STUDIES ON THE REPRODUCTIVE ENDOCRINOLOGY OF THE PENAEID PRAWN, *PENAEUS INDICUS* H. MILNE EDWARDS

THESIS SUBMITTED
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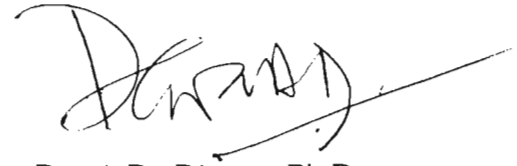
1989

C E R T I F I C A T E

This is to certify that the thesis entitled 'Studies on the reproductive endocrinology of the penaeid prawn, Penaeus indicus H. Milne Edwards' is the bonafide record of the research work carried out by Shri K. Sunilkumar Mohamed, under my guidance and supervision at the Centre of Advanced Studies in Mariculture, Central Marine Fisheries Research Institute (CMFRI), and that no part thereof has been presented for the award of any other degree.



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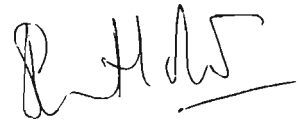
D E C L A R A T I O N

I hereby declare that this thesis entitled 'Studies on the reproductive endocrinology of the penaeid prawn, Penaeus indicus H. Milne Edwards' has not previously formed the basis of the award of any degree, diploma, associateship or other similar titles or recognition.



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PREFACE

Penaeid prawns constitute about 13% of the total marine fish landings in India and earn a yearly foreign exchange to the tune of Rs.4500 million by means of export. In recent times, due to the shortfall in prawn production from the sea, the development of coastal aquaculture of prawns has gained tremendous importance. Among the penaeid prawns identified for culture in our country, *Penaeus indicus* is considered as the most ideal candidate species due to its short life-span, fast growth and excellent survival rates.

Of late, the aquaculture technology of penaeid prawns has developed remarkably and at present these prawns are cultured on a large scale in captivity. One of the most important aspect of any culture operation is the controlled breeding of the animal to obtain seeds, and this is done in some prawn hatcheries by using the technique of eyestalk ablation or extirpation. This technique which is now used worldwide, leads to the accelaration of gonadal maturation. However, there is very little information with regard to the physiological changes occurring in the neuroendocrine system

of the animal, although neuroendocrine factors are known to control the process of gonadal maturation.

An essential prerequisite for intensive aquaculture is the information on basic physiology of the candidate species. Such basic information is, however, scanty among dendrobranchiate crustaceans in general and penaeids in particular. Hence, the present investigation on the reproductive endocrinology of P. indicus was taken up to understand the fundamentals of the various aspects of reproductive physiology in relation to the changes occurring in the neuroendocrine system of the animal and to gain knowledge about the mechanism involved in the technique of eyestalk ablation for induced maturation. The following aspects of study were covered during the present work.

1. Investigations on the process of oogenesis and spermatogenesis and the mechanism of spermatophore,formation in the vas deferens.

2. Detailed investigation on the identification of neuroendocrine centres and distribution and mapping of the neurosecretory cells and their chemical characteristics.

3. Studies on the neuroendocrine control of reproduction based on eyestalk extirpaton, eyestalk extract injection and central nervous system extract injection experiments and histological analysis to correlate the architectural changes in the neurosecretory cells during the different phases of maturation.

4. Investigations on the biochemical changes during the process of gonadal development in different tissues like ovary, hepatopancreas, muscle and haemolymph.

The results of these studies are embodied in the thesis which consists of five chapters with a preface in the beginning. The first chapter is a general introduction to the topic of research and it highlights the present status of the knowledge on the subject. Subsequent chapters have each an introduction, followed by materials and methods, results and discussion. Introduction of each chapter highlights the importance of the particular aspect of study covering a review of the literature. Under materials and methods, the materials and the techniques used, and in results the data obtained are presented. Each chapter is concluded with a discussion followed by a short summary.

The second chapter deals with the process and events leading to the maturation of the ovary and testis in the animal. Investigations on the process of oogenesis and spermatogenesis were made by using histological, histochemical and electron microscopic methods. Ovarian maturation was classified into five developmental stages based on the changes in colour, diameter of oocytes and nuclei and gonadosomatic index. Further the sequential manner of yolk deposition in the oocytes was classified into five vitellogenic phases. Spermatogenesis was found to involve the progressive reduction in cytoplasmic volume and condensation of chromatin matter leading to the formation of a unistellate sperm. Attempts have been made to study the structure of the sperm using electron microscopy. The structure of the spermatophore and the process of its formation in the vas deferens is also described.

The third chapter is on the identification of the neurosecretory cells in the different neuroendocrine centres like eyestalk, supraesophageal, tritocerebral, subesophageal, thoracic and abdominal ganglia. Four morphological types of neurosecretory cells were identified using histological methods and their positions in each ganglion was

mapped. Electron microscopic techniques revealed the size and structure of the elementary neurosecretory granule. The chemical nature of the neurosecretory material was investigated using histochemical methods. Eyestalk ablation and central nervous system extract injection experiments revealed the existence of factors responsible for the control of gonadal growth in these ganglionic masses.

In the fourth chapter investigations on the involvement of metabolites like protein, carbohydrate, lipid, cholesterol, moisture, carotenoids, RNA and DNA during the process of gonadal maturation in female prawns are described. These biochemical components were estimated in the haemolymph, ovary, hepatopancreas and muscle of the animals and profound changes were observed in their concentration during the different maturity stages, especially in the ovary, hepatopancreas and haemolymph.

Fifth chapter is on the general summary and conclusions drawn from the present study. References used in the investigation are also given at the end.

CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Reproductive biology is central to biological science and an understanding of it is vital for proper animal resource management. The technologies of controlled reproduction, induction of spawning, sex reversal, artificial fertilization, sterilization and preservation of gametes are increasingly applied in acquculture to obtain quality fish seed, better stock and increased yields. With the advent of such practices in intensive aquaculture of useful marine invertebrates such as prawns,crabs and molluscs, not only a basic knowledge of the reproductive processes of these animals but also an experimental approach to exogenous and endogenous factors controlling their reproduction has become in imperative need.

Among crustaceans, the main area in which the experimental studies have been concentrated is the endocrine manipulation of reproductive activities such as gonad maturation and spawning. Gametogenesis, the central event of the reproductive cycle, has been shown to be under the control of endocrine processes (Highnam and Hill, 1977). Since reproductive adaptations are in accordance with the lifestyle of the organisms concerned, it is difficult to generalize on the reproductive processes and their endocrine control among crustacean groups. Therefore, a separate study

on the sparsely investigated and commerically important genus of Penaeus appears warranted.

The Indian white prawn Penaeus indicus is a tropical penaeid having a wide range of distribution in the Indo-Pacific region and has been identified as the most favourable candidate species for intensive aquaculture in India. A synopsis of biological information on the species has been given by Mohamed (1968) and the reproductive biology of the species in relation to fisheries has been studied by Rao (1968). However, studies on the reproductive physiology of P. indicus are very few and preliminary in nature (Gopalakrishnan, 1953; Subrahmanyam, 1965; Read and Caulton, 1980 and Sasikala and Subramoniam, 1987). Hence in the present study attempts have been made to investigate the detailed process of gametogenesis, the neuroendocrine system and its control on reproduction and the changes in bio-chemical components in [redacted] maturation in [redacted]

Although the reproductive and breeding cycles of many crustacean groups have been studied by several workers (Ryan,1967; Rahman, 1967; Haefner, 1976; Zucker,1978), similar studies on penaeids are very few and limited to gross morphological descriptions and light microscopic observations of the gonad (King,1948; Rao,1968; Pillay and


Penaeus indicus

Nair,1973; Thomas,1974; Joshi et al.,1982; Yano,1988). So far the process of vitellogenesis in penaeid prawns has not been a subject of detailed investigation and consequently little is known about the cytological changes which occur during oogenesis and yolk deposition in penaeids. The modern technique of electron miscroscopy has helped in unveiling many of the cytological processes occurring during oogenesis in crabs and lobsters (Beams and Kessel, 1963; Hinsch and Cone, 1969). The present state of the knowledge on these aspects of reproductive biology has been reviewed by Norrevang (1968), Adiyodi and Subramoniam (1983) and Adiyodi (1985) and they have noted the paucity of information regarding gametogenesis in dendrobranchiate group of crustaceans in general.

Such studies on the male reproductive organs and process of spermatogenesis are even less and therefore information on the reproductive physiology of male penaeids is scanty. The current developments in the field of spermatogenesis and the morphology of spermatozoa in Crustacea has been reviewed by Pochon-Masson (1983) and Adiyodi (1985). The processes of spermatogenesis have been documented on the basis of observations on gross morphology and histology of the testis in lobsters (Aiken and Waddy, 1980), crabs (Ryan, 1967; Kon and Honma, 1967) and prawns (King, 1948; Subrahmanyam, 1965; Lu et al., 1973). In most crustaceans sperm transfer during

copulation is accomplished by making use of a specialized sperm packet called spermatophore. The spermatophores of crustaceans has been studied by only very few investigators (Shaikhmahmud and Tembe, 1958; Malek and Bawab, 1974; Uma and Subramoniam, 1979; Kooda-Cisco and Talbot, 1982; Radha and Subramoniam, 1967) inspite of its diversity in structure and morphology among different species reflecting their reproductive adaptability. Detailed studies on spermatogenesis and morphology of spermatophores among penaeids are very few.

Much of our understanding of hormonal control of moulting and reproduction and their temporal inter-relationships is based on the experimental work done on crabs and lobsters (Passano, 1960; Adiyodi and Adiyodi, 1970; Nagabhushanam et al., 1980; Quackenbush, 1986; Fingerman, 1987). The extent towhich this information is applicable to a sparsely investigated group like penaeids remains unclear. Experimental studies involving the removal of the eyestalks and implantation of neurosecretory organs such as brain and thoracic ganglia have revealed that reproduction in Crustacea, in general, is under the control of the active principles contained in the neurosecretory cells in these ganglia. The morphological details of the various neuroendocrine areas like eyestalk, brain and thoracic ganglia has been investigated by many workers in different groups of

crustaceans. However, such studies on penaeids are few and preliminary in nature (Dall, 1965; Nakamura, 1974; Nanda and Ghosh, 1985; Nagabhushanam et al., 1986). Although eyestalk ablation is known to stimulate vitellogenesis (Panouse, 1943), only few attempts have been made to correlate the changes in the neuroendocrine organs with those in the gonad (Joshi, 1989). Likewise, gonadal growth has been reported to be stimulated by the implantation or injection of the brain and thoracic ganglia, eventhough the changes taking place in the neurosecretory cells are not precisely known.

Crustacean endocrinology has benefited greatly from the recent advances in peptide chemistry and immunoassay techniques. Moreover immunocytochemical analysis of neuro-endocrine centres has demonstrated several new classes of peptide neurohormones considered responsible for regulation and control of various metabolic processes (Quackenbush, 1986). Attempts for purification of these crustacean peptides controlling reproduction are being made with a view to understand the interrelations of the various classes of crustacean hormones (Fingerman, 1987).

Investigations on changes in biochemical constituents relative to the gonadal cycle of invertebrates has been pioneered by Giese and his coworkers. However, studies on biochemical changes in the various body components of

crustaceans related to gonadal maturation are few compared to such studies on moulting. The variations in the concentration of major organic reserves of the yolk like protein, lipid and carbohydrates in relation to different stages of maturity in various groups of crustaceans have been fairly well studied (Barnes et al., 1963; Pillay and Nair, 1973; Diwan and Nagabhushanam, 1974; Varadarajan and Subramoniam, 1982). But the variations in metabolites like cholesterol, carotenoids, DNA and RNA in relation to the process of reproduction has not been a subject of detailed studies and consequently very little is known about the involvement of these metabolites in the reproductive process.

The hepatopancreas or the midgut gland has been identified as the major organic reserve organ in crustaceans (Chang and O'Connor, 1983), nevertheless the qualitative and quantitative changes of metabolites taking place in it has not been investigated in detail, particularly with reference to reproduction. Similarly the role of the haemolymph in transporting metabolites from the storage site to the gonads has not been fully delineated although it is well known that haemolymph is responsible for this vital function. Complete studies on changes in biochemical constituents related to the maturity stages are significant both from the standpoint of basic studies and for enabling a more detailed account of

the translocation of various materials during development and growth of the gonads.

CHAPTER II

HISTOMORPHOLOGY OF THE REPRODUCTIVE SYSTEM AND THE PROCESS OF GAMETOGENESIS

INTRODUCTION

In order to study the regulation and control of gonadal activity in malacostracan Crustacea, it is of prime importance to know the morphology and physiological aspects of the gonads (Charniaux - Cotton, 1985). It was remarked in as late as 1980 by Aiken and Waddy that the state of the art regarding reporductive physiology of crustaceans is primitive by comparison with what is known about moulting physiology, and the science involved is just now emerging from the descriptive phase. The morphology of the reproductive organs of crustaceans has been fairly well investigated, but as might be expected of such a diverse group, there are major differences in the morphology to be resolved.

Knowledge about the cytological changes which occur during gametogenesis in Arthropoda in general, and insects in particular is extensive. However, such studies are very few in Crustacea and among the penaeids they are by any limits scanty. Owing to the quantum of energy involved and the dramatic fashion in which the ovary matures, the female reproductive system and the process of oogenesis has been very often a subject of more detailed investigations as compared to the male reproductive system.

The breeding cycles of crustaceans has been studied extensively by many workers in different parts of the world (Knudsen, 1964; Chandran, 1968; Kon and Honma, 1970; Pillay and Nair, 1970; Diwan and Nagabhushanam, 1974; Haefner, 1977; Jones, 1977; Zucker, 1978; Subramonian, 1979; Aiken and Waddy, 1980.). Comparitively studies on the breeding cycles of penaeid prawns are very few. Moreover most investigations on penaeid prawns have been restricted to the description of spawning seasons and spawning areas of several commerically important species(Cummins, 1961; Rao, 1968; Thomas, 1974; Penn, 1980; Kennedy and Barber, 1981 and Motoh, 1981). Spawning seasons of penaeid prawns have been studied either by using changes in the percentage of ripe females in catch (Cummins, 1961 and Rao, 1968) or by using changes in the gonad index (Thomas, 1974 and O'Connor, 1979).

The anatomy of the female reproductive organs in various species of crustaceans has been studied by few investigators. Using histological techniques the anatomy of the reproductive organs has been described in crabs (Ryan, 1967; Chandran, 1968; Pillay and Nair, 1970 and Diwan and Nagabhushanam, 1974) and in prawns (Hudinaga, 1942; King,

1948; Sheikhmahmud and Tembe, 1958; Cummins, 1961 and Subrahmanyam, 1965). However the process of vitellogensis in prawns has not been subjected to detailed investigations.

According to Papathanassiou and King (1984) the process of vitellogenesis or the development of the oocytes in Crustacea could be divided into several distinct stages. First, a stage of germ cell division and formation of the oogonia, followed by a cytoplasmic growth stage, a stage of yolk deposition (vitellogenesis) and finally a post-vitellogenic stage during which egg membranes are laid. Among penaeids, oogenesis has been partially studied by light and electron microscopy by few investigatiors like King (1948) in Penaeus setiferus, Cummins (1961) in P. duorarum, Duronslet et al. (1975) in P.aztecus and P. setiferus, Kennedy et al.(1977) in Sicyonia breviorstris, Anderson et al. (1984) in S.ingentis and Yano (1988) in P.japonicus. So far Indian penaeids have not been investigated in detail on these lines. The reproductive organs and process of gametogenesis in Parapenaeopsis stylifera from Bombay has been described briefly by Shaikhmahmud and Tembe (1958) and in detail by Joshiet al,(1982). Using ova diameter measurements and cytological methods Subrahmanyam (1965) described the reproductive cycle of P. indicus from Madras.

