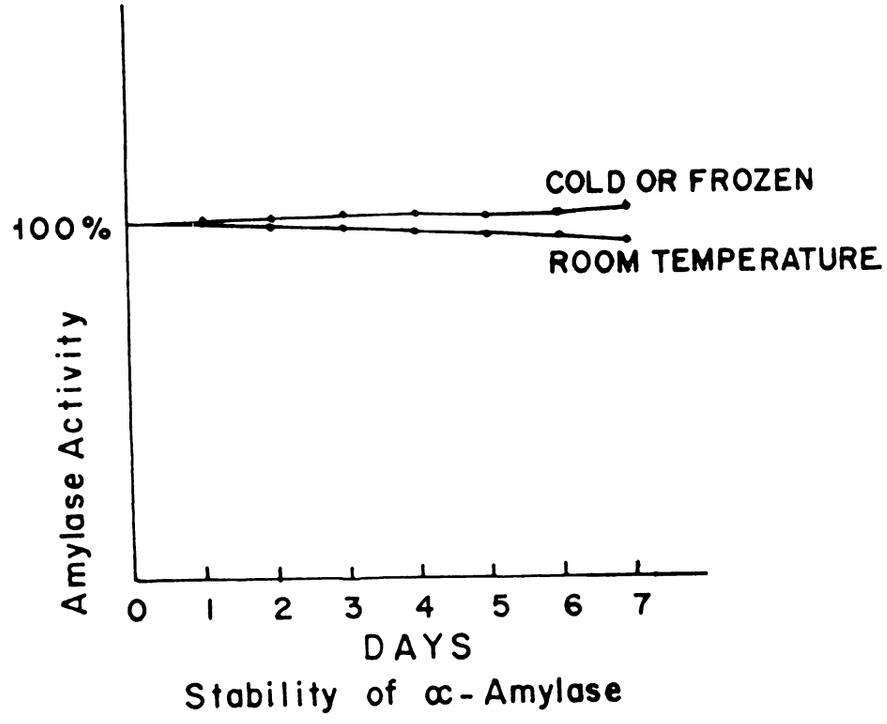

COLD OR FROZEN
100%
ROOM TEMPERATURE
Amylase Activity
0 1 2 3 4 5 6 7
DAYS
Stability of α-Amylase

FIG. 16

Fig.17. The effect of incubation period and enzyme concentration on amylase activity in Penaeus indicus. Values are given as Mean ± S.D.

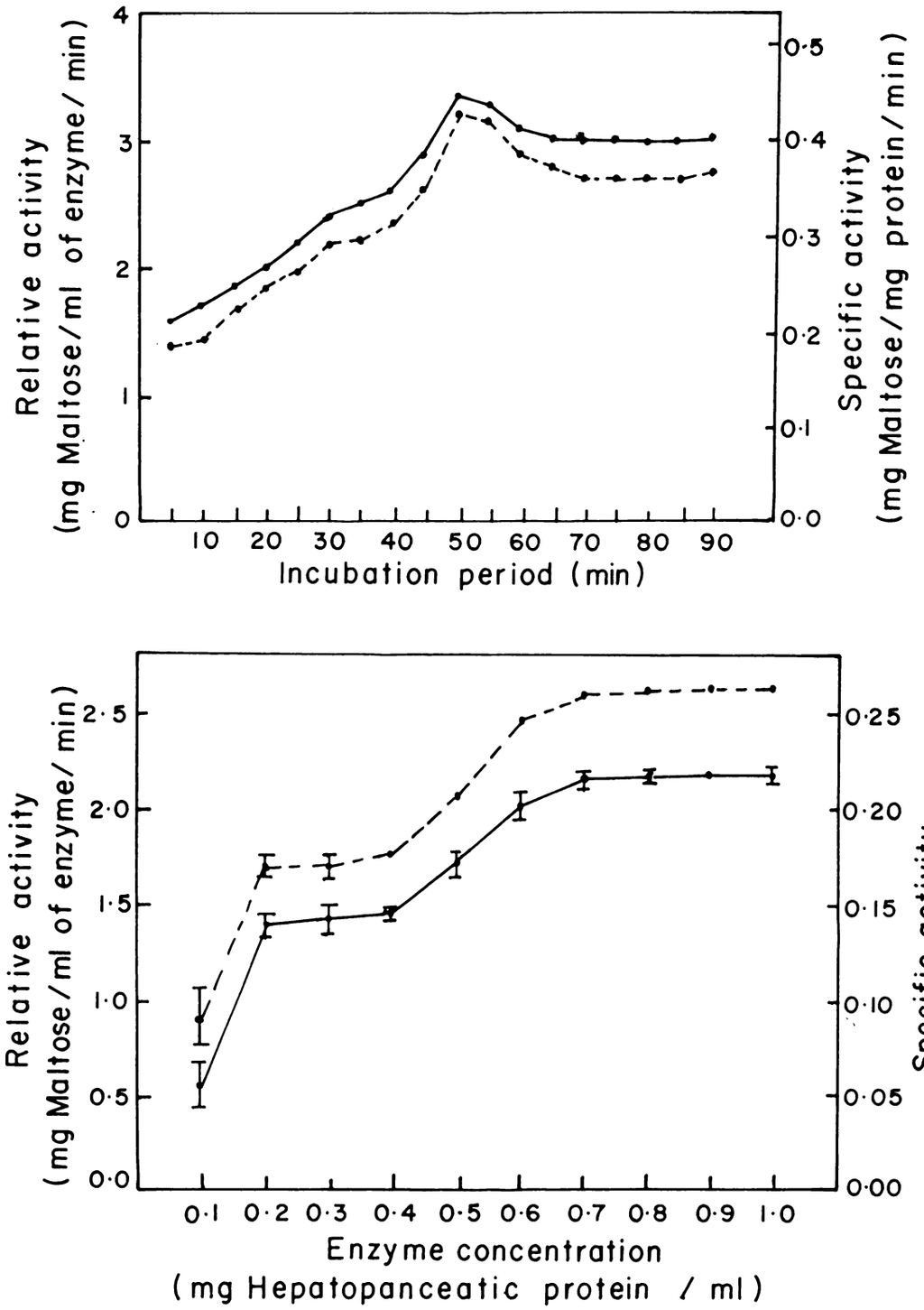

Relative activity
(mg Maltose/ml of enzyme/ min)
Specific activity
(mg Maltose/mg protein/min)
Incubation period (min)
Enzyme concentration
(mg Hepatopanceatic protein / ml)

FIG. 17

Fig. 18. Effect of substrate concentration on the hepatopancreatic amylase. Values are given as Mean ± S.D. of six independent determinations.