The process of oogenesis in crustaceans in general has been reviewed by Raven (1961), Norrevang (1968) and Adiyodi and Subramoniam (1983). The origin of the yolk in crustaceans has been investigated using biochemical and electron microscopic methods by Beams and Kessel (1963), Hinsch and Cone (1969),Lui and O'Connor (1977),Varadarajan and Subramoniam (1982), Papathanassiou and King (1984) and Charniaux-Cotton (1985). The chemical nature of the crustacean yolk has been studied by using cytochemical methods in Artemia salina (Fautrez-Firlefyn,1957),Palaemon adspersus (Bonina, 1974), Orchestia gammarella (Zerbib, 1976), Balanus amphitrite (Fyhn and Costlow, 1977) and Clibanarius clibanarius (Varadarajan and Subramoniam, 1980). These studies revealed the complexity of yolk as well as the manner in which yolk substances are synthesized in crustacean oocytes. Surprisingly there are no comparable studies on penaeids covering these aspects and therefore knowledge on the nature and orgin of penaeid yolk is lacking.

In crustaceans the male reproductive biology and its physiology is not as well understood as that of the female (Aiken and Waddy, 1980). Unlike the females, maturity in

males is particularly difficult to define, as there are no striking external criteria for male maturity. Studies on the anatomy of male reproductive organs are few (Fasten, 1918; Cronin, 1947; Ryan, 1967), however, based on the histology of the testis many researchers have described the spermatogonial, spermatocyte and spermatid stages in the formation of the decapod sperm (Mathews, 1951; King, 1948; Pillai, 1960; Kon and Honma, 1970; Amato and Payen, 1978 and Chow et al., 1982). The present state of the knowledge on spermatogenesis and sperm function in Crustacea has been reviewed by Pochon-Masson (1983) and Adiyodi (1985) with emphasis on spermatozoan morphology. The nature of the organic reserves in the sperm cells has also been studied by histochemical methods by Fautrez-Firlefyn and Fautrez (1955) in Artemia and Barnes (1962) in the cirripede Balanus balanus.

Most crustaceans transfer sperm from male to the female during copulation via a specialized sperm packet known as spermatophore. Spermatophores are comprised of sperms surrounded by layers of acellular secretions produced by the vas deferens (Aiken and Waddy, 1980). The process of their formation in the vas deferens has been investigated by Malek and Bawab (1974 a and b) in Penaeus kerathurus, Subramoniam (1984) in Albunea symnista and Radha and Subramoniam (1985) in the spiny lobster Panulirus homarus.

The structure and chemical composition of various crustacean spermatophores has been studied by Uma and Subramoniam (1979) in Scylla serrata, Kooda-Cisco and Talbot (1982) in Homarus americanus, Subramoniam (1984) in A.symnista and Emerita asiatica, and Radha and Subramoniam (1987) in P. homarus. Among penaeids the structure and chemical composition of spermatophores has not been studied in detail and hence their male reproductive biology and physiology are poorly understood.

The present investigation has been carried out to understand the process and events leading to maturation of the ovary and testis in the Indian white prawn Penaeus indicus. The study includes the classification of the ovarian maturity stages based on its colour, gonadosomatic index, oocyte diameter and morphological changes in the oocyte. Further the process of oogenesis has been investigated using light and electron microscopic techniques. A histochemical study of the ovary has also been carried out to determine the sequence in which yolk substances are synthesized or sequestered in the oocytes and also to elicit the nature of the penaeid yolk material.

The process of spermatogenesis and the development of

the spermatophore has been studied in detail using light and electron microscopic methods. In addition a brief histochemical study on the testis was also made to understand the nature of the organic reserves in the sperm cells.

MATERIALS AND METHODS

1. Collection of animals

Live adult prawns ranging in size from 120-170 mm total length in various maturity stages were used in the present study. They were collected from the sea off Cochin using the Central Marine Fisheries Research Institute's trawler boats, M.V.CADALMIN I and IX. After hauling the net, prawns of the species P. indicus were immediately seperated from the catch and kept in a fibre glass tank with seawater. They were then carefully transported to the laboratory in transportation bags. Later they were segregated according to sex and maturity stages, and kept in plastic pools containing seawater with a sub-gravel biological filter working on an air-lift mechanism. All prawns were fed daily with frozen clam meat, until their utilization.

2. Dissection and fixation

Prawns were dissected to study the internal morphology of the reproductive system and the process of oogenesis was studied in detail by subjecting the gonads to histological and histochemical investigations. Dissections were generally carried out in crustacean saline (Smith and Ratcliffe, 1980) between 1600 and 1800 h to avoid the interference of circadian changes in the maturation process if any.

Female prawns in different maturity stages, identified on the basis of external features like gonadal size and colour were weighed separately and then their cephalothorax and abdomen was cut open to expose the ovary. Gross morphological observations of the ovary and oviducts were made with the aid of stereoscopic dissection miscroscope. The exposed ovary was then carefully excised out and weighed to the nearest milligram. The gonadosomatic index (GSI) was calculated as per the method described by Giese and Pearse (1974).

$$GSI = \frac{\text{Wet weight of ovary x 100}}{\text{Wet weight of animal}}$$

For conducting routine histological studies one of the middle lobes of the ovary was cut and fixed in Bouin's fluid for 24-48 h.

Male prawns in various maturity stages were dissected in the manner described above and the exposed testes and seminal ducts were examined under a dissection microscope to study their anatomical features. For general histological studies, to understand the process of spermatogenesis, parts of the testicular lobes, proximal vas deferens, mid vas deferens, distal vas deferens and terminal ampoule were fixed in Bouin's fluid for 24-48 h. In order to study the structural details of the sperm and spermatophore, spermatophore extrusion was induced by electrically stimulating the base of the fifth walking leg with a 12 V current delivered through the electrodes of an electrocautery apparatus. Upon stimulation, a single spermatophore was extruded from each terminal ampoule and they were joined together immediately in the surrounding seawater. Spermatophores thus extruded were subjected to gross morphological observations under a dissection microscope and then fixed in Bouin's fluid for detailed histological studies.

Histochemical tests for detecting protein and

carbohydrate materials were performed with ovaries and testes of different maturity stages and extruded spermatophores fixed in 10% neutral buffered formalin (Pearse, 1968) for a period of 24 - 48 h. For lipid histochemical studies freshly frozen tissues or those fixed in Baker's formol-calcium for a period of 24 h were used. Generally the volume of the fixative used was 20 times to that of the tissue.

3. Processing and sectioning

All tissues fixed in Bouin's fluid were washed overnight in running tapwater to remove the excess picric acid. Both Bouin's and formalin fixed tissues were dehydrated using an alcohol series (30% to 100% ethanol) and cleared in methyl benzoate. The tissues were further cold impregnated overnight with wax using benzene and wax shavings in a 1:1 ratio. Subsequently the solvent was evaporated by placing the tissues in an oven at 58°C. The tissues were then transferred through two changes of fresh molten wax (Paraffin wax with Ceresin, BDH, MP. 58-60°C). Tissue blocks were prepared by using paper boats or small glass troughs after proper orientation.

Serial sections of the blocks were cut at approximately 6 - 8 μ thickness using a rotary microtome. Sections were

affixed on clean glass slides using fresh Mayer's egg albumin and flattened by placing on a slide warmer with a drop of distilled water. Subsequently the water was drained off and the slides were allowed to dry. These slides were then used for histological and histochemical staining .

Frozen sections of fresh unfixed tissues or those fixed in Baker's formol-calcium were cut using an American Opticals Cryostat microtome at 8 - 10 μ thickness and used for lipid histochemical studies. Tissues were placed on the block holder with a few drops of crustacean saline and the copper heat extractor was placed on top of it to speed up the freezing process. Frozen blocks were cut at -20°C and the sections were then processed for staining with lipid histochemical techniques.

4. Staining

Routine staining for gross morphological observations was carried out using Harris or Heidenhains hematoxylin stain with 1% aqueous Eosin as the counterstain. Sections to be stained were first deparaffinized in two changes of xylene and then hydrated through a down series of ethanol grades. Sections were blued using tap water or 1% lithium

carbonate. Eosin stained sections were repeatedly washed in 95% alcohol to remove the excess eosin. Slides were further dehydrated in absolute alcohol and cleared in xylene and mounted with DPX or Canada Balsam of neutral pH. Mounted slides were examined under a monocular research microscope.

5. Micrometric measurements

Since the size increase of the oocytes is a function of oogenesis, the micrometric measurements of the oocytes in different stages of maturation were taken using an ocular micrometer calibrated with a stage micrometer. As oocytes strongly deviate from a spherical shape, the largest and smallest axes of the oocyte diameter was taken and the average used as the actual oocyte diameter. Spermatogonial and sperm cell diameters were also recorded.

6. Histochemical tests

Histochemical tests with appropriate controls were carried out to elucidate the chemical nature of the ova and yolk and also to find out the pattern of the yolk accumulation. Broadly, the histochemical tests were performed to detect proteins, nucleic acids, carbohydrates and lipids in the oocytes and sperm cells. The intensity of

the staining was recorded as '+++' (intense postive reaction), '++' (strong positive reaction), '+' (positive reaction), '±' (very mild postive reaction of doubtful nature) and '-' (negative reaction).

6.1. **Tests for detection of proteins**

For the detection of general proteins and different amino acid and groups the following detailed histochemical tests were carried out.

a) Mercuric Bromophenol Blue test for general proteins

(Mazia *et al*., 1953)

The mercuric ions of bromophenol blue solution react with acidic, sulphydryl and aromatic residues of protein to give a blue colour.

b) Aqueous Bromophenol blue test for basic proteins

(Pearse, 1968).

The acidic groups of bromophenol blue react with basic groups of protein to give a blue colour. Deamination with nitrous acid was carried out as control.

c) Toluidine blue test for acidic groups. (Pearse, 1968)

Toluidine blue which is a basic dye in aqueous medium reacts with acidic groups of protein to give a blue colour. Mild methylation using Methanol-HCl mixture was used to block acidic end groups.

d) Ninhydrin Schiff test for amino groups. (Pearse, 1968)

The stable tissue aldehydes produced in the course of oxidative deamination with ninhydrin are coloured with Schiff's reagent. The reaction was blocked by deamination before the stage of oxidative deamination.

e) Ferric ferricyanide method for -SH groups (Pearse, 1968)

This method depends on the reduction of a fresh solution of ferricyanide in acid solution at pH 2.4 by sulphydryl groups in the tissues. The resulting ferrocyanide combines with ferric ion (in ferric sulphate) to give an insoluble prussian blue precipitate. Mercaptide block with saturated mercuric chloride at 30°C for 1 hour was performed as control.

f) Performic acid Alcian blue test for S-S groups

(Adams and Sloper, 1956)

Performic acid which is an oxidising agent splits the disulphide groups into sulphonic and sulphinic acid aldehyde. The sulphonic and sulphinic acid combines with alcian blue to give a greenish blue colour. Slides treated directly with alcian blue served as the control.

g) Millon's test for tyrosine (Pearse, 1968)

Millon's reagent, a mixture of mercurous and mercuric nitrates and excess of nitric acid, reacts with phenolic groups (tyrosine) to give a reddish or yellowish red colour. Slides iodinated with Gram's iodine and 3% ammonia were used as the control.

h) DMAB - Nitrite method for tryptophan (Pearse, 1968)

The aldehyde component of p-dimethylamino-benzaldehyde (DMAB) solution reacts with tryptophanyl reactive sites and forms a blue coloured compound called B - carboline pigment. Sodium nitrate solution was used to intensify the colour of the pigment. Slides pretreated with 40% formalin were used as control.

i) Alkaline fast green test for histones (Pearse, 1968)

This is a standard method for staining the basic proteins of nucleus and cytoplasm. Basic proteins stain bright green at pH 8.0 to 8.1 due to their arginine and lysine content.

6.2 Tests for detection of nucleic acids

RNA and DNA were detected using the following histochemical tests.

a) Methyl Green-Pyronin test for DNA and RNA (Pearse, 1968)

This test depends on the affinity of the basic dyes to nucleic acids. Methyl green is specific for DNA and Pyronin with which it is combined is specific for RNA. Methyl green was repeatedly washed with choloroform to remove methyl violet with which it is contaminated. Pretreatment of slides with 10% perchloric acid at 4 C for 12 hours proved satisfactory as the control.

b) Fuelgen test for DNA (Pearse, 1968)

The hydrolysis of the purine-deoxypentose linkage of DNA with 1 N HCl at 60°C exposes the aldehyde groups which are then coloured purple to magenta by Schiff's reagent.

This test was very specific to nuclear chromatin. Control slides were pretreated with cold 10% perchloric acid for 12 hours.

6.3. **Tests for detection of Carbohydrates**

The following histochemical tests were performed to detect the different types of carbohydrate substances.

a) Periodic acid Schiff (PAS) reaction

(McManus and Mowry, 1960)

This reaction is based on the fact that aqueous periodic acid will oxidize 1,2 glycol groups in tissues, largely in materials that comprise of carbohydrates, to produce aldehydes that are coloured by Schiff's reagent. Stained sections were placed under running tap water briefly to enhance the colour.

A number of control slides were run to confirm the presence of the variety of PAS positive materials present. In order to detect the interference of free aldehydes in the reaction a slide was stained with Schiff alone without prior oxidation. Malt diastase digestion at 37°C was used to confirm the presence of glycogen. Absence of PAS positivity after acetylation in a acetic anhydride pyridine mixture for

12 hours served to confirm the presence of 1,2 glycol groups. Further deacetylation by treatment with alkali restored the PAS reactivity. Deamination prior to the PAS test removed the Schiff reactive aldehyde in tissue proteins. To affirm the presence of glycolipids slides were pretreated with chloroform/methanol and then subjected to the PAS test.

b) Best's Carmine test for glycogen (Pearse, 1968)

The active principle of the natural dye carmine is carminic acid which at a pH on the alkaline side of its isoelectric point (4.0 - 4.5) is negatively charged and behaves like an acid dye staining 1,2 glycol groups. Malt diastase treated slides were used as the control.

c) Critical electrolyte concentration method for Acid mucopolysaccharides (AMP) (Pearse, 1968)

Both sulphated and carboxylated mucins bind with alcian blue in situ in the presence of low concentrations of electrolytes (below 0.3 M magenesium chloride), whereas only sulphated mucosubstances do so with higher concentration (above 0.8 M).

d) Bracco-Curti's test for sulphated AMP (Pearse, 1968)

This method was specific for sulphated AMPs. Benzidine

in 2% boric acid reacts with the sulphate groups of AMP to form benzidine sulphate. Potassium dichromate oxidizes the benzidine sulphate to give a blue colour indicating the presence of sulphated AMP.

e) Alcian Blue - PAS method (McManus and Mowry, 1960)

Sections were stained first in 1% alcian blue and subsequently with the PAS technique. Acid mucopolysaccharides (AMP) are stained blue. Neutral mucopolysaccharides such as glycogen give a magenta colour.