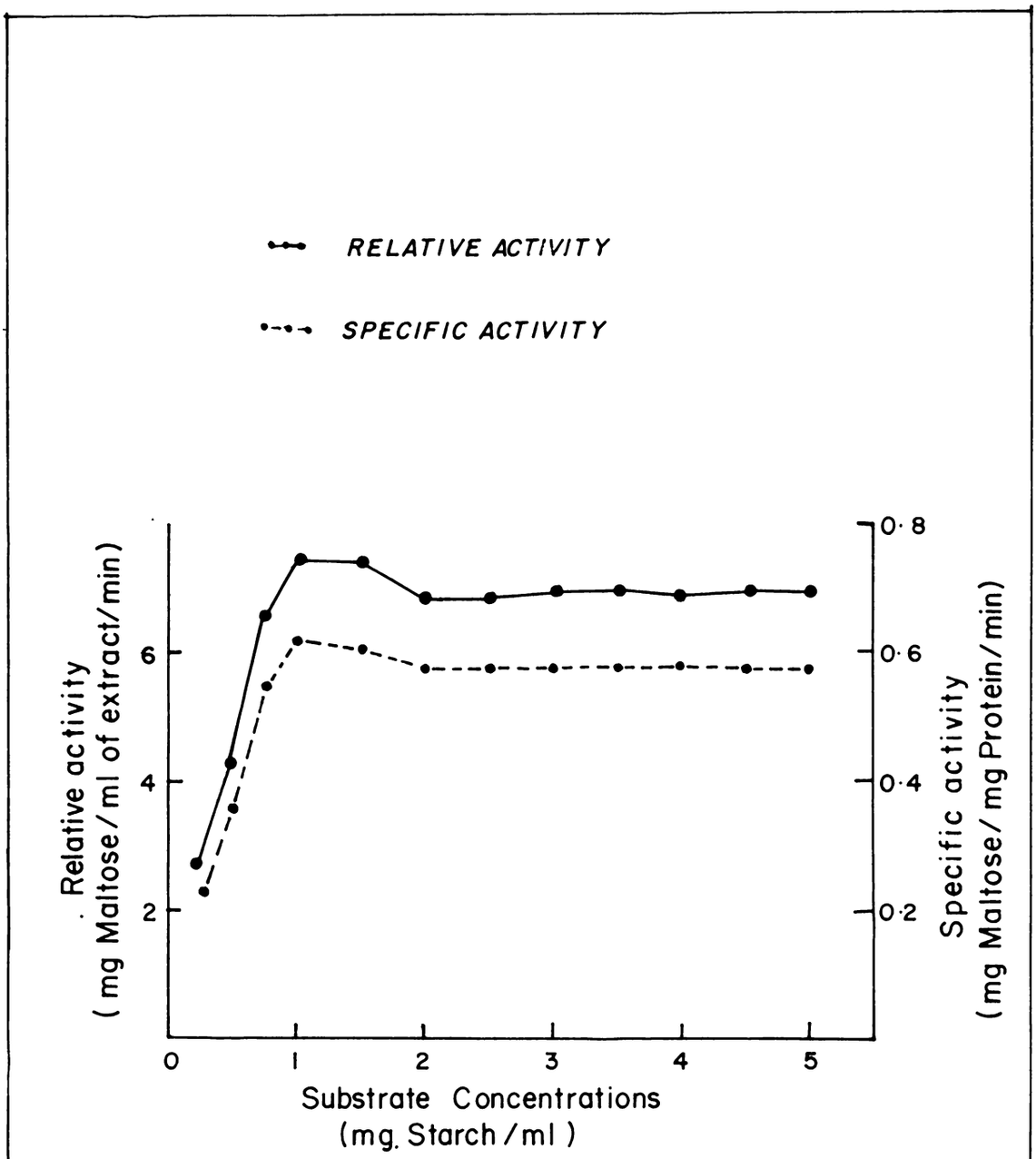

RELATIVE ACTIVITY
SPECIFIC ACTIVITY
Relative activity
(mg Maltose/ ml of extract/min)
Specific activity
(mg Maltose/ mg Protein/min)
0·8
0·6
0·4
0·2
2
4
6
0
1
2
3
4
5
Substrate Concentrations
(mg. Starch / ml)

FIG. 18

Fig. 19. Line weaver-Burke plot for Michaelis constant (Km value) of hepatopancreatic amylase.

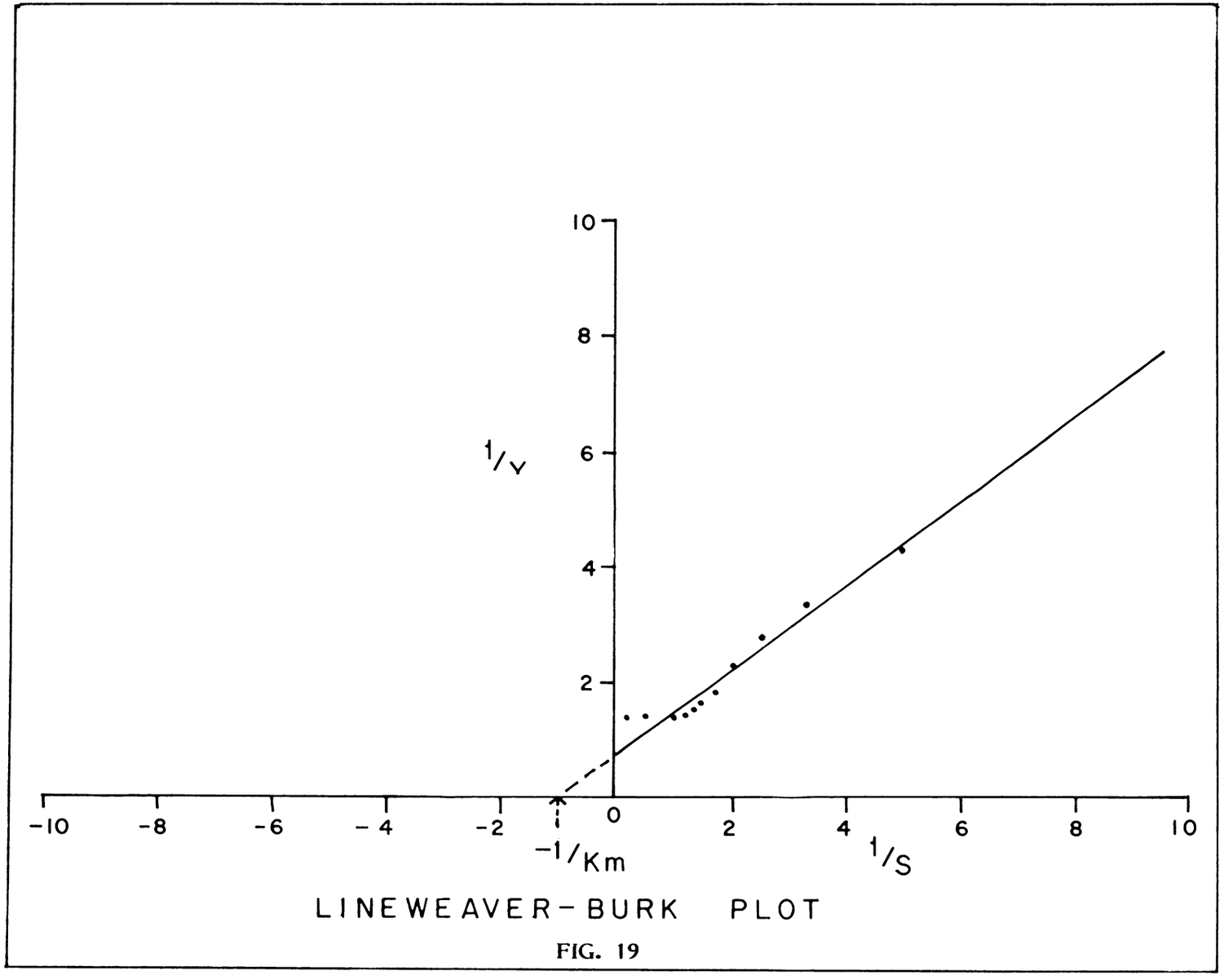


FIG. 19

Fig. 20. Effects of chlorides of aluminium, ammonium, antimony, barium, calcium and cobalt at 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 molar concentrations on amylase activity.

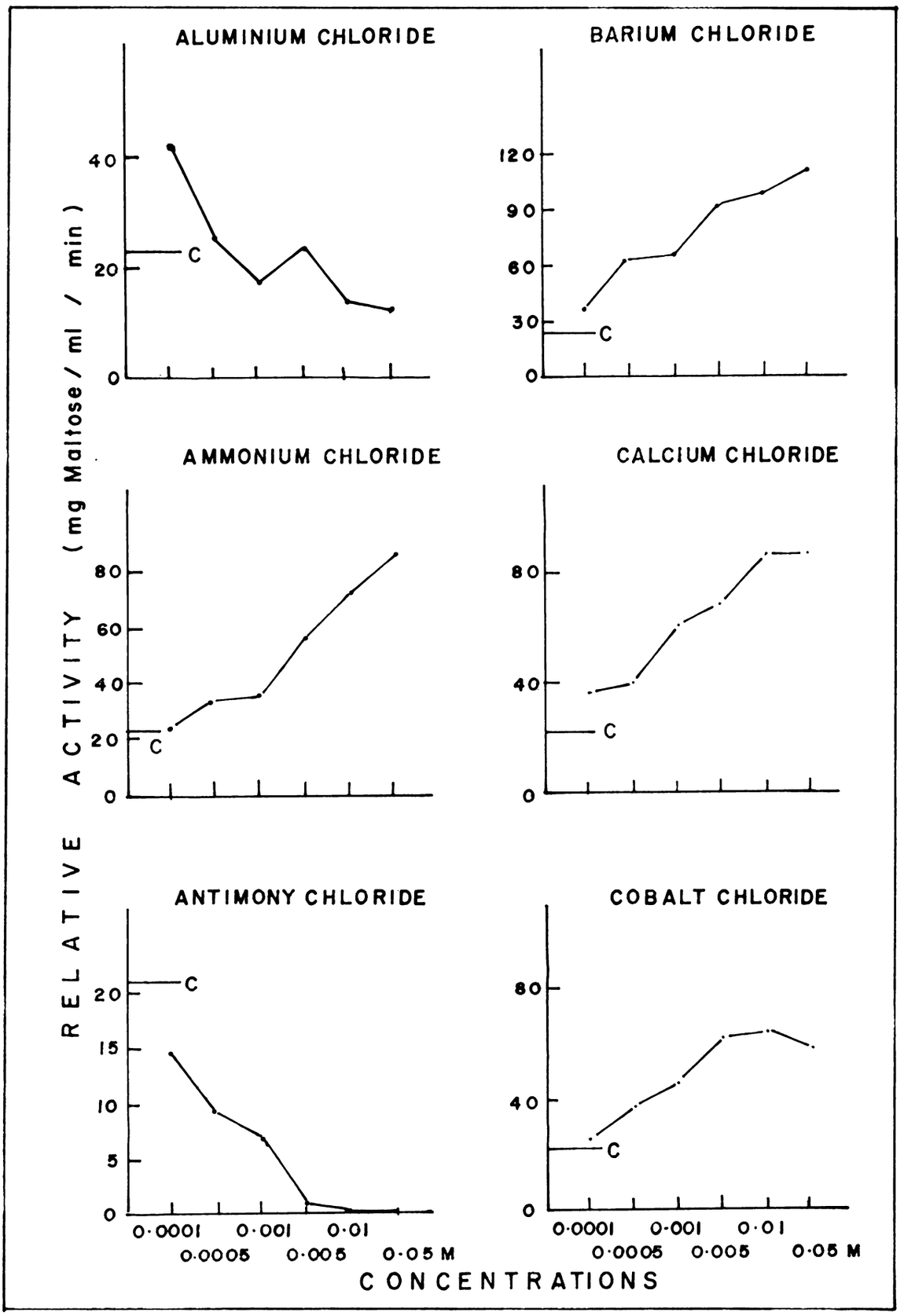

ALUMINIUM CHLORIDE
BARIUM CHLORIDE
AMMONIUM CHLORIDE
CALCIUM CHLORIDE
ANTIMONY CHLORIDE
COBALT CHLORIDE
RELATIVE ACTIVITY (mg Maltose / ml / min)
CONCENTRATIONS
C
0
20
40
30
60
90
120
80
15
10
5
0·0001
0·0005
0·001
0·005
0·01
0·05 M

FIG. 20

Fig.21. Effects of chlorides of copper, iron, magnesium, manganese, mercuric and nickel at 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 molar concentrations on amylse activity .

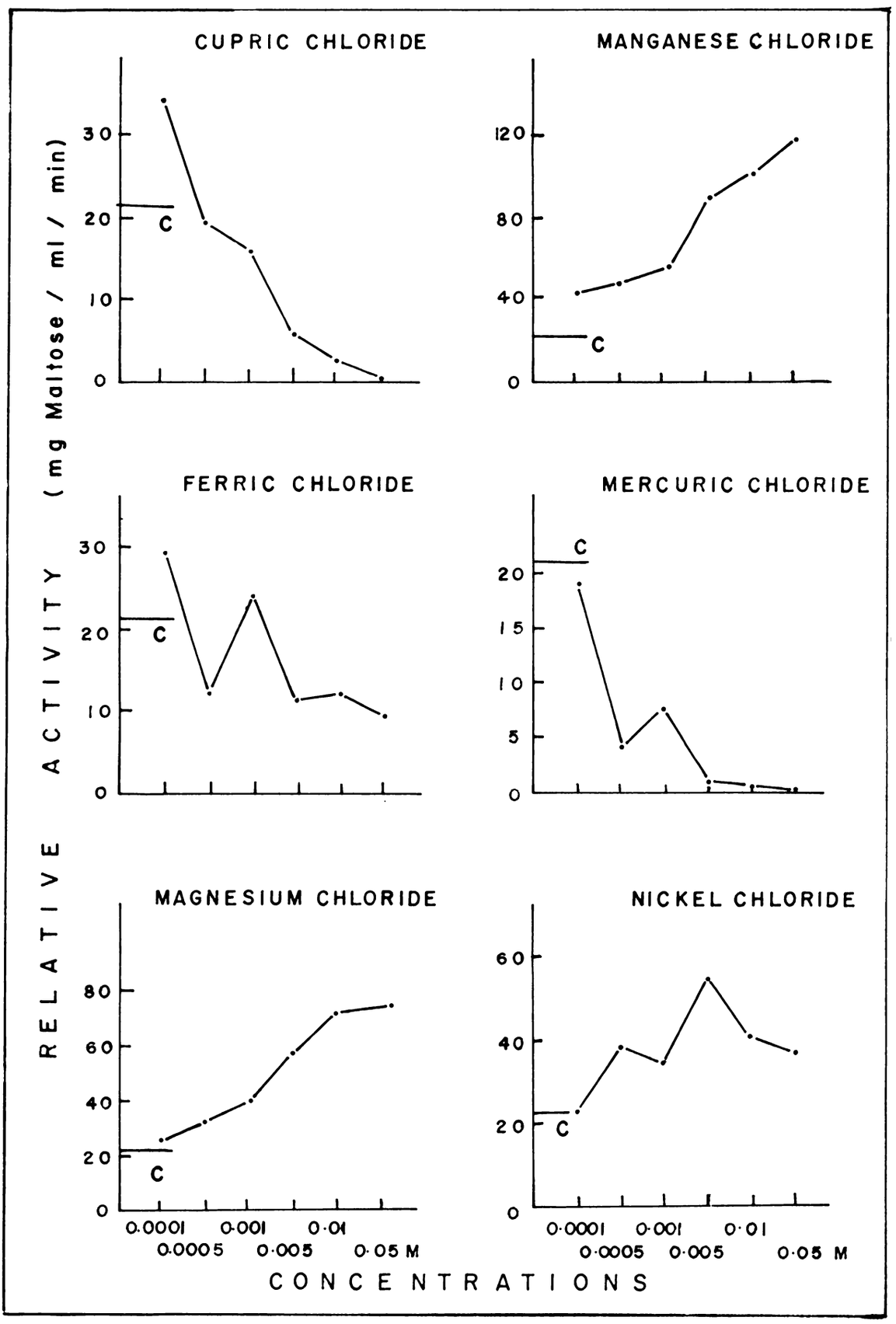

CUPRIC CHLORIDE
MANGANESE CHLORIDE
FERRIC CHLORIDE
MERCURIC CHLORIDE
MAGNESIUM CHLORIDE
NICKEL CHLORIDE
RELATIVE ACTIVITY (mg Maltose / ml / min)
C
0
10
20
30
40
60
80
120
5
15
0.0001
0.0005
0.001
0.005
0.01
0.05 M
CONCENTRATIONS