6.4. Tests for the detection of Lipids

The following histochemical tests were carried out to detect the different classes of lipids.

a) Sudan black B test for lipids (Pearse, 1968)

Sudan black B being a diazo dye, and being slightly basic, combines with the acidic groups of compound lipids to give a black or blue colour. Delipidized control slides were prepared by treatment with a chloroform:methanol (1:1) mixture for 18 hours at 60°C.

b) Nile blue method for neutral and Acidic Lipids

(Pearse, 1968)

Nile blue sulphate method stained neutral fats red, acidic lipids dark blue and cytoplasm pale blue. Chloroform methanol extraction was carried out for use as the control.

c) Baker's Acid Hematein test for phospholipids

(Pearse, 1968)

This test depended on the treatment of the blocks of tissue with formaldehyde-dichromate mixtures by which phospholipids are oxidized and combined with chromium. Frozen sections were cut and the presence of combined chromium was subsequently shown by the black colour produced by combination with acid hematoxylin. Hot pyridine extracted material was used as control.

d) Oil Red O method for neutral lipids (Pearse, 1968)

Oil Red O in isopropanol stains the neutral lipids red without forming dye precipitates. Chloroform methanol extraction was performed to delipidize control slides.

e) UV Schiff reaction for Unsaturated lipids (Pearse,1968)

Frozen sections were subjected to long and short wave

(254 nm) irradiation for 3-4 hours and then treated with Schiff's reagent. Control slides were not exposed to UV. The difference in staining intensity demonstrated the number of double bonds saturated by oxidation.

7. Photomicrography

Photomicrographs of the histological and histochemical preparations of the ovary and testis were taken using an Olympus Universal Research microscope (Vannox model PM 10 AD) equipped with an automatic exposure system. Black and white 35mm film (ORWO, 100 ASA) and colour 35mm (KODAK COLOUR, 100 ASA) film were used.

8. Ultrastructural studies

Ultrastructural investigations on the ovary and spermatophore using an electron microscope was carried out at the Electron microscope laboratory of the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore.

The gonadal tissues in different maturity stages and spermatophore were fixed in ice cold (4°C) 4% gluteraldehyde solution prepared in Millonig's phosphate buffer at pH 7.2

for 12 hours. Fixed tissues were then washed in cacodylate buffer (pH 7.4) for 15 minutes and post-fixed in 1% osmium tetroxide for about 1 h until fully osmicated. Tissues were again washed in the buffer (cacodylate) and then dehydrated through an ascending series of ethanol grades. Tissues were cleared in two changes of propylene oxide (15 min. each) and then kept overnight in a rotator with a mixture of propylene oxide and Araldite. Further the tissues were transferred to pure Araldite for 6 hours and then embedded in plastic moulds. Polymerization was done in an oven at 55°C for 48 hours.

Polymerized blocks were trimmed for ultramicrotomy and silver to grey ultrathin (50 to 150 nm) sections were cut in a LKB Ultrotome IV with glass knives. Ultrathin sections were collected on uncoated copper grids (300 mesh) and post-stained in uranyl acetate (Watson, 1968) and lead citrate (Reynolds, 1963). After drying, the grids were examined in a JEOL-JEM 100 CX II Electron microscope at 80 KV. Areas of interest were photographed using AGFA plate film.

RESULTS

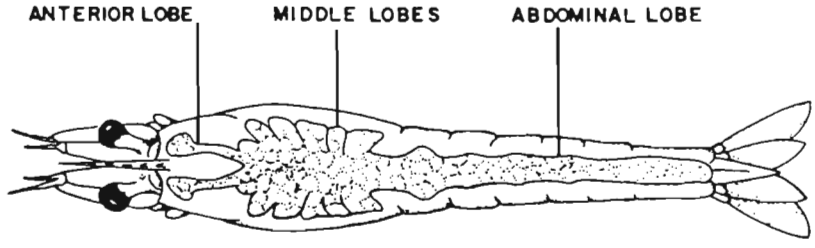
1. Female reproductive system and gross morphology of the ovary

The female reproductive system of P. indicus consisted of a dorsally located paired ovary, paired oviducts and a single ventral thelycum. The thelycum which functions as a receptacle for mature sperm was found to be present externally on the ventral surface between the fourth and fifth coxapoditżes. Morphological observations revealed that the ovaries are bilaterally symmetrical, partly fused along the inner margin and extend the entire length of the animal from the base of the rostrum to the telson (Fig. 1A). Three main lobes were observed on each half of the ovary. The anterior lobe occupied the cephalic region, the middle lobe with 6 to 7 finger-like lateral lobules filled the cardiac and thoracic regions and the posterior lobe extended the entire length of the abdomen upto the telson (Fig. 1A).

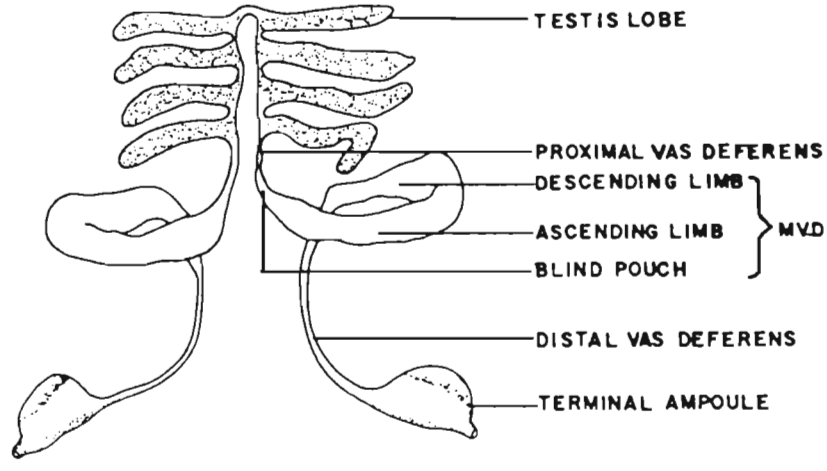
The oviducts were observed to be short, narrow tubes leaving the ovaries at the tips of the 6th pair of lateral lobes and opens through the genital pores at the coxae of the third pair of pereiopods.

FIG .1

A . FEMALE REPRODUCTIVE SYSTEM



B . MALE REPRODUCTIVE SYSTEM



In P. indicus, as in other crustaceans, ovarian maturation was accompanied by distinctive colour changes as well as increase in volume of the ovary and diameter of the oocytes and nuclei. based on these factors five different stages (Stage I to V) of maturity were recognized, viz., immature, early maturing, late maturing, mature and spent (Table I A).

In the immature stage (Stage I), the ovaries were found to be thin, smooth, translucent and unpigmented. Moreover they were not visible externally through the dorsal exoskeleton. Early maturing (Stage II) ovaries were observed to be pale cream in colour. During this stage the anterior and middle lobes of the ovary increase in size and the ovarian surface appeared as granular. The ovary loses its transparency and now appeared as opaque, and was visible externally through the dorsal exoskeleton upon careful examination. Development of a light green colour and increased granular appearance were the prominent characteristics observed in late maturing ovaries (Stage III). All the lobes were seen to increase in dimension and fill up the body cavity. During the mature or ripe stage (Stage IV) the ovarian lobes are so distended that they fill all the available space and appeared to crowd the other organs of the body cavity. The ovary develops a dark green

Table - I A : Classification of maturity stages in female *P. indicus* based on colour of the ovary, GSI and oocyte diameter.

OVARY STAGE	COLOUR AND APPEARANCE OF OVARY	GONADO-SOMATIC INDEX ± S.D	OOCYTE DIAMETER ± S.D	NUCLEUS DIAMETER ±S.D
STAGE I	Translucent	0.435 ± 0.272		
Immature ovary	Smooth			
Primary oogonial cells			7.203 ± 2.225	5.500 ± 0.823
Secondary oogonial cells			21.475 ± 4.396	13.206 ± 1.883
Primary oocytes			39.130 ± 10.045	20.935 ± 6.349
STAGE II				
Early maturing ovary	Pale cream Smooth-granular	2.207 ± 0.619	125.832 ± 22.695	44.299 ± 7.627
STAGE III				
Late maturing ovary	Light green granular	4.527 ± 1.499	187.364 ± 19.476	60.016 ± 7.025
STAGE IV				
Mature/Ripe ovary	Dark green granular	7.312 ± 1.059	241.345 ± 16.704	66.485 ± 3.693
STAGE V				
Spent/spent - recovering ovary	Pale cream granular-flaccid	1.042 ± 0.413	25.185 ± 4.951	17.619 ± 2.659

colour and was clearly visible through the dorsal exoskeleton. A characteristic of this stage was the triangular shape of the ovary as seen through the first abdominal somite caused by the distension of the abdominal lobe at that point. The spawned ovary designated as spent (stage V) had a loose and flaccid appearence and was creamy in colour. However it retained its opacity and was visible through the dorsal cuticle.

1.1. **Gonadosomatic index (GSI).**

During the present investigation, GSI values were determined for female prawns in all stages of maturity and the mean values obtained are presented in Table I A. In P. indicus the GSI values ranged from 0.435 ± 0.272 in immature females to 7.312 ± 1.059 in ripe females. The transition from stage I to stage II showed more than four fold increase in GSI values (from 0.435 to 2.207). Subsequently in stage III and IV the increase was only two fold (4.527 and 7.312). In spent animals the fall in values was remarkable and the values ranged between that of stage I and II (1.042 ± 0.413).

2. Oogenesis

2.1 Histology of the ovary

An examination of the histological sections of the ovary in different stages of maturity revealed the process of oogenesis and the graded manner in which oocytes developed and accumulated yolk. Based on the changes evident in the cytoplasm and nucleus of the oocytes, the complete development of the oocyte was classified into five different phases, viz., previtellogenic, early vitellogenic, late vitellogenic, vitellogenic and spent oocytes (Table I B). These oocyte phases correspond to the stages I to V described earlier based on morphological characters.

The ovary in P. indicus was found to be encompassed by a thin ovarian wall having two distinct layers (Fig. 4). In cross sections, the outer layer of pavement epithelium was moderately basophilic with hematoxylin and eosin and the inner layer was composed of loose connective tissue which was eosinophilic. Blood capillaries were also noticed in the peripheral regions of the ovary (fig. 4 and 5). The germinal zone or the germarium was observed in all sections as a thin band along the innermost layer of the ventral ovarian wall (fig. 2 and 6). This zone of proliferation was observed to

Table - I B : Distinctive changes in the cytological structure of the oocytes in relation to vitellogenesis in *P. indicus*.

OOCYTE PHASE	CYTOPLASM	NUCLEUS	NUCLEOLI	FOLLICLE CELLS
Stage I Previtellogenic	Homogenous, Basophilic due to the presence of RNA; mitochondria and rough endoplasmic reticulum present.	Vesicular with numerous nuclear pores	5 - 10 Electron dense; arranged along peripheral margin of the nucleus.	Tall with conspicous nuclei.
Stage II Early vitellogenic	Granular; Perinuclear halo of nucleolar RNA; mitochondria and R.ER present; vesicular primary yolk.	Vesicular with nuclear pores.	5-10; Arranged in a circular ring along the nuclear wall.	Decrease in cell height; hypertrophied nucleus and nucleoli.
Stage III Late vitellogenic	Granular and acidophilic; lipid globules and yolk platelets present. ER and mitochondria not apparent; oolemma with micropinocytotic vesicles.	Faintly stained	Nucleoli number reduced to 2-3.	Flattened around oocyte; R.ER and mitochondria present.
Stage IV Vitellogenic	Full of yolk platelets; disulphide rich cortical bodies with feathery matrix.	Faintly stained	Not apparent	Very flattened and stretched around oocyte.
Stage V Early vitellogenic (Spent)	Deeply basophilic with signs of pycnosis.	Vesicular	5-10; arranged along the perinuclear margin.	Intensely PAS positive and hypertrophied.

persist in all maturity stages. The smaller primary oogonial cells were found at the periphery of the germinal zone and the larger secondary oogonial cells were seen distributed towards the centre of the ovary in a graded manner (fig. 6). The primary oocytes are formed by a reduction division of the secondary oogonial cells.

The primary oogonial cells had a mean cell diameter of 7.2 $\pm$ 2.2 μ and possessed a large conspicuous nucleus with a diameter of 5.5 $\pm$ 0.8 μ. The cytoplasm was palely eosinophilic (fig. 3). The nucleus stained lightly with hematoxylin and the chromatin matter was distributed uniformally. The nucleoli number was high ranging from 10 to 20. The secondary oogonial cells are formed by the mitotic division of the primary oogonial cells. Their average diameter was found to be 21.4 $\pm$ 4.3 μ and the nuclear dimensions ranged between 12 to 15 μ (Table I). As the oogonial cells were crowded together in the narrow germinal zone, the cytoplasmic boundaries were ill defined and the follicle cells were apparently absent in this stage (fig. 3).

2.2. **Pre-vitellogenic oocytes**

Primary or previtellogenic oocytes are formed by the

meiotic division of the secondary oogonial cells and enter into meiotic prophase of the first reduction division. These oocytes had a strikingly basophilic cytoplasm with increased cytoplasmic volume (fig. 2). In a stage I ovary these oocytes occupied the entire lumen in clusters surrounded by follicle cells (fig. 4 and 5). Pre-vitellogenic oocytes measured 39.13 $\pm$ 10.04 μ in diameter and their vesicular nuclei had a diameter of 20.9 $\pm$ 6.3 μ (Table I A). The nuclei were observed to be lightly stained with hematoxylin and had within them 5-10 darkly stained nucleoli arranged along the peripheral margin (fig. 5). The deeply basophilic cytoplasm which was homogenous and agranular was surrounded by a layer of follicle cells. The follicle cells in this stage were found to be highly vacuolated and tall with a conspicuous nuclei (fig. 5).

Ultrastructurally, the nucleoli of the previtellogenic oocytes appeared as electron dense bodies along the inner periphery of the nuclear wall (fig. 22). Numerous gaps or nuclear pores were evident on the nuclear wall through which the electron dense nucleolar material was observed to diffuse into the cytoplasm (fig. 23). Cytoplasmic organelles like mitochondria and endoplasmic reticulam were also evident.

The histochemical studies on the immature ovary showed that the nucleus and nucleolii of oogonial cells were apparently devoid of any carbohydrate substances (Table 2). The thin rim of cytoplasm in the oogonial cells was found to be positive to Best's carmine test indicating the presence of moderate quantity of glycogen. Alcianophilia at low molar concentrations showed the presence of sulphated mucosubstances and this was confirmed with the Bracco-Curtis test. The previtellogenic oocytes had similar chemical constitution apart from the increased postivity to PAS (fig. 9) and Bracco-Curtis test. The follicle cells at this stage were strongly positive to the PAS test due to the presence of 1, 2 glycol groups.

The oogonial cells displayed intense positivity to proteins of both basic and acidic nature (Table 3). Amino and tyrosyl groups were present in moderate amounts. The ooplasm of previtellogenic oocytes were positive to tests for -S-S groups, tyrosine, tryptophan and amino groups. Both the ooplasm and nucleoli were seen to be intensely pyroninophilic indicating that the cytoplasmic basophilia observed with hematoxylin was due to the presence of large amounts of RNA (fig.19). Fuelgen positivity was found to be more in the oogonial nucleus than in the nucleus of primary oocytes.

Table - 2 : Histochemical responses of the ovary for Carbohydrates during Previtellogenic phase in *P. indicus*.

HISTOCHEMICAL TESTS	EPITHEHAL TISSUE	CONNECTIVE TISSUE	OOGONIAL CELLS			PRE-VITELLOGENIC OOCYTES			FOLLICLE CELLS
			CY	NU	NUL	CY	NU	NUL	
1. Schiff alone	-	-	-	-	-	±	-	-	-
2. Periodic Acid Schiff (PAS)	++	-	±	-	-	+	-	-	++
Deamination	+	-	±	-	-	-	-	-	++
Acetylation	-	-	-	-	-	±	-	-	-
Deacetylation	++	-	±	-	-	+	-	-	++
Delipidation	++	-	±	-	-	+	-	-	++
Diastase digestion	+	-	-	-	-	±	-	-	+
3. Best's Carmine test	+	-	+	-	-	+	-	+	++
Diastase digestion	-	-	-	-	-	+	-	-	±
4. Alcian Blue -C-E-C-Method									
Molar concentrations									
0.1 M	+	±	+	-	-	-	-	-	+
0.2 M	-	-	-	-	-	+	-	-	-
0.6 M	-	-	-	-	-	-	-	-	-
0.8 M	-	-	-	-	-	-	-	-	-
1.0 M	-	-	-	-	-	-	-	-	-
5. Bracco - Curti's test	-	±	+	-	-	++	-	-	±

Key : CY = Cytoplasm; NU = Nucleus; NUL = Nucleolus; CB = Cortical Bodies; FC - Follicle cell;
- = Negative reaction; ± Very mild reaction of doubtful nature; + = Positive reaction;
++ = Strongly positive reaction; +++ Intense positive reaction.

Table - 3 : Histochemical responses of the ovary for proteins and nucleic acids during pre-vitellogenic phase in *P. indicus*.

HISTOCHEMICAL TESTS	EPITHELIAL TISSUE	CONNECTIVE TISSUE	OOGONIAL CELLS			PRE-VITELLOGENIC OOCYTES			FOLLICLE CELLS
			CY	NU	NUL	CY	NU	NUL	
Mercuric Bromophenol Blue	++	+	+++	+	+	++	+	++	+
Aq.Bromophenol Blue	+	-	+++	+	+	+	+	++	+
Deamination	-	-	+	-	-	-	-	-	-
Aq.Toluidine Blue	-	±	++	-	-	++	-	+++	+
Methylation	-	-	-	-	-	-	-	-	-
Ferric Ferri-cyanide	+	-	+	-	-	+	++	++	+
Mercaptide	-	-	-	-	-	+	-	+	-
Performic Acid Alcian Blue	+	-	-	-	-	+	-	-	++
Alcian blue alone	±	-	-	-	-	-	-	-	+
Million's test	+	-	++	+	+	+	-	±	-
Iodination	-	-	-	-	-	-	-	-	-
DMAB - Nitrite test	+	-	±	±	-	++(PN)	-	+	-
Formaldehyde	-	-	-	-	-	-	-	-	-
Ninhydrin - Schiff	+	-	++	-	+	+	-	+	+
Deamination	-	-	-	-	-	-	-	-	-
Methyl Green Pyronin	-	-	+(R)	±(G)	±(R)	+++(R)	±(G)	+++(R)	++(R)
10% Perchloric acid	-	-	-	±(G)	-	-	±(G)	-	-
Fuelgen Reaction	-	-	-	+++	±	-	++	+	+
10% Perchloric acid	-	-	-	-	-	-	-	-	-

PN - Perinuclear, R - Red, G - Green

Table - 4 : Histochemical responses of the ovary for lipids during pre-vitellogenic phase in *P. indicus*.

HISTOCHEMICAL TESTS	EPITHELIAL TISSUE	CONNECTIVE TISSUE	OOGONIAL CELLS			PRE-VITELLOGENIC OOCYTES			FOLLICLE CELLS
			CY	NU	NUL	CY	NU	NUL	
Sudan Black B	±	-	±	±	±	+(GB)	-	-	±
Delipidation	-	-	-	-	-	-	-	-	-
Nile blue method	±	-	+	-	+	+++(BL)	±	-	+(BL)
Delipidation	±	-	±	-	-	+	±	-	-
Baker's Acid Hematein	+(BR)	-	+(BR)	±	+	++(BB)	+	+	±
Pyridine extraction	-	-	±	-	-	-	-	-	-
Oil Red O method	±	-	-	-	-	±	-	-	-
Delipidation	-	-	-	-	-	-	-	-	-
UV Schiff reaction	-	-	+	-	+	±	-	-	±
non UV	-	-	-	-	+	+	-	-	±

BL - Blue, Br - Brown, BB - Blue Black, GB - Greyish black.

Follicle cells gave positive reaction to all classes of proteins excepting tyrosine and tryptophan. Moderate quantities of RNA was also detected in these cells. Oogonial cells as well as follicle cells were uniformly poor in lipids (table 4). Previtellogenic oocytes displayed intense positivity to acidic lipids and moderate positivity to the test for phospholipids.

2.3. Early Vitellogenic oocytes

This phase of the maturation of oocytes was characterized by a three-fold increase in oocyte diameter to 125.8 $\pm$ 22.6 μ. The nucleus also increased greatly in size and dimensions ranged from 35 - 50 μ (Table I A). The cytoplasm was observed to loose some of its basophilia and now appeared as granular (figs. 6 and 7). The granular appearance was mainly due to the presence of the vesicular primary yolk (fig. 7). A perinuclear halo of nucleolar material was observed in this phase with the nucleoli arranged as a circular ring around the periphery of the nucleus (fig, 8). The follicular investment around the individual oocytes or folliculogenesis was completed during this phase (fig. 7). Since the oocyte volume had increased, the follicle cells were considerably stretched and there was

an apparent decrease in its height (fig. 8). The follicle cells were seen to have increasingly conspicuous and hypertrophied nucleus and nucleoli (fig. 8).

Electron microscopic observations of the early vitellogenic oocytes revealed the abundance of mitochondria in the perinuclear region of the cytoplasm (Fig. 24). Numerous free ribosomes and rough endoplasmic reticulum were also seen in the cytoplasm. The ooplasm showed the presence of the primary yolk bodies in the form of swollen reticular elements. A nucleolonema was observed in the nucleoplasm in this stage (Fig. 28). These are modified nucleoli with a filamentous appearance.

Histochemically, the ooplasm in this phase was intensely positive to the PAS test in patches (Table 5 and fig. 9). The PAS positivity which was only partially removed by deamination was totally removed by acetylation. Deacetylation restored fully the PAS positivity indicating the presence of 1,2 glycol groups and glycoproteins. Carmine positivity observed in the cytoplasm indicated the presence of glycogen. The presence of carboxylated and sulphated acid mucopolysaccharides (AMP) was denoted by the alcianophilia at low molar concentrations upto 0.2 M. The nucleus and

nucleolus which were PAS negative gave positive reaction to the carmine test for glycogen. The nucleus displayed alcianophilia at 0.6 M level indicating a preponderance of sulphated AMP whereas the nucleoli contained only carboxylated AMP. Follicle cells showed decreased PAS positivity and contained both carboxylated and sulphated AMPs.

Almost all classes of proteins were present abundantly in early vitellogenic oocytes (Table 6). The cytoplasm gave a strong positive reaction to tests for tyrosine, tryptophan and amino groups. Sulphur containing amino acids were present in moderate amounts. A progressive decrease in the positivity to pyronin was observed but the reaction was confined to the perinuclear region. The nucleoli, however, remained intensely pyroninophilic. The karyoplasm was positive to tests for sulphydryl and tryptophanyl groups. The fuelgen reaction was of doubtful nature. Follicle cells gave an intense positive reaction to tests for acidic proteins and moderate positive reaction to tests for -S-S, -SH and -NH2 groups.

Early vitellogenic oocytes displayed an increase in positivity to tests for acidic and phospholipids (Table 7). Neutral lipids and lipids with unsaturated bonds were

Table - 5 : Histochemical responses of the ovary for Carbohydrates during Veitellogenic phases in *P. indicus*.

HISTOCHEMICAL TESTS	EARLY VITELLOGENIC OOCYTES				LATE VITELLOGENIC OOCYTES				VITELLOGENIC OOCYTES				SPENT OOCYTES			
	CY	N	NUL	FC	CY	N	NUL	FC	CY	N	FC	CB	CY	N	NUL	FC
1. Schiff alone	±	-	-	-	±	-	-	-	±	-	-	-	-	-	-	-
2. Periodic acid Schiff (PAS)	+++	-	-	+	+++	-	-	±	++	-	-	+	±	-	-	+++
Deamination	++	-	-	+	+	-	-	±	++	-	-	±	-	-	-	++
Acetylation	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Deacetylation	+++	-	-	+	+++	-	-	±	++	-	-	+	±	-	-	+++
Delipidation	+++	-	-	±	++	-	-	±	+	-	-	+	±	-	-	+++
Diastase digestion	++	-	-	+	++	-	-	±	+	-	-	+	±	-	-	++
3. Best's Carmine test	++	+	++	+++	++	-	+	++	++	-	+	-	+	-	++	++
Diastase digestion	+	-	+	++	++	-	+	+	+	-	-	-	+	-	+	+
4. Alcian blue C-E-C-method.																
Molar concentrations																
0.1 M	+	+	+	++	++	+	+	++	+	-	-	-	-	-	-	+++
0.2 M	+	++	-	++	+	±	-	+	+	-	-	+	-	-	-	++
0.6 M	±	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
0.8 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1.0 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5. Bracco-Curti's test	++	+++	++	+	+	+	-	-	+	-	-	+	±	+	+	-

Table - 6 : Histochemical responses of the ovary for proteins and nucleic acids during vitellogenic phases in *P. indicus*.

HISTOCHEMICAL TESTS	EARLY VITELLOGENIC OOCYTES				LATE VITELLOGENIC OOCYTES				VITELLOGENIC OOCYTES				SPENT OOCYTES			
	CY	N	NUL	FC	CY	N	NUL	FC	CY	N	FC	CB	CY	N	NUL	FC
Mercuric Bromophenol Blue	+++	++	+++	+++	+++	+	+++	+	+++(PN)	+	++	+(PP)	++	+	+++	+
Aq.Bromophenol Blue	+++	+	++	+	+++	+	++	+	+++	+	+	±	+	-	+	-
Deamination	++	-	+	-	++	-	±	-	++	-	-	-	-	-	-	-
Aq.Toluidine Blue	+	-	+++	+++	+(PN)	-	+	+	-	+	++	-	+++	-	++	±
Methylation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ferric Ferri-cyanide	+	++	+	++	++(PP)	+	-	++	++(PP)	+	++	+++	+	-	+	++
Mercaptide	-	+	+	+	+	-	-	-	++	-	-	+	-	-	±	+
Performic acid Alcian Blue	+	-	-	++	++(PN,PP)	-	-	+++	+	±	++	+++	±	-	-	+++
Alcian blue only	-	-	-	±	-	-	-	+	-	-	+	-	-	-	-	+
Millon's test	++	-	+	+	++	-	±	++	++	-	+	+	±	-	+	-
Iodination	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DMAB-Nitrite test	++	+	++	+	+++	+	++	++	++	+	+	+	±	-	-	-
Formaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nixhydrin-Schiff	++	-	++	++	+++	+	++	+	++	+	-	+	±	-	+	-
Deamination	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-
Methyl green pyronin	++(R)	±(G)	+++(R)	++(G)	±(R)	±(G)	+(R)	++(G)	-	±(G)	+(G)	±(R)	-	±(G)	+(G)	++(G)
10% Perchloric	-	±	±(R)	+(G)	-	±	-	+(G)	-	±	-	-	-	-	-	±(G)
Fuelgen Reaction	-	±	±	++	-	±	-	++	-	-	+	-	-	+	+	++
10% Perchloric	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

PN - Perinuclear, PP - Peripheral, R - Red, G - Green.

Table - 7 : Histochemical responses of the ovary for lipids during Vitellogenic phases in P. indicus.

HISTOCHEMICAL TESTS	EARLY VITELLOGENIC OOCYTES				LATE VITELLOGENIC OOCYTES				VITELLOGENIC OOCYTES				SPENT OOCYTES			
	CY	N	NUL	FC	CY	N	NUL	FC	CY	N	FC	CB	CY	N	NUL	FC
Sudan Black B	++	-	-	±	+++(GB)	±	-	±	+++(GB)	-	-	-	+	-	-	±
Delipidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nile blue method	++(BL)	-	±	+	++(BL)	-	-	+	++(BL)	-	-	-	+(BL)	±	±	+
Delipidation	-	-	-	±	±	-	-	-	+	-	±	±	-	-	-	±
Baker's Acid Hematein	++(BB)	-	-	+	+++(BB)	-	-	+	+++(BB)	-	+	+	+	+	+	±
Pyridine extraction	±	-	-	-	±	-	-	-	±	-	-	±	±	-	-	-
Oil Red O Method	+	-	-	±	+++	-	-	-	+++	-	±	-	-	-	-	±
Delipidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UV Schiff reaction	+	-	-	+	++	-	-	-	++	-	±	-	±	-	-	±
non UV	+	-	-	±	±	-	-	-	+	-	-	-	-	-	-	-

GB - Greyish black, BL - Blue, BB - Blue Black.

present in small amounts only. The nucleus and nucleoli were observed to be devoid of lipids.

2.4.Late Vitellogenic oocytes

During this phase the oocytes were found to have an average diameter of 187.3 ± 19.4 μ and the nucleus diameter ranged from 40-70 μ (Table 1A). Characteristic of this phase was the rough granular cytoplasm which was wholly eosinophilic having lost the basophilia (fig. 10). The nucleus was palely stained with hematoxylin and the nucleoli number was greatly reduced (fig. 10). The granular nature of the cytoplasm was mainly due to the formation of dense yolk platelets and accumulation of lipid globules (fig. 12). The fully formed yolk platelets were dark blue and partially formed ones were light blue in colour with toluidine blue stain. Lipid droplets were not stained (fig.13) and their size ranged between 1 - 5 μ. Because of the increase in oocyte volume, the follicle cells were stretched further and appeared as a narrow band of flattened cells encompassing the oocytes (fig. 13).

Electron microscopic studies revealed that the yolk platelets were spherical to oval in shape and electron dense (fig. 25). The formation of the yolk platelets was

apparently achieved through two sub-stages. The initial yolk vesicle was almost electron lucent and filled with tiny electron dense flocculent granules. Through progressive differentiation these granules aggregated to form a moderately electron dense yolk sphere which finally devolved into the yolk platelet (fig. 25). The whole of the ooplasm was filled with yolk spheres and platelets and consequently the ER and mitochondria were not apparent in this stage. The oolemma, although smooth in the early vitellogenic phase, now appeared as broken with the presence of numerous micropinocytotic vesicles (fig. 26). These vesicles appeared on the oocyte wall in close apposition to the follicle cells. The follicle cells in this phase possessed a hypertrophied nucleus, rough ER and mitochondria denoting the heightened synthetic activity taking place within them.

Histochemically the yolk spheres and yolk platelets in this phase were seen as deeply PAS positive (fig. 11), denoting the presence of 1,2 glycol groups, glycoproteins and glycolipids (Table 5). Alcianophilia at low molar concentrations indicated the presence of both carboxylated and sulphated AMP in the yolk platelets. The nucleus and nucleoli showed continued negativity to PAS test. Follicle cells were alcianophilic and carmine positive. Yolk platelets were positive to all classes of proteins tested

(Table 6). Intense positive reaction was observed with tests for basic proteins, tryptophan and amino groups. Positive reactions were observed in the peripheral and perinuclear regions of the ooplasm with tests for -S-S and -SH group amino acids. Yolk spheres were negative to the tests for the detection of RNA. The nucleus was generally poor in positivity to proteins and nucleic acids. The nucleoli retained its positivity to pyronin with moderation denoting the presence of RNA. Follicle cells reacted strongly to the tests for sulphur containing amino acids.

Yolk platelets were intensely sudanophilic and acid hematein positive denoting the abundance of lipids in general and phospholipids in particular (Table 7). Yolk bodies stained blue with Nile blue method indicating the presence of acidic lipids. The presence of neutral lipids was revealed by the intense positivity to Oil red O . Unsaturated lipids were also detected in yolk platelets. The nucleus and nucleoli were found negative to lipid tests.

2.5. **Vitellogenic oocytes**

Ripe or stage IV ovaries were filled with vitellogenic oocytes which measured 241.3 $\pm$ 16.7 μ in diameter and their nucleus diameter ranged from 60-70 μ (Table I A).