FIG. 21

Fig.22. Effects of chlorides of potassium, sodium, strontium at 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 molar concentrations on amylase activity (six replicates). Effects of amino acids like alanine, aspartic acid, aminobutyric acids, at six different concentration, on amylase activity.

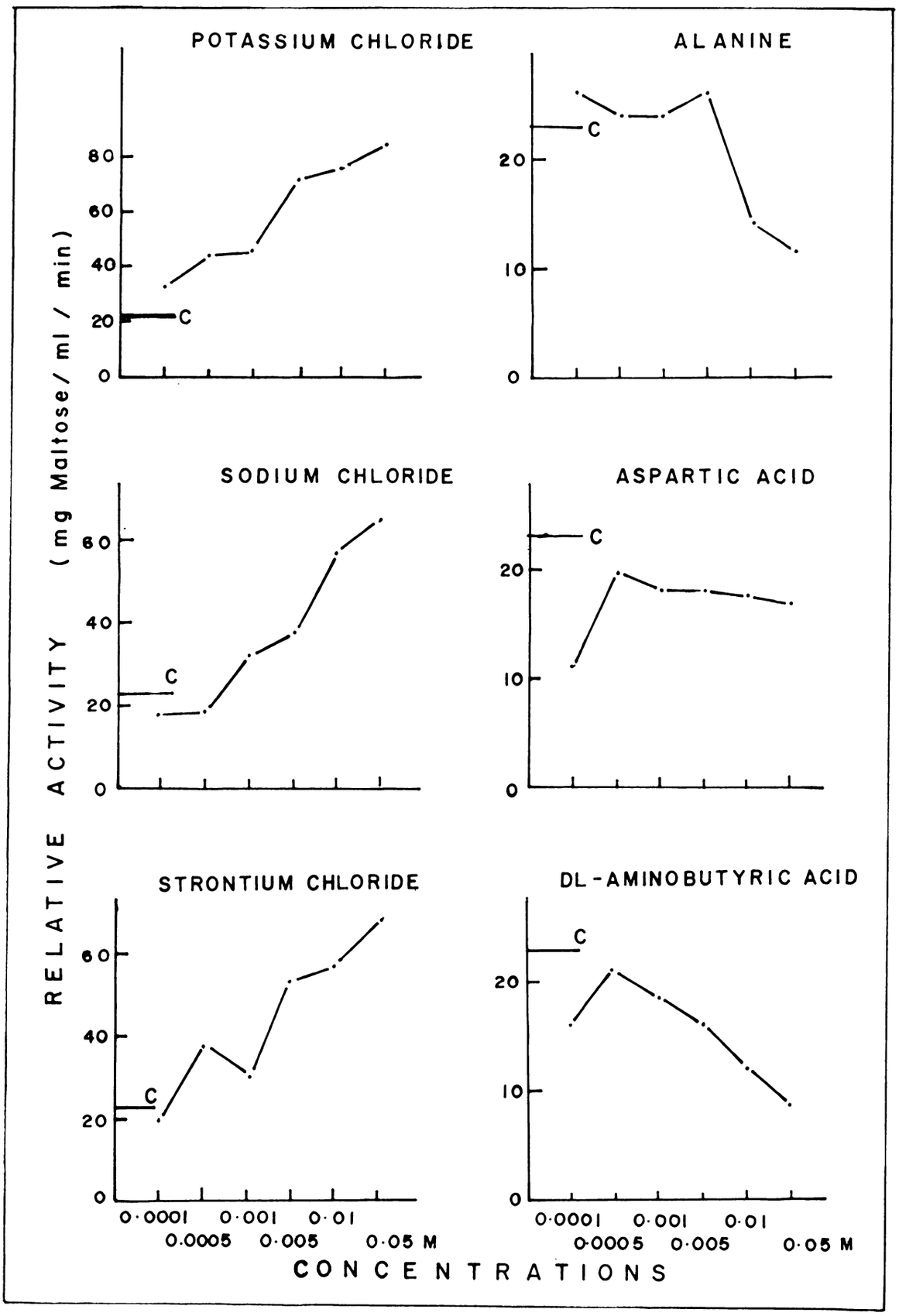

POTASSIUM CHLORIDE
ALANINE
SODIUM CHLORIDE
ASPARTIC ACID
STRONTIUM CHLORIDE
DL-AMINOBUTYRIC ACID
RELATIVE ACTIVITY (mg Maltose/ml/min)
C
0
20
40
60
80
10
0·0001
0·0005
0·001
0·005
0·01
0·05 M
CONCENTRATIONS

FIG. 22

Fig.23. Effects of arginine, cysteine, dihydroxy phenyl alanine, phenyl alanine, glutamic acid glycine and histidine at 0.0001, 0.0005,0.001, 0.005, 0.01, 0.05 molar concentrations on amylase activity (six replicates).