Vitellogenic oocytes were characterized by the abundance of mature yolk platelets filling the entire ooplasm and the appearance of specialized elongated rod-like cortical bodies on its peripheral margin adjacent to the oolemma (figs. 14, 15 and 16). The cortical bodies measured 34.71 $\pm$ 3.6 μ in length and 13.8 $\pm$ 2.8 μ breadth. The nucleus was stained vary faintly and the nucleoli were not apparent at this stage. The follicle cells were further flattened and were present only as a thin covering (fig. 16). The cortical bodies were palely eosinophilic (fig. 14) and lightly stained with 1% toluidine blue (fig. 16).

The ultrastructure of late vitellogenic oocytes and vitellogenic oocytes were similar with the exception of the presence of cortical bodies in the latter. The cortical bodies exhibited a distinct sub-structure (fig. 27). The matrix of these bodies were packed with minute feather-like strands arranged randomly.

Histochemical tests showed that the yolk platelets in vitellogenic oocytes had a slightly reduced PAS positivity and moreover the reaction was not wholly blocked by acetylation and diastase digestion (Table 5). Carboxylated and sulphated AMP were present in the ooplasm as well as yolk platelets (fig. 15) as indicated by the alcianophilia at 0.2

M concentration. The nucleus of the oocytes and follicle cells at this stage were observed to be devoid of any carbohydrate material. The cortical bodies were seen as weakly PAS positive and also positive to the test for sulphated AMP. Vitellogenic oocytes did not exhibit any major differences in the reactivity to tests for proteins with that of late vitellogenic oocytes (Table 6). However acidic proteins were absent in yolk platelets at this stage. Interestingly the cortical bodies were intensely positive to tests for -SH and -S-S groups (fig. 20). The were also mildly pyroninophilic denoting small quantities of RNA. Reactions to lipid tests were also unchanged when compared to late vitellogenic oocytes (Table 7). Cortical bodies were mildly acid hematein positive due to presence of phospholipids.

2.6. **Spent oocytes**

Oocytes of spawned prawns showed characters indentical to that of previtellogenic and early vitellogenic oocytes (Table 1). Microscopically, the spent ovary displayed empty follicles, sites of oocyte resorption and areas of proliferative growth of oocytes (fig. 17). Empty follicles from which ova have been released have hypertrophied follicle cells. Pycnotic oocytes which have deeply basophilic

cytoplasm and nucleus were also observed in conjunction with hypertrophied follicle cells. Proliferation of fresh batches of oocytes with characteristics of previtellogenic oocytes like nuclear halo was also observed (fig. 18).

Spent oocytes were found to be negative to all histochemical tests employed for the detection of carbohydrates (Table 5). A striking feature was the intense PAS positivity of the follicle cells (fig. 21), which was removed by acetylation and restored by deacetylation, indicating the presence of 1,2 glycol groups. Follicle cells were also deeply alcianophilic at 0.1 M level denoting large amounts of carboxylated AMP. The cytoplasm of spent oocytes were interestingly positive to aqueous toluidine blue denoting the abundant presence of acidic proteins (Table 6). Follicle cells had a profusion of sulphur rich amino acids and DNA. The ooplasm of spent oocytes were weakly sudanophilic and positive to acidic lipids (Table 7). Follicle cells were observed to be negative to tests for lipids.

From the foregoing account it is clearly evident that yolk in the ovary of P. indicus is a glycolipoprotein complex, with proteins being the first to be associated with the yolk complex, followed by carbohydrates and finally

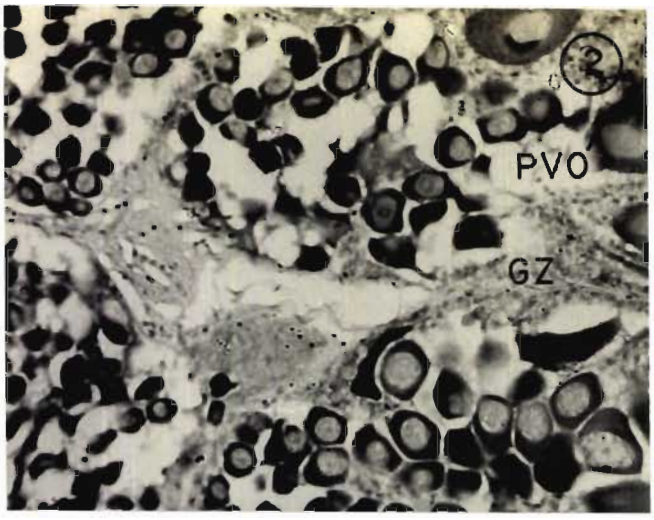
Fig.2 Photomicrograph of a stage I ovary with deeply basophilic previtellogenic oocytes (PVO). The germinal zone (GZ) is apparent as a thin band of cells extending from the ovarian wall. Haematoxylin and Eosin. x50.

Fig.3 Semithin section of the germinal zone showing primary (PG) and secondary oogonial (SG) cells. Note the absence of follicle cells. Toluidine blue. x200.

Fig.4 Semithin section of a stage I ovary showing previtellogenic oocytes (PVO) surrounded by tall follicle cells (FC). Note the thin ovarian wall (OW) composed of two layers. Toluidine blue. x100.

Fig.5 Detail of the previtellogenic oocytes (PVO) showing vaculated follicle cells (FC) and hypertrophied nucleoli (NUL) around the nuclear membrane. N - nucleus. Toluidine blue. x200.

Fig.6 Cross section of stage II ovary with the germinal zone (GZ) and early vitellogenic oocytes (EVO). Toluidine blue. x50.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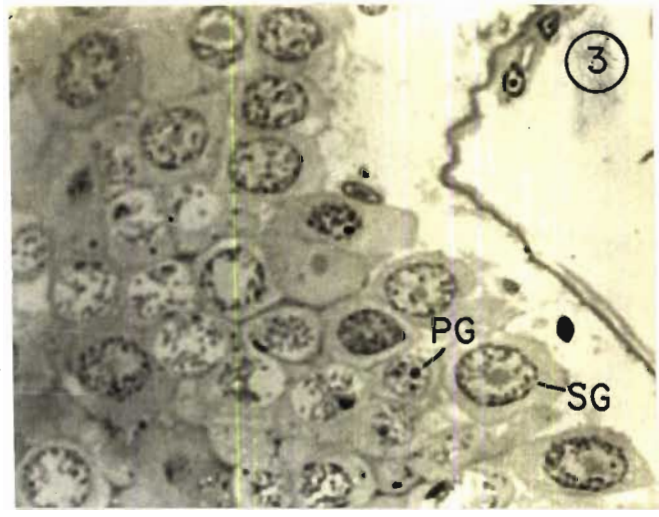
PVO
GZ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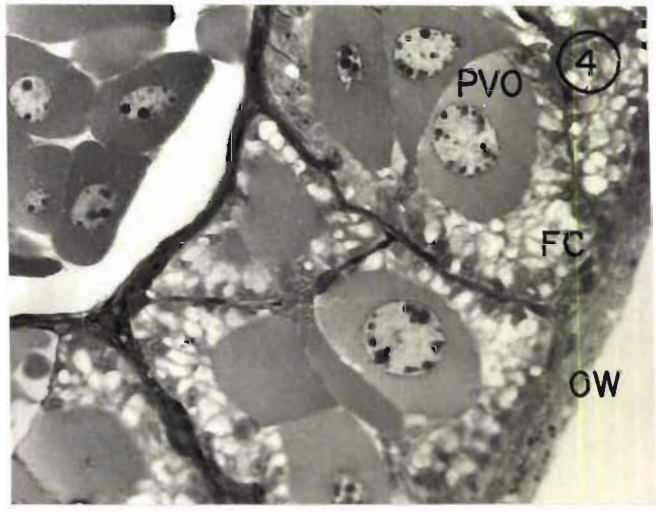
PG
SG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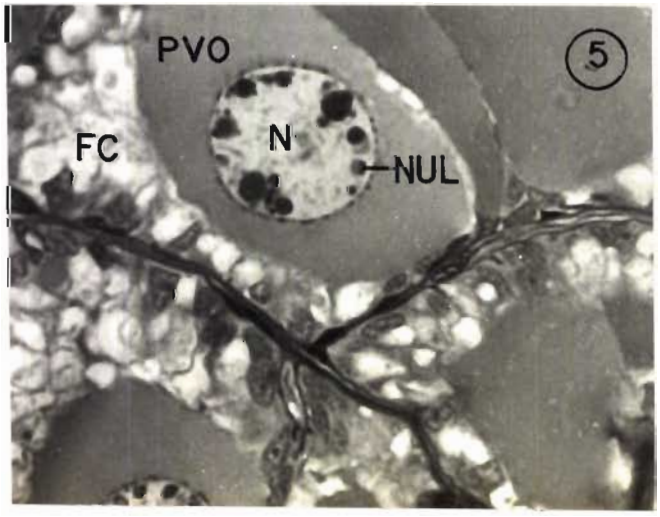
PVO
FC
OW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PVO
FC
N
NUL

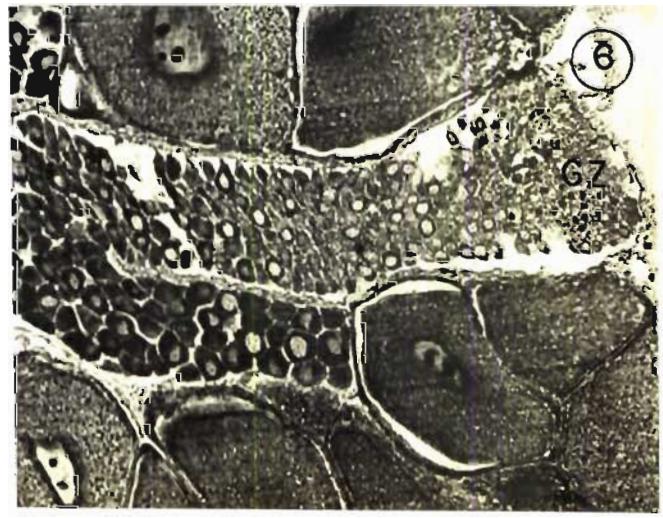

6
GZ

Fig.7 Detail of early vitellogenic oocytes showing granular cytoplasm and the complete encircling of the follicle cells (FC). Toluidine blue stain. x100.

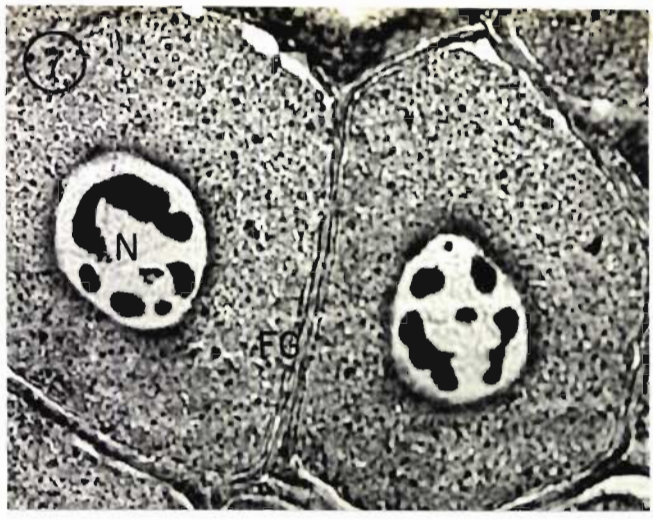
Fig.8 Semithin section of EVO showing perinuclear halo of nucleolar material (NM) and granular cytoplasm. The follicle cells are flattened and have hypertrophied nucleus (FCN). Toluidine blue stain. x200.

Fig.9 The PAS reaction in stage II ovary showing the darkly stained primary yolk (EVO). The previtellogenic (PVO) oocytes are also positive to the PAS test. x50.

Fig.10 Photomicrograph of stage III ovary showing late vitellogenic oocytes (LVO). Note the granular cytoplasm filled with yolk platelets and lipid bodies. Haematoxylin and Eosin. x100.

Fig.11 PAS reaction in late vitellogenic oocytes. The yolk platelets are deep magenta while the lipid globules are PAS negative. x50.

Fig.12 Semithin section of LVO showing the darkly stained yolk platelets, lightly stained yolk vesicles and unstained lipid globules. Toluidine blue stain. x200.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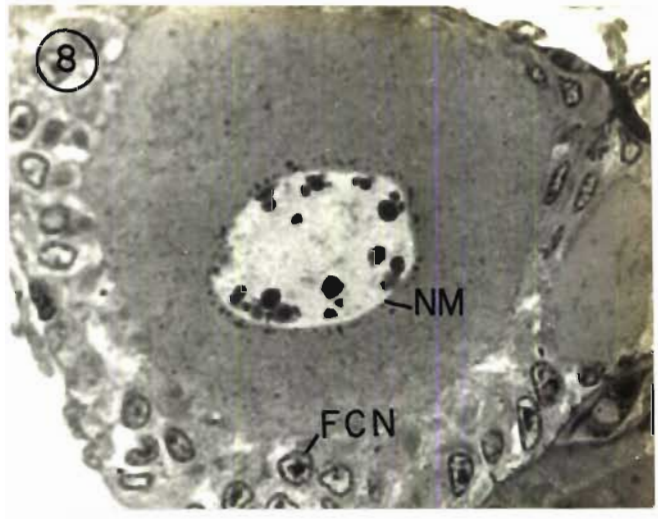
N
FC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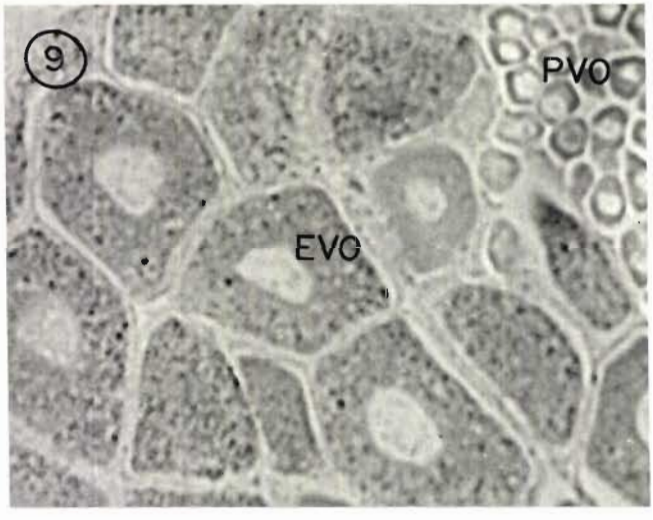
NM
FCN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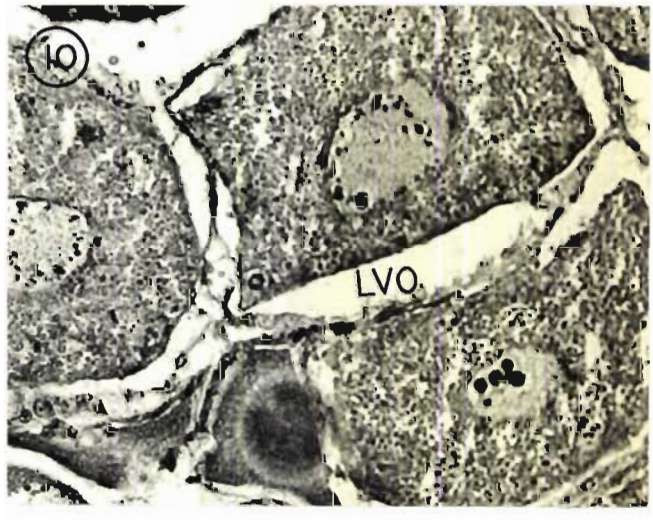
PVO
EVO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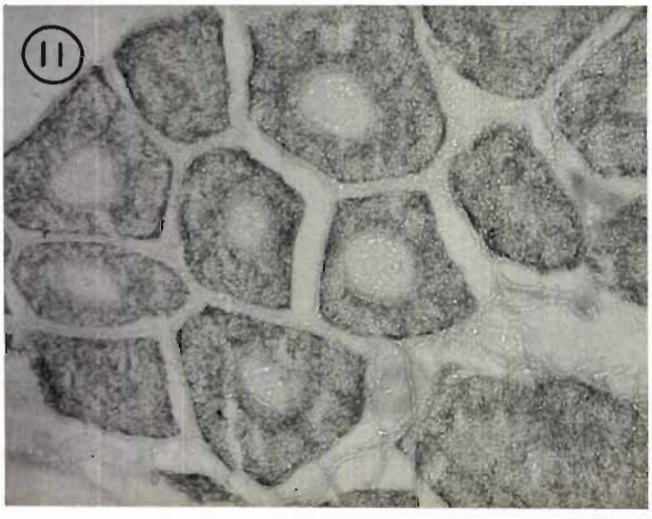
LVO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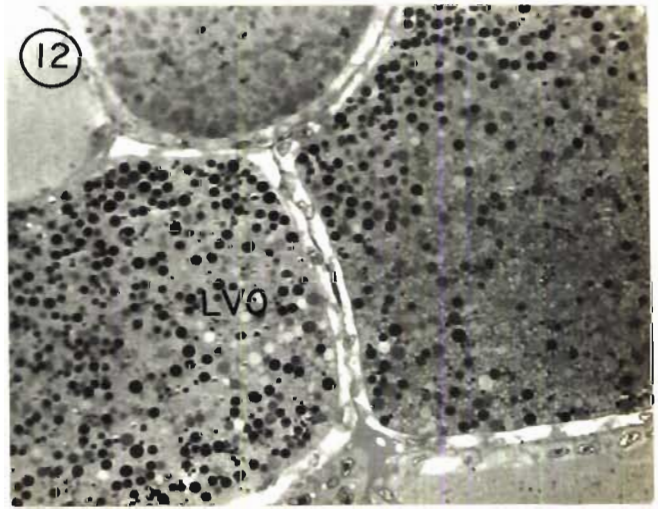

12
LVO

Fig.13 Semithin section, detail of LVO indicating the flattening of follicle cell layer (FC) and dark yolk platelets. Toluidine blue stain. x200.