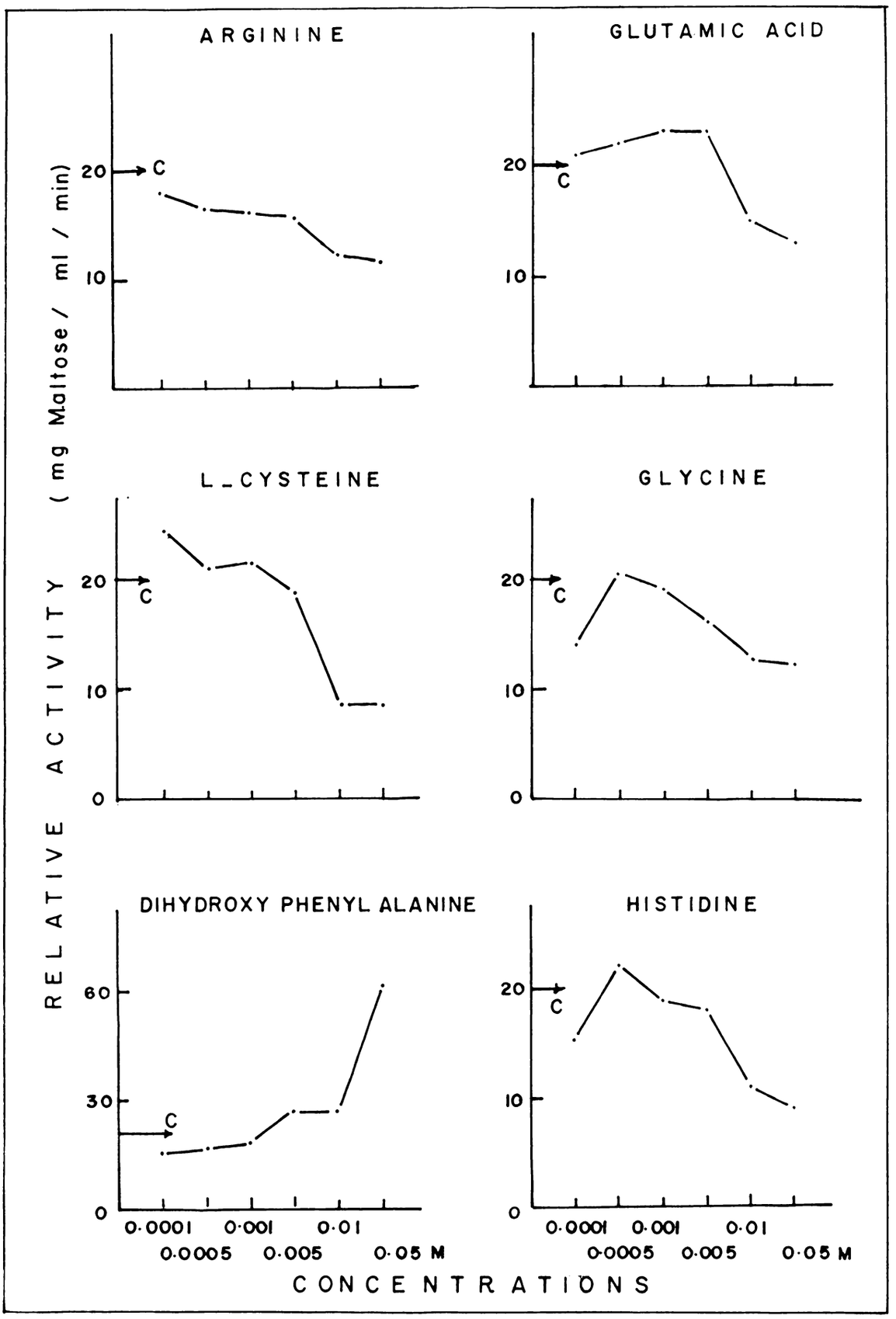

ARGININE
GLUTAMIC ACID
L_CYSTEINE
GLYCINE
DIHYDROXY PHENYL ALANINE
HISTIDINE
C
20
10
0
60
30
RELATIVE ACTIVITY (mg Maltose / ml / min)
0·0001
0·0005
0·001
0·005
0·01
0·05 M
CONCENTRATIONS

FIG. 23

Fig.24. Effects of isoleucine, leucine, lysine, methionine,norleucine and ornithine at 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 molar concentrations on amylase activity.

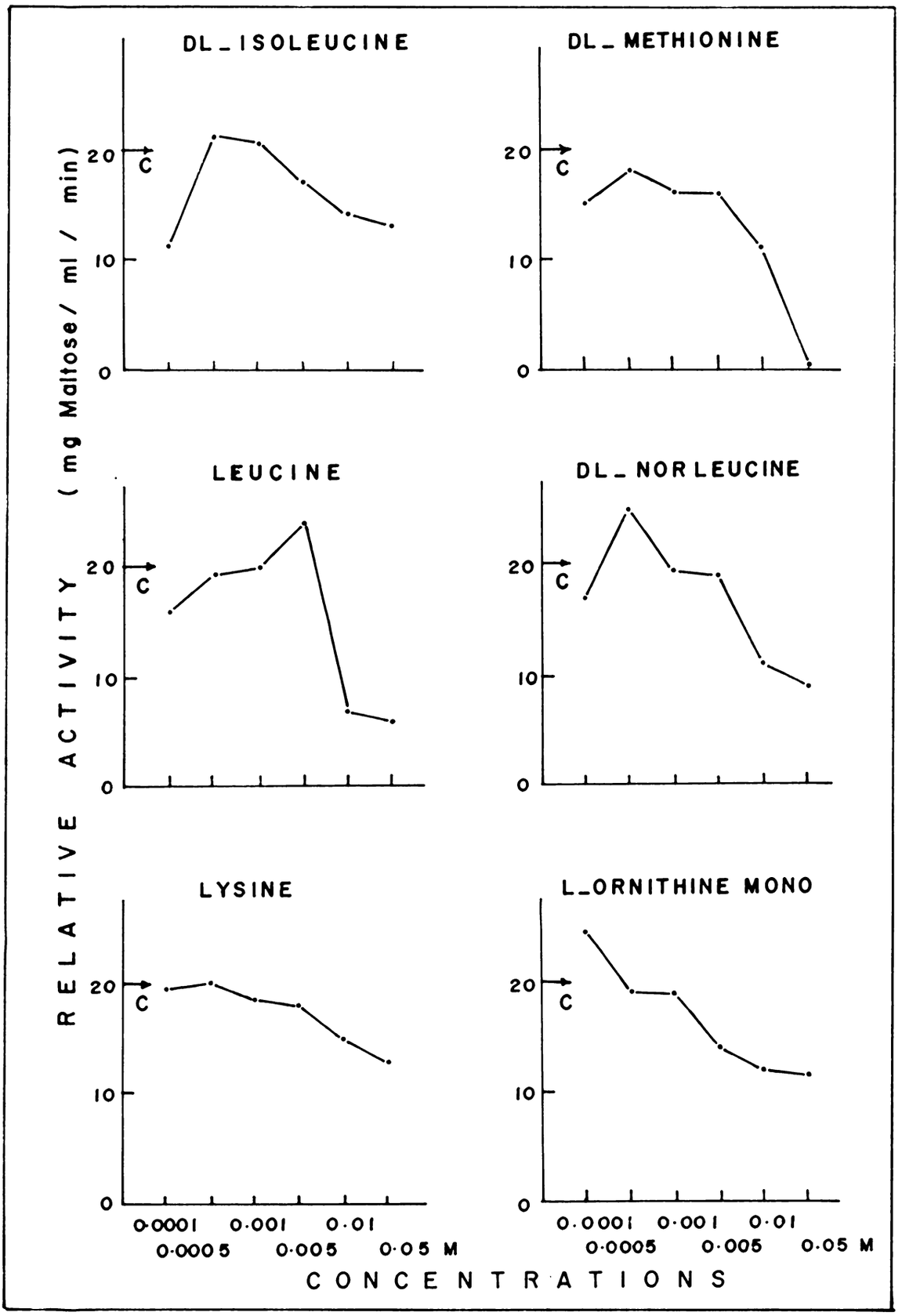

DL_ISOLEUCINE
DL_METHIONINE
LEUCINE
DL_NORLEUCINE
LYSINE
L_ORNITHINE MONO
RELATIVE ACTIVITY (mg Maltose/ml / min)
C
20
10
0
0·0001
0·0005
0·001
0·005
0·01
0·05 M
CONCENTRATIONS

FIG. 24

Fig. 25. Effects of proline, serine, threonine, tryptophan, tyrosine & valine at 0.0001, 0.0005, 0.001, 0.005, 0.01 & 0.05 molar concentrations on amylase activity (6 replicates)