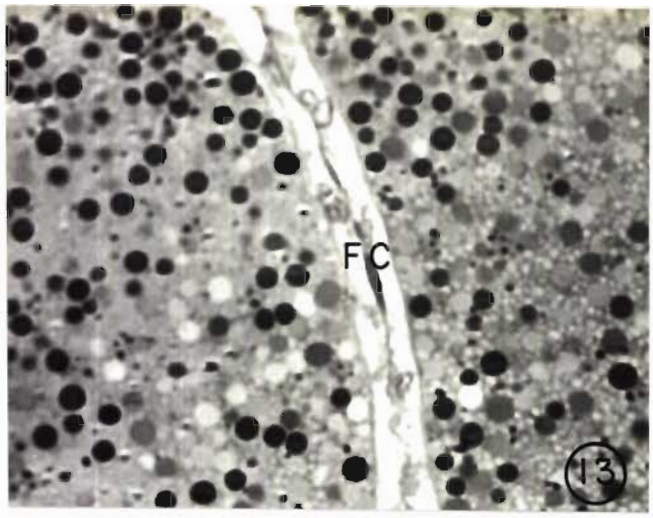
Fig.14 Cross section of the fully mature stage IV ovary showing the lightly stained nucleus(N) without inclusions and cytoplasm with yolk platelets (YP) and cortical bodies (CB). Haematoxylin and Eosin. x50.

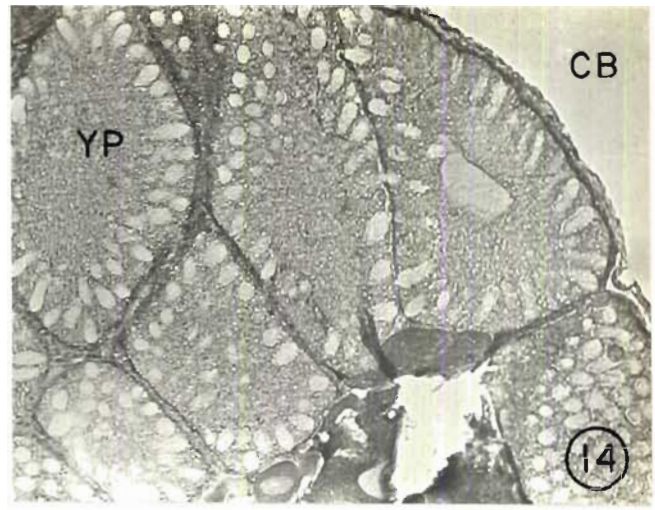
Fig.15 Photomicrograph of the vitellogenic oocyte. The peripheral cortical bodies are close to the oolemmal margin (CB). Toluidine blue stain. x100.

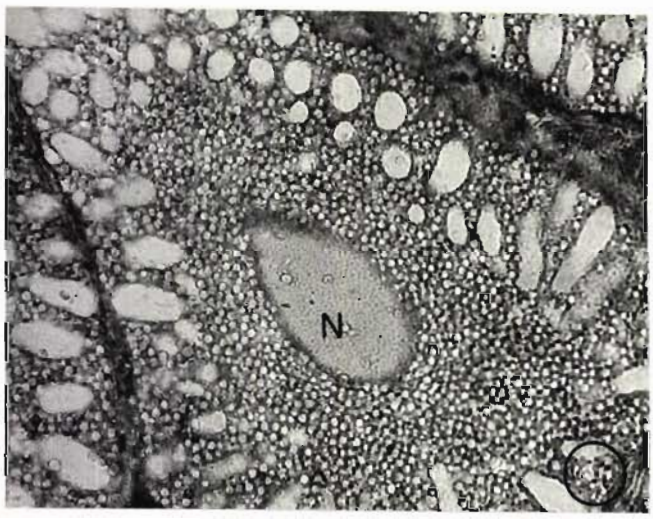
Fig.16 Semithin section of vitellogenic oocyte. The fully formed yolk platelets are deeply toluidine blue positive. The follicle cells (FC) are present only as a thin covering. Toluidine blue stain. x100.

Fig.17 Photomicrograph of the spent (stage V) ovary. Resorbing oocytes (RO) as well as oocytes in the previtellogenic (PVO) phase are seen. Haematoxylin and Eosin. x50.

Fig.18 Spent ovary displaying oocytes in the early vitellogenic phase (EVO) in the proliferative zone. Haematoxylin and Eosin. x100.


FC
13


CB
YP
14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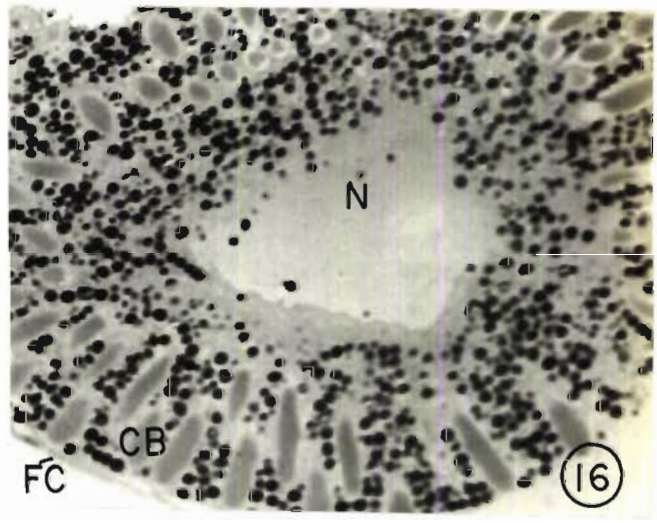
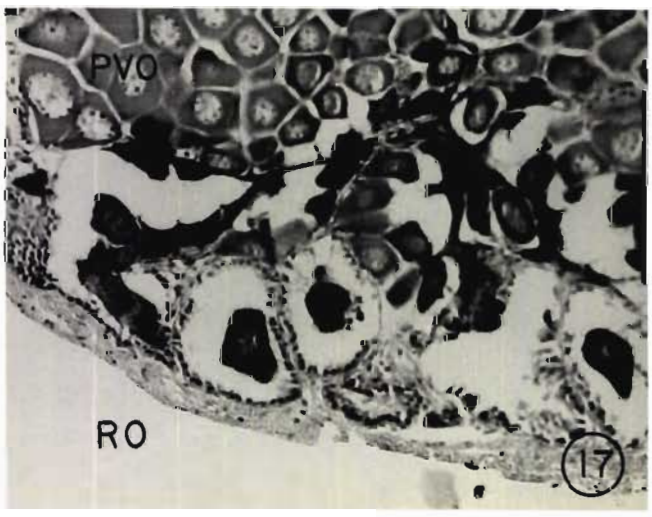

N
CB
FC
16


PVO
RO
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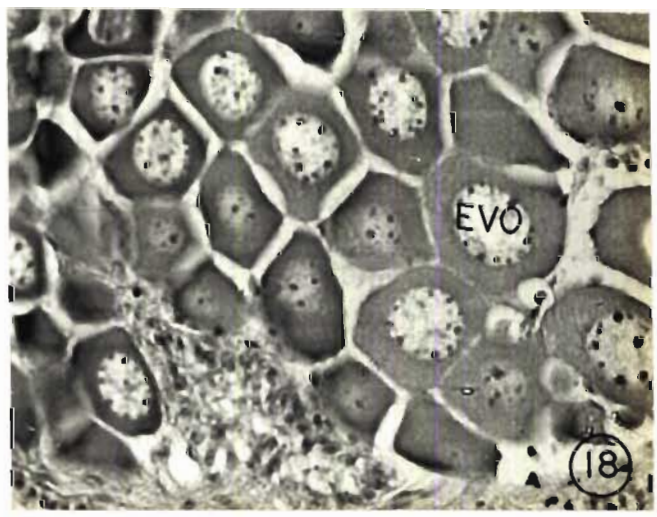
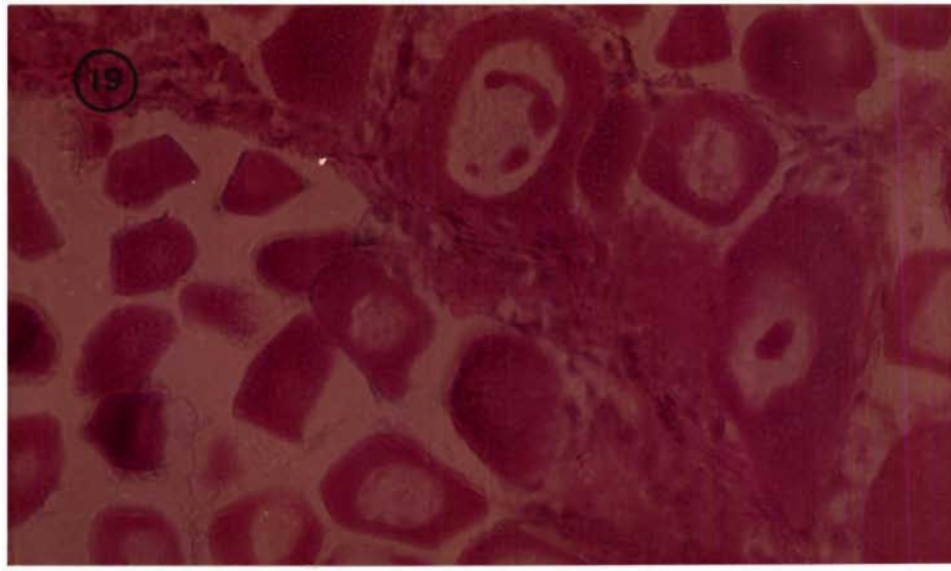

EVO
18

Fig.19 The Methyl green pyronin reaction in previtellogenic oocytes. Note the deeply pyroninophilic ooplasm and nucleoli. MGP reaction. x100.

Fig.20 The performic acid -alcian blue reaction in vitellogenic oocytes showing the disulphide group rich cortical bodies (CB). Other parts of the ooplasm are only faintly positive. x100.

Fig.21 The PAS reaction after deacetylation in spent ovary. Note the intensely positive follicle cells (FC). The oocytes are negative (SO). x100.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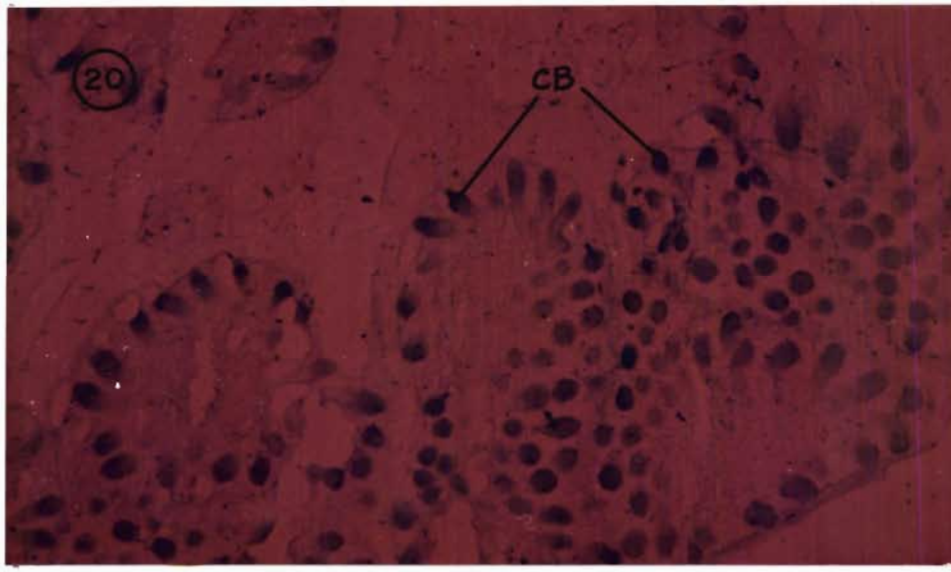
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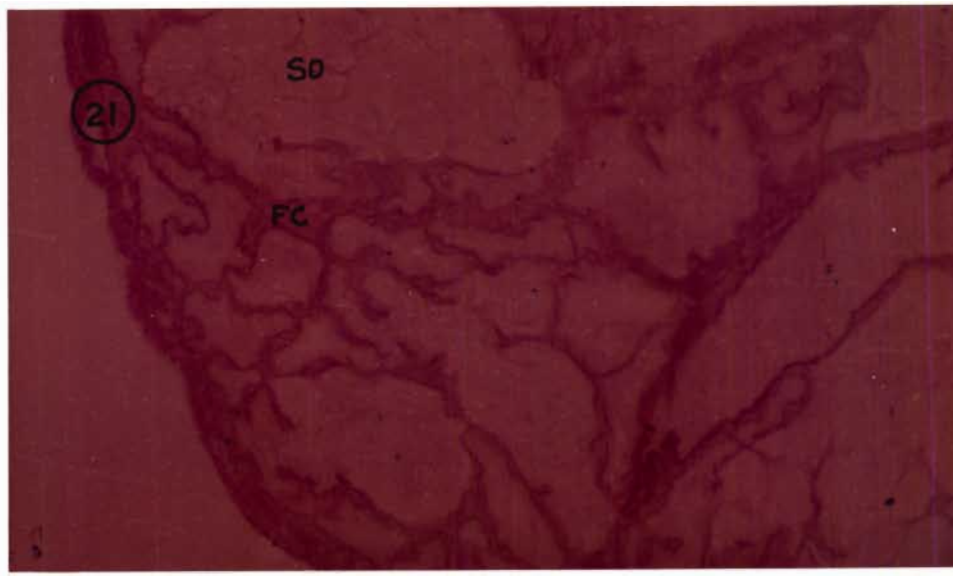
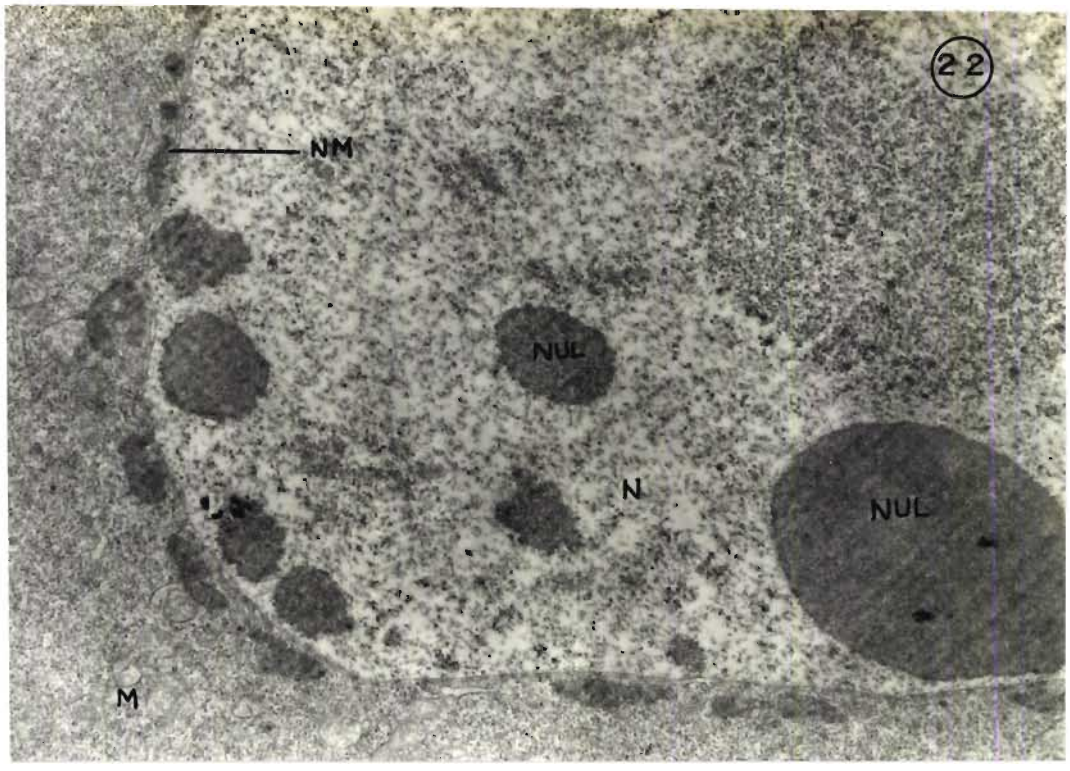