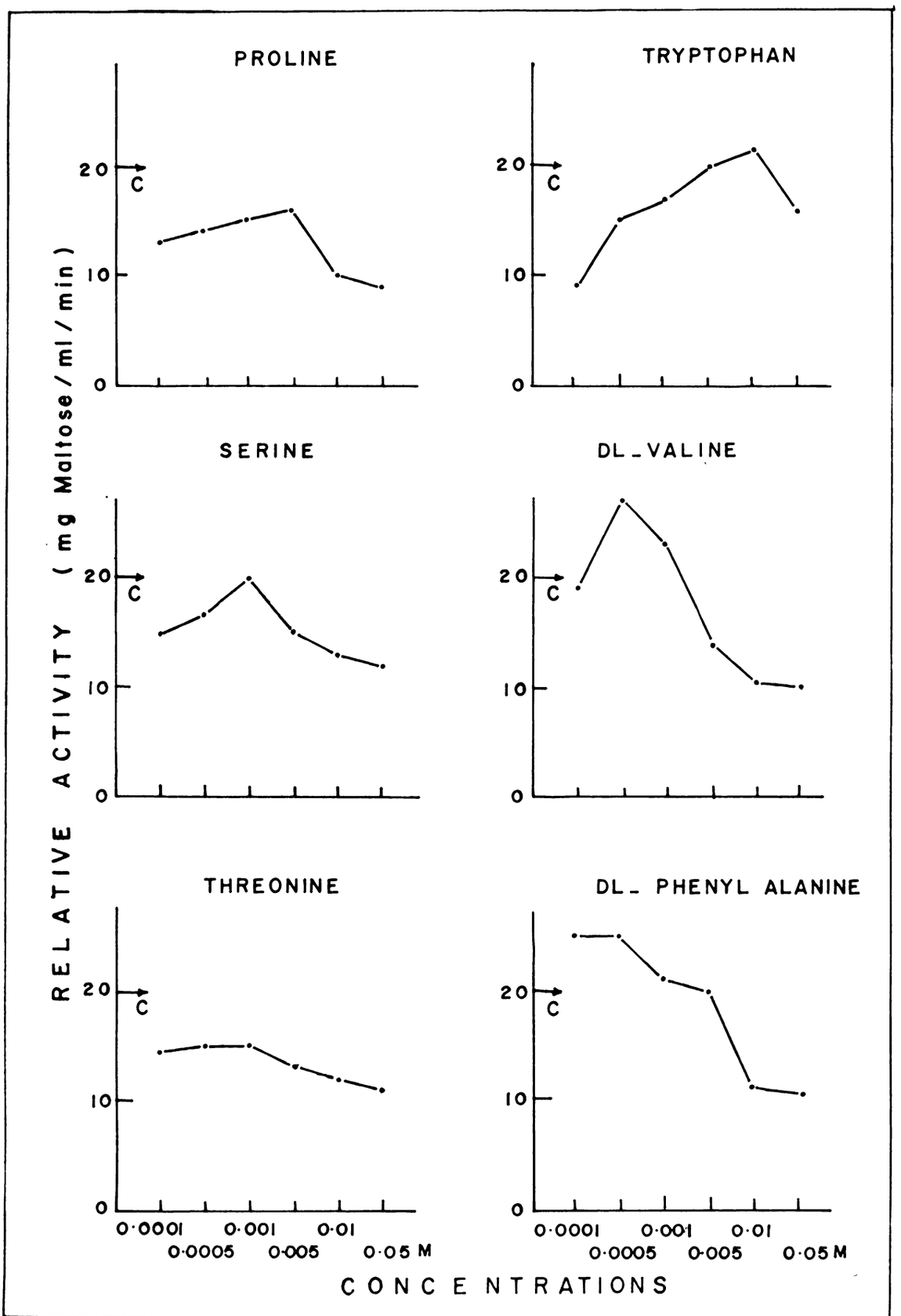

PROLINE
TRYPTOPHAN
SERINE
DL_VALINE
THREONINE
DL_ PHENYL ALANINE
RELATIVE ACTIVITY (mg Maltose / ml / min)
20
10
0
C
0·0001
0·0005
0·001
0·005
0·01
0·05 M
CONCENTRATIONS

FIG. 25

Fig. 26. Effects of selected vitamins; ascorbic acid, choline, inositol, calciferol, nicotinic acid and riboflavin at 0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 molar concentrations on amylase activity (six replicates)

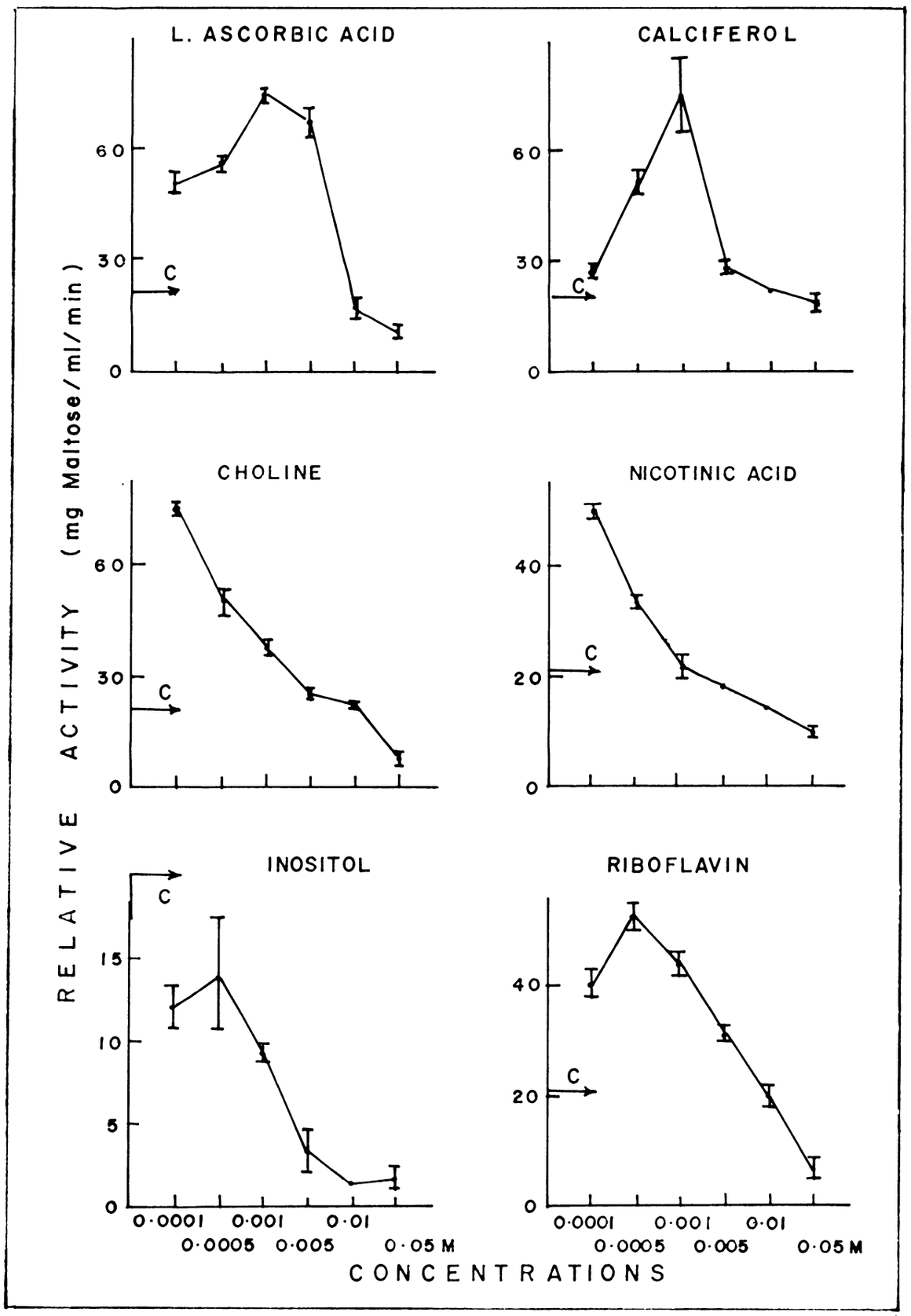
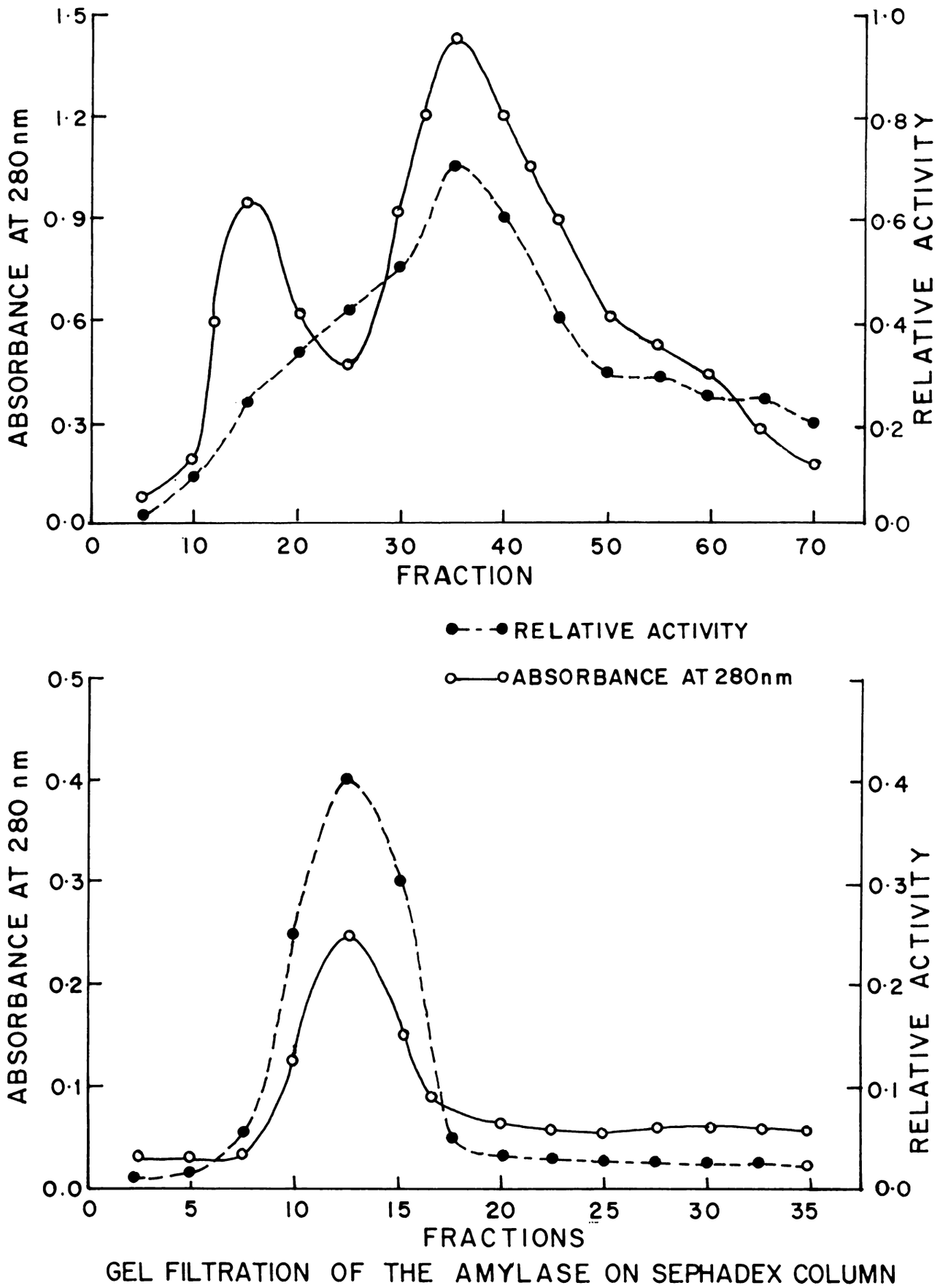

L. ASCORBIC ACID
CALCIFEROL
CHOLINE
NICOTINIC ACID
INOSITOL
RIBOFLAVIN
RELATIVE ACTIVITY (mg Maltose/ml/min)
C
0
30
60
20
40
5
10
15
0·0001
0·0005
0·001
0·005
0·01
0·05 M
CONCENTRATIONS

FIG. 26

Fig. 27. Gel filtration of the crude hepatopancreatic enzyme extract of Penaeus indicus on sephadex G-25 (40 x 1.5cm). Active fractions between 30-40 were pooled and dialyzed in 0.02M phosphate buffer (pH 7.0). The concentrated solution from the above step was applied to sephadex G.100 gel column (90 x 2.5cm) equilibrated with phosphate buffer pH 7.0. Elution was performed with the same buffer. A flow rate of 12 ml/hr was maintained and the active fractions 10-15 were collected at one hour interval.

●--●--●- relative activity of amylase

0—0—0 absorbance at 280 nm



GEL FILTRATION OF THE AMYLASE ON SEPHADEX COLUMN

FIG. 27

Fig. 28. Disc electrophoretic patterns of preparations of amylase enzyme partially purified from the hepatopancreatic extract of Penaeus indicus

Disc electrophoresis was performed according to Ornstein and Davis (1964) on 7% acrylamids gel at pH 9.5. A current of 3 mA per tube was applied. Protein bands in the gel were stained with Kenacid blue (0.25% in methanol, water and acetic acid mixture (5:5:1) ratio.

A: Electrophoretic pattern of general proteins in the crude hepatopancreatic extract.

B: Protein bands (1, 2, 3, 4) in the active fractions obtained from Sephadex G-100 column (10-15 fractions).

C: Amylase band pattern representing the isoenzymes obtained in the purified fraction.

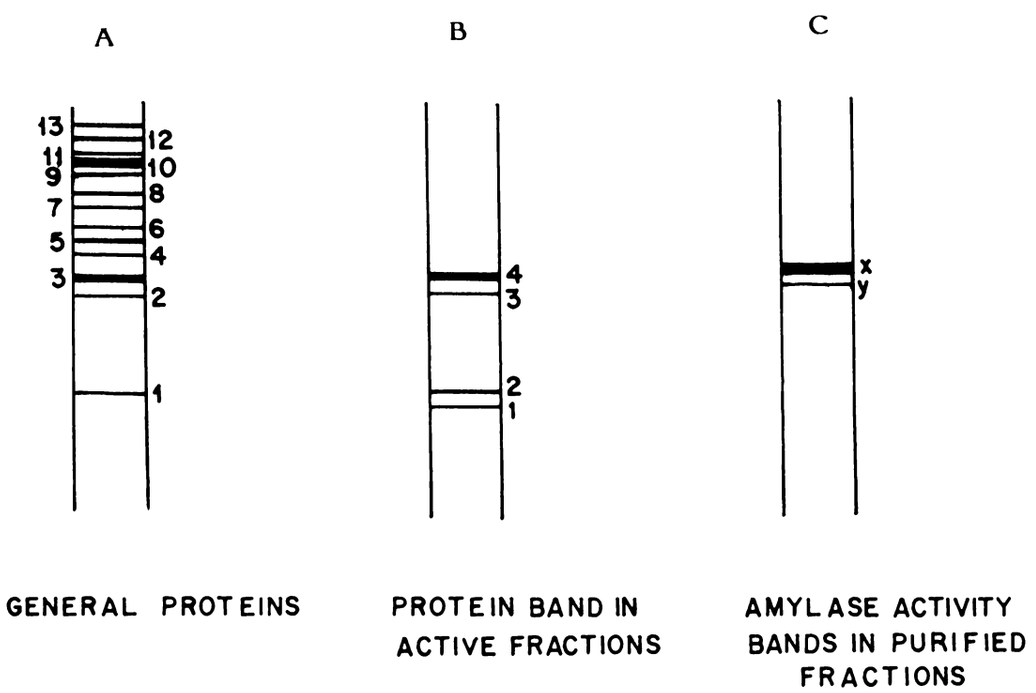

A
B
C
13
12
11
10
9
8
7
6
5
4
3
2
1
4
3
2
1
x
y
GENERAL PROTEINS
PROTEIN BAND IN ACTIVE FRACTIONS
AMYLASE ACTIVITY BANDS IN PURIFIED FRACTIONS
ELECTROPHOROGRAMS