21
SD
FC

Fig.22 Electronmicrograph of a previtellogenic oocyte showing the electron dense nucleoli (NUL) lining the nuclear membrane (NM). N - nucleus, M - mitochondrion. x4000.

Fig.23 Higher magnification of the nuclear membrane showing the nuclear pores (arrow heads) and the diffused nucleolar material (NLM). M - mitochondrion, N - nucleus. x8000.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NM
NUL
N
NUL
M

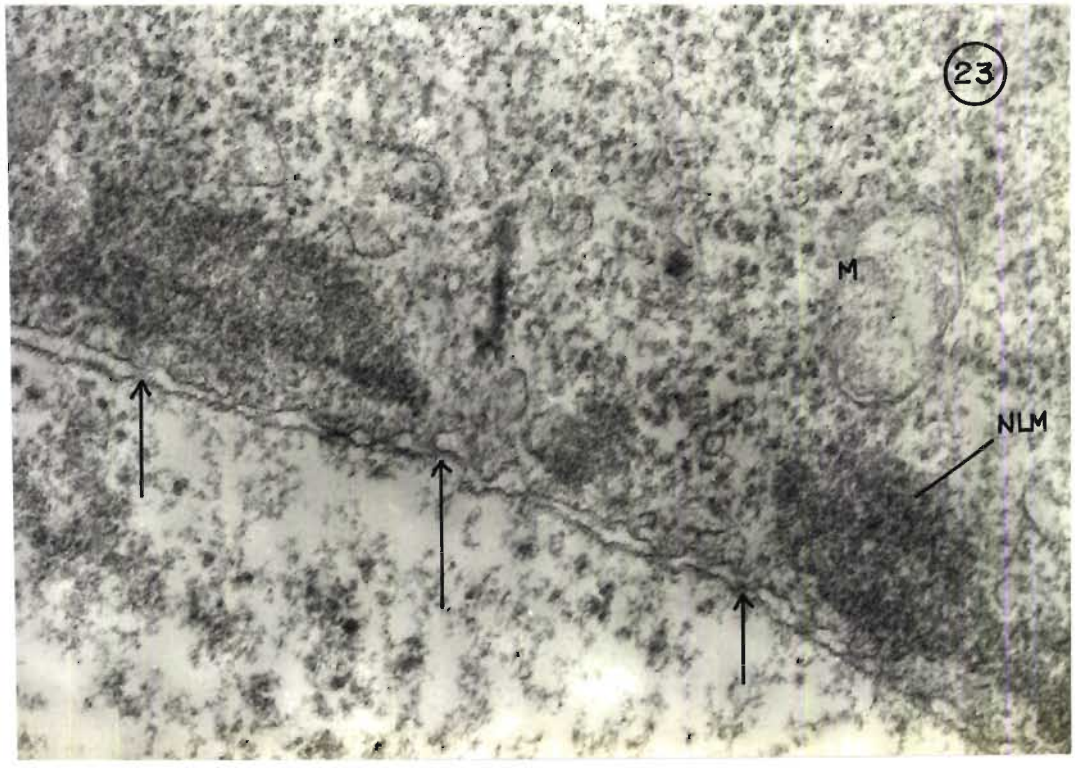
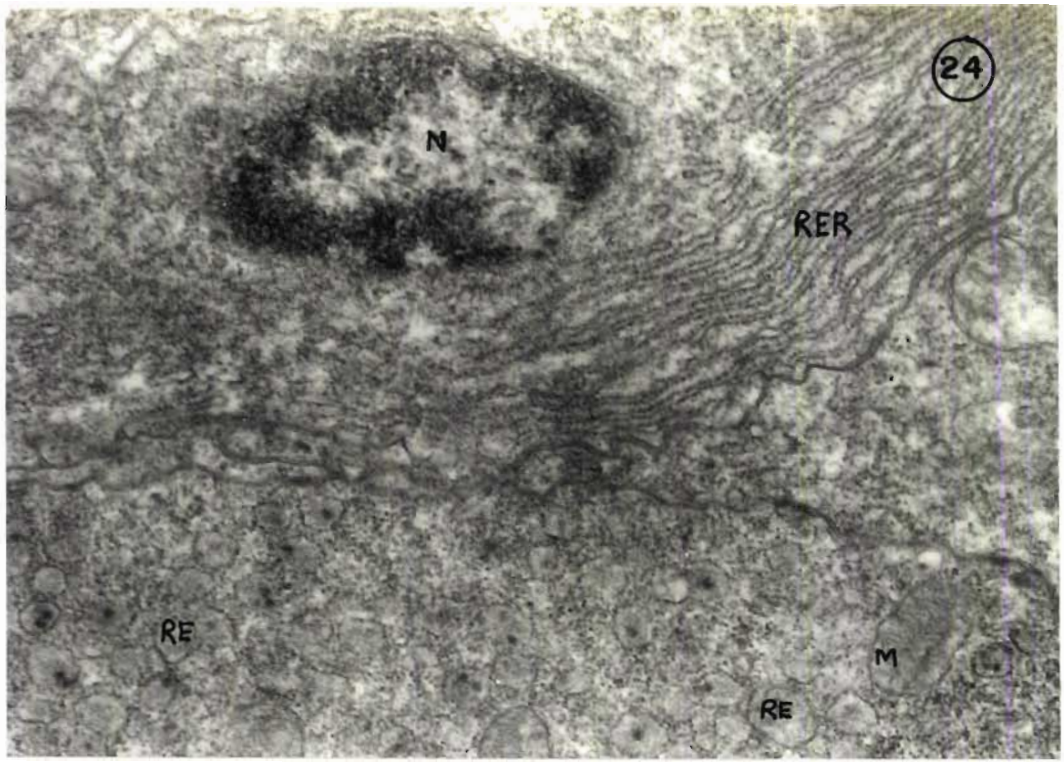

23
M
NLM

Fig.24 Electron micrograph of the perinuclear region of an early vitellogenic phase oocyte displaying abundant mitochondria (M), rough endoplasmic reticulum (RER), swollen reticular elements (RE) and numerous free ribosomes. x8000.

Fig.25 Electron micrograph of the cytoplasm of late vitellogenic oocyte showing the formation of yolk. Evident are the Yolk vesicles (YV), yolk spheres (YS), and the fully formed yolk platelets (YP). x4000.


24
N
RER
RE
M
RE

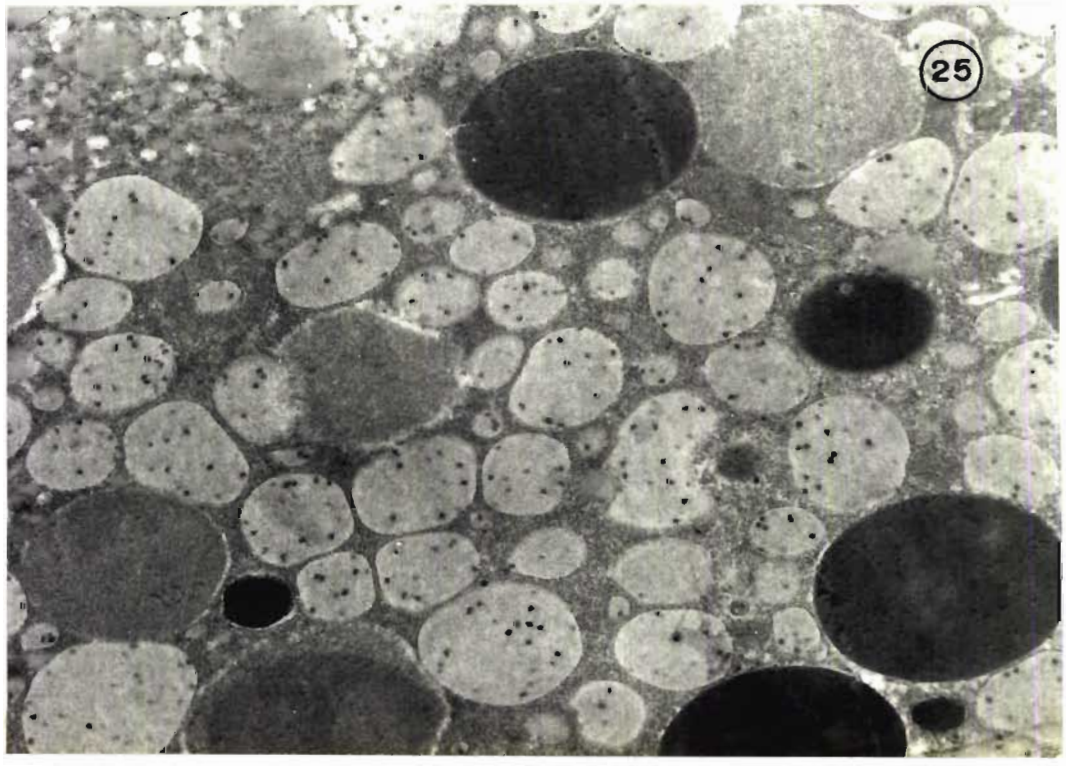

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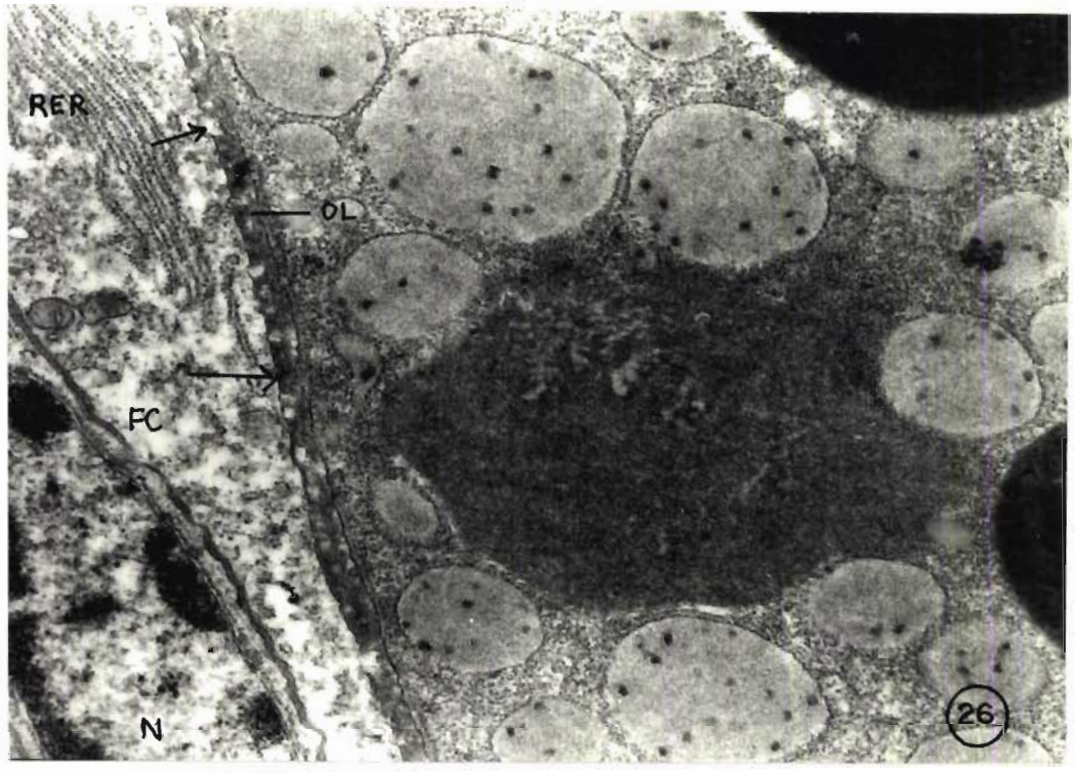
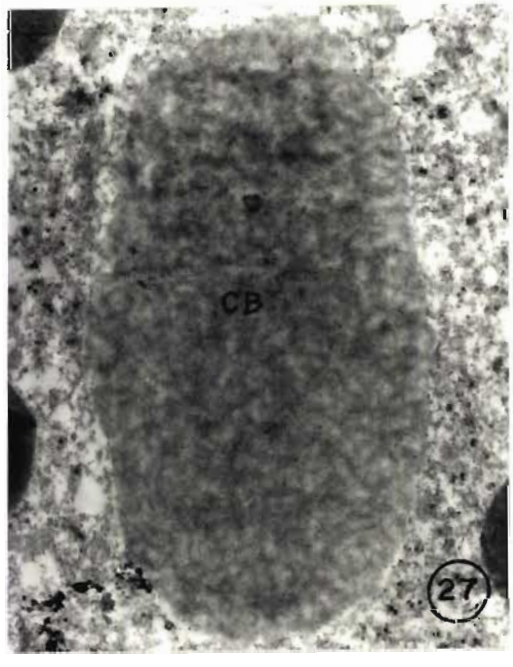
Fig.26 Electron micrograph of late vitellogenic oocyte showing the oolemma (OL) and follicle cells (FC). The oolemma shows micropinocytotic vesicles (arrow heads). The FC has a hypertrophied nucleus (N) mitochondria (M) and rough endoplasmic reticulum (RER) in its cytoplasm. x8000.