FIG. 28

SUMMARY

SUMMARY

In intensive prawn culture operations, one of the foremost requirements is the availability of proper formulated practical feeds for the different stages of the prawn. These practical feeds should contain adequate levels of the essential nutrients to provide maximum growth, survival and best conversion efficiencies. The efficacy of a diet not only depends on its nutrient composition and nutrient balance, but also on the effective utilization of the dietary nutrients. The digestive enzymes play the crucial role of catalysing the hydrolytic reactions splitting the macromolecules into simple absorbable molecules. The activity of these biocatalysts is regulated by so many biological, chemical and physical factors and thus any shift from the optimum conditions necessary for these enzymes may affect their activity, thereby correspondingly modify the digestibility of the nutrients supplied to the animals. Therefore, information on the digestive enzymes and their preferential conditions of activity would greatly help in rationally adjusting the quality and quantity of feed supplied to prawns during the different stages in their life cycle. Besides, knowledge of the structure of the alimentary tract and its associated organs would help in understanding the mechanism of food ingestion, digestion and absorption. With this in view the present study has been carried out in the Indian white prawn, *Penaeus indicus*.

In *Penaeus indicus* the alimentary canal shows three distinct regions, the foregut, midgut and the hindgut. The mouth is situated on

the ventral side. The oesophagus is a very short tube running vertically dorsalwards and leads in to the stomach. The cardiac and pyloric divisions of the stomach serve different functions. The midgut is a long and narrow tube with slender walls, runs almost the entire length of the abdomen. The hindgut is a very short tube with a thick muscular wall connecting the midgut and the anus. The brownish-red coloured massive gland hepatopancreas occupies a major portion of the cephalothoracic cavity and is made up of loosely arranged tubules. Tubules in cross-section show the presence of a connective tissue layer called tunica propria, a basement membrane, and an epithelial layer. Light microscope studies have shown that the epithelial cells at the blind end of the tubules are embryonic cells. In the middle region are found the absorptive and secretory cells, and at the distal end of the tubule secretory cells are widely located. This corresponds to the distribution of various cell-types in many other crustaceans.

The histochemical studies of the epithelial layers of the alimentary canal have shown that the various biochemical components are distributed and localised according to the function of the gut layers. Thus the secretory and absorptive nature of the different regions has been elucidated in the present investigation by histochemical tests.

The ultrastructural study of the hepatopancreas has revealed that there are four types of cells namely, the E-cell, F-cell, B-cell, and R-cell. These cells show differences in their cellular and cytoplasmic make-up and are similar to the cell types reported in other Natantians. The histochemical properties of the different cell-types have also been characterized.

Preliminary experiments conducted with the tissue extracts of stomach, hepatopancreas, midgut and hindgut revealed that hepatopancreas is the main organ associated with the secretion of different types of enzymes. Other regions showed rather weak activity or total absence of certain enzymes. From the study of the properties of the hepatopancreatic extract of *Penaeus indicus*, it appears that carbohydrates, proteins and fats are all effectively digested. The existence of separate enzymes in the digestive gland conforms to that of many other crustaceans.

Digestive enzyme complement in the three different size groups showed a varied picture. Amylolytic and proteolytic activity increased steadily from post-larval, stage to the adult sta e, with the proteolytic enzymes showing a sharp increase. Nevertheless lipolytic activity, shot up only from the juvenile to adult stage.

Starvation induced a significant influence on the activity of amylase and proteases. During starvation, a decline in the amylase and protease activity was observed with apparently slight change in the pattern of lipolytic activity.

Starch levels in the diets significantly influence the activity of amylase. A linear increase in amylase was observed with the starch level in the diet upto the optimum at 20% starch in the diet and beyond that level activity was greatly reduced. Proteases and lipases were not significantly affected by the dietary levels of starch. Further studies on digestibility of carbohydrates, proteins and lipids are suggested for a better understanding of the physiological abilities of the organisms.

Eyestalk ablation studies revealed that neuroendocrines play a definite role in the secretion of the enzymes. Eyestalk removal resulted in enhanced starch hydrolysis when, compared to the intact animals. Amylase activity was found to be 15% more in the bilaterally ablated prawns and 10% more activity was recorded in the unilaterally ablated ones than the control. Proteases showed significantly decreased activity in the ablated prawns than in the normal prawns. Whereas lipases showed slightly increased activity in the ablated ones than in the normal ones. Thus increased food uptake and enhanced metabolic rate reflect upon the acceleration of the digestive enzyme mechanism.

Detailed studies on the characterization of α-amylase indicated the important role of this enzyme in carbohydrate digestion. From the data obtained it has been confirmed that this particular enzyme has a pH optimum of 7.0, and is fairly active between the pH range 6.0-8.0. The optimum temperature recorded is 40°C, and is fairly active between 30°C-50°C. It remains stable in cod and frozen conditions for more than a week. It may be indicated that the diet formulated for culture operations should have the same pH for normal activity of the enzyme.

α-Amylase shows maximum activity at an incubation period of 50 minutes, when active hydrolysis of starch took place. The optimum substrate concentration determined is 1 mg starch/ml and the Michaelis-Menten (Km) value is 1 mg/ml. The optimum enzyme concentration was found to be between 0.7 mg hepatopancreatic protein/ml of enzyme extract.

From the data obtained it is clear that of the 15 metallic chlorides tested, chlorides of ammonium, barium, cobalt, magnesium, manganese potassium, sodium and strontium activate the amylase system at the highest concentration of 0.05 M. Whereas aluminium, cupric, ferric and nickel resulted in maximum activation at lower molar concentrations. However, antimony and mercuric chloride caused inhibition at all concentrations and thus acted as enzyme inactivators in the reaction system.

Of the twenty one amino acids tested, alanine, aspartic acid, amino butyric acid, arginine, cysteine, dihydroxy phenyl alanine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, nor-leucine, ornithine, phenylalanine, proline, serine, threonine, tryptophan and valine activated the amylase at lower concentrations. Whereas at higher concentrations, their activity was significantly reduced. Nevertheless, amino butyric acid, cysteine, histidine, leucine, methionine, norleucine, proline, valine showed more than fifty percent inhibition at 0.05 M concentrations.

Among the vitamins ascorbic acid, choline, calciferol, nicotinic acid and riboflavin activated the amylase at lower molar concentrations and more than 40% inhibition occurred at higher concentrations of these solutions. Inositol, even at the lowest concentration of 0.0001 M concentration resulted in the inhibition of the amylase.

In the present study, purification of α -amylase enzyme by gel filtration chromatography has been undertaken and the purity of the enzyme established by polyacrylamide gel electrophoresis. The electrophoretic pattern has indicated the presence of two closely associated bands. Thus the presence of isoenzymes of α -amylase has been reported for the first time in this tropical prawn.

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