Fig.27 Electronmicrograph of the cortical body (CB) in vitellogenic oocyte. Note the feathery matrix of the CB. x4000.

Fig.28 A filamentous nucleolonema in the nucleoplasm of an early vitellogenic oocyte. x6700.


RER
OL
FC
N
26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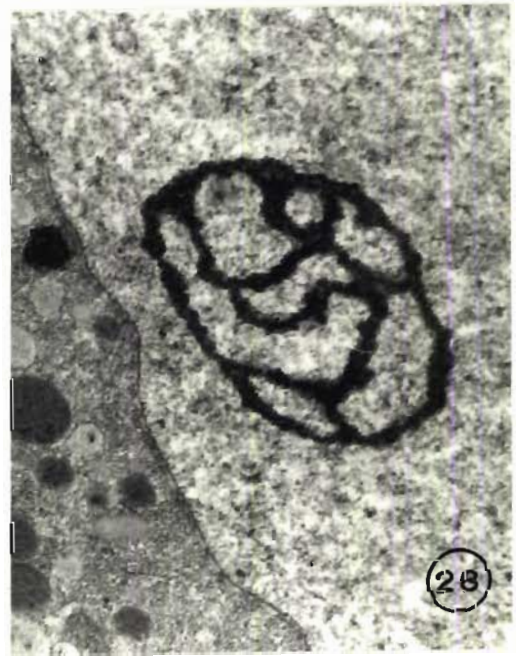
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lipids. Yolk formation apparently takes place by the selective deposition and sequestration of organic material both from within (autosynthesis) and without (heterosynthesis). During the early vitellogenic phase of the oocytes, the emphasis is on intraoocytic synthesis of proteins and polysaccharides. From late vitellogenic phase onwards extraoocytic sequestration of organic reserves, especially lipids is prevalent.

3. Male reproductive system and gross morphology of the testis

The male reproductive system consisted of internal organs, viz. paired testis, paired vas deferens and paired terminal ampoules and external organs viz. a petasma and a pair of appendix masculina. The testis was found to be an unpigmented translucent organ, composed of four lateral lobes located in the cardiac region dorsal to the hepato-pancreas. The testis lobes were connected to each other at their inner ends and lead to the vasa deferentia (fig. 1 B). Each vas deferens consisted of three portions: a short and narrow proximal vas deferens (PVD), a thick and large mid vas deferens (MVD), and a long, narrow distal vas deferens (DVD). The MVD consisted of a descending limb, a blind pouch and an ascending limb which continued as the DVD. The DVD continued into the short, muscular and bulbous terminal

ampoule (TA) which opened at the base of the 5th pereiopods.

The testes were observed as extremely delicate transparent structures in young prawns. In larger males the testes were seen as bigger in size and somewhat opaque. Transverse sections revealed that the testis was surrounded by a thin wall of epithelial cells (fig. 31). No muscle tissue was observed along the wall. The testes was composed of a mass of minute convoluted seminiferous tubules or acini in which the developing sperm cells were seen.

4. **Spermatogenesis**

Spermatogenesis or the formation of the mature spermatozoa takes place in the lumen of the testicular acini. In cross sections a strand-like germinal zone was apparent adjacent to the acinar wall (fig. 31 and 32). The germinal zone consisted of primary gonial cells, viz. spermatogonial cells and nurse cells (figs. 32 and 33). Spermatogenesis was observed to progress towards the central lumen of the acini and therefore subsequent developmental stages like spermatocytes and spermatids were formed in the central regions of the acini (fig. 31). Each spermatogonium passed through a period of quick growth to become a primary spermatocyte. A meiotic division results in two secondary sperma-

tocytes. These divide again to produce four spermatids which develop without further division into spermatozoa.

Since spermatogenesis involves the progressive reduction of cytoplasm and condensation of chromatin to produce the spermatozoa, the spermatogonial cells were observed as larger than spermatocytes, which in turn were larger than spermatids (fig. 30). The cytoplasmic limits of the gonial cells were not clearly apparent under light microscope and therefore only the diameters of the nuclei were used to classify the gonial cells as given below.

GONIAL STAGE	NUCLEUS DIAMETER	NATURE OF CHROMATIN
1. Spermatogonial cells	5.0 - 7.0	Diffuse
2. Spermatocytes	4.0 - 5.0	Condensed
3. Spermatids	3.0 - 4.0	Condensed
4. Spermatozoa	3.0	Condensed

Histological sections showed that the spermatogonial cells had a round vesicular nucleus with diffused chromatin matter which stained weakly with hematoxylin (fig. 33). The cytoplasm was observed to be eosinophilic, although their boundaries were not clearly demarked. The nurse cells were

Fig.30 Transverse section of the testis showing spermatogonia (SG), spermatocyte (SC) and spermatids (ST) in different lobes. Haematoxylin and Eosin. x50.

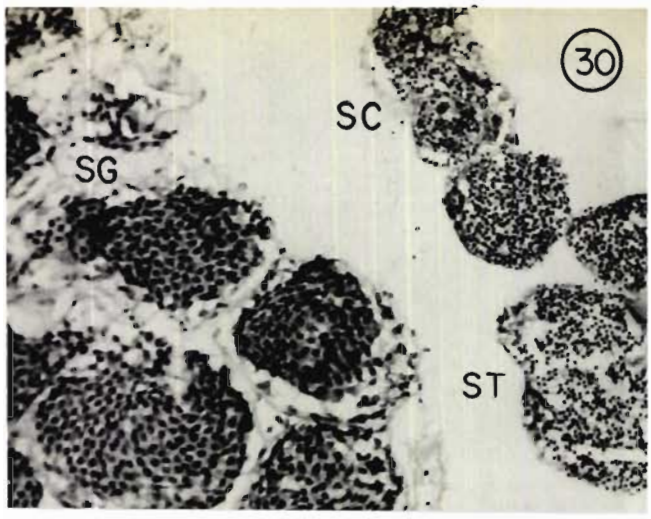
Fig.31 Photomicrograph of the testicular acini showing the strand-like germinal zone (GZ) with spermatogonial cells (SG) and spermatocytes (SC) in the lumen. Methyl green pyronin stain. x100.

Fig.32 Semi-thin section showing spermatogonia (SG) in the germinal zone and spermatocytes (SC) in the lumen. Note diakinetic stages among the spermatocytes (arrow heads). Toluidine blue stain. x100.

Fig.33 Detail of the germinal zone. Spermatogonia (SG) with vesicular nucleus and diffused chromatin matter. Nurse cells (NC) are also seen inbetween the SG. Toluidine blue stain. x200.

Fig.34 The fuelgen reaction in the acini of the testis. Spermatogonia (SG) are lightly stained and spermatids (ST) very deeply. x200.

Fig.35 Cross section of proximal vas deferens (PVD) with an outer connective tissue sheath (CT) and circular muscle fibres (CMF). L - lumen. Haematoxylin and Eosin. x50.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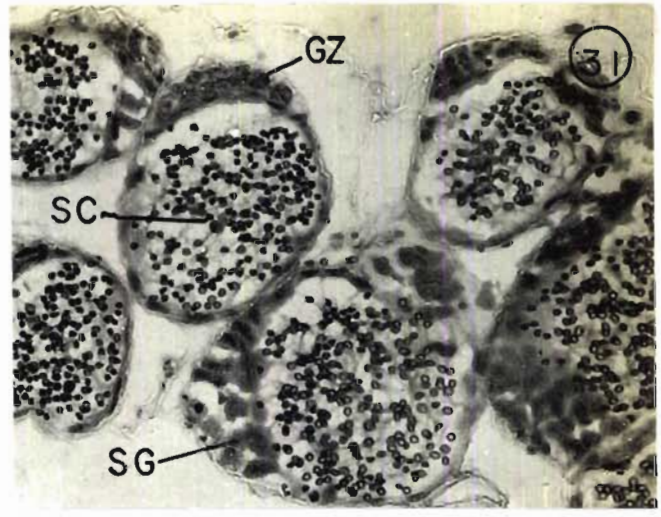
SC
SG
ST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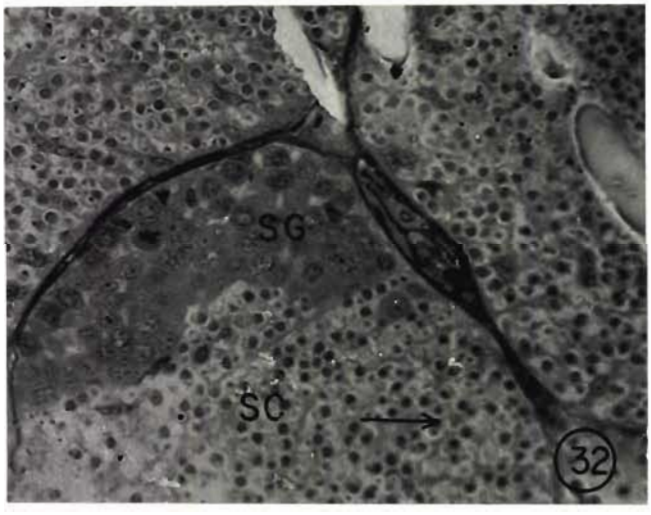
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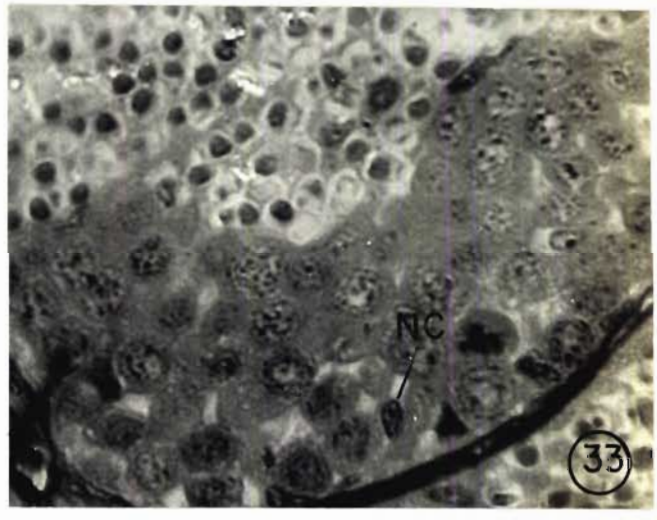
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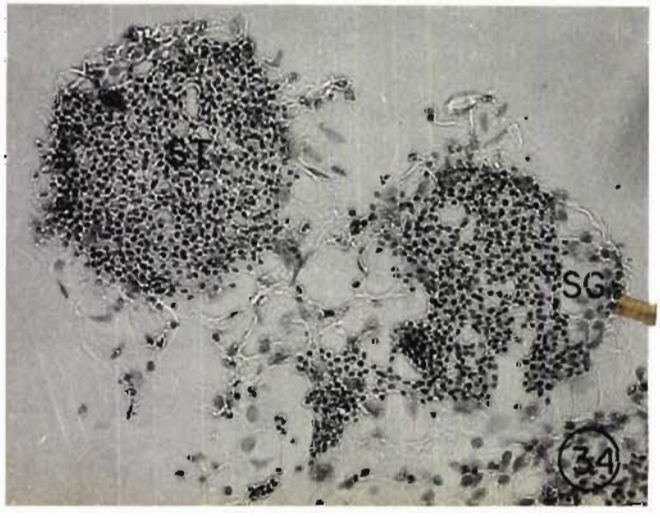
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SG
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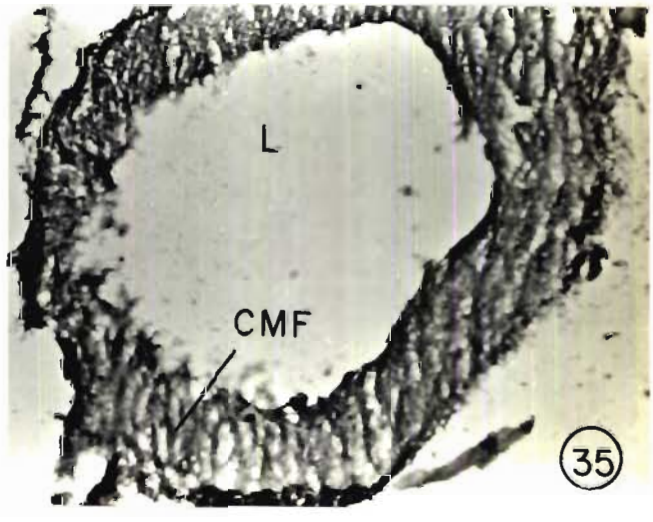
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CMF
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found dispersed in between spermatogonial cells (fig. 33). These small elongate cells were seen to have a prominent nuclei and measured 8 - 12 μ in length and 3 - 5 μ in width. By virtue of their close association with the spermatogonial cells, it is assumed that they have nutritive and supportive role. The spermatocytes were observed to have a more condensed nucleus with a diameter range of 4 - 5 μ (fig. 32). As is common among dividing cells, diakinetic stages were frequently seen among spermatocytes. Spermatids were seen to undergo further reduction in size and condensation of chromatin matter. The spermatozoa developed from these cells through cellular differentiation without further reduction in size. The mature sperm cells were almost spherical in outline with condensed chromatin matter. A 'Y' shaped acrosome vesicle and a short spike were apparent at the apical region of the main body (fig. 51).

4.1. Ultrastructure of the sperm

Electron microscopic studies on the spermatophore revealed that the sperm was embedded in an amorphous sperm matrix (fig.55). The sperm was composed of a spherical main body that was partially encompassed by a morphologically diverse cap region containing the acrosomal complex, from which extended a single spike (fig. 54 and 55). The electron

micrograph (fig. 54) revealed that the main body had a diffuse nuclear region containing chromatin that was partially surrounded by a cytoplasmic band. The nucleus was seen to be separated from the acrosomal complex by an electron dense plate and a less electron dense latticed matrix (fig. 54) . The electron dense spike measured approximately 5 - 7 μ in length. The cap and spike were seen to be bound by a double plasma membrane. Thus the sperm in P.indicus was composed of a nucleus bound by a cytoplasmic band, an electron dense acrosome and a spike.

4.2. Histochemical studies on the testis

The histochemical tests applied to the testis revealed the complex chemical nature of the sperm cells in P. indicus.The cytoplasm of the spermatogonial cells were found to be negative to the PAS test, but small amounts of sulphated and carboxylated AMPs were indicated (Table 8). The nucleus however, showed moderate positivity to the PAS reaction due to the presence of 1,2 glycol groups. The presence of glycogen in the nucleus was revealed by its positivity to Best's Carmine. Both PAS and carmine positivity increased substantially in spermatocytes and

spermatids, with the maxima observed in spermatids and spermatozoa. With the AB-PAS test all the spermatogenic cells except spermatozoa gave a magenta reaction indicating presence of neutral mucopolysaccharides. In the sperm cells only the acrosome gave a magenta reaction and the nucleus gave a blue reaction denoting the presence of AMP, particularly hyaluronic acid and sialomucins. Alcianophilia at low molar concentrations with the critical electrolyte method confirmed the presence of AMPs. The nurse cells responded to the tests in a manner similar to that of spermatogonial cells.

The cytoplasm of the spermatogonial cells were intensely positive to the ferric-ferricyanide test for -SH proteins (Table 9). The cytoplasm was also weakly pyroninophilic indicating the presence of RNA, which however, was not detected in the nucleus or nurse cells. The nuclei were intensely positive to tests for basic and acidic proteins and to amino groups and tyrosine. Due to the diffused nature of the chromatin, fuelgen reaction was very weak in these cells. Spermatocytes and spermatids recorded decreased positivity to all tests for protein end groups. In contrast, progressively increased positivity was observed in the nuclei of spermatocytes and spermatids with the alkaline fast green test for basic proteins. This was presumably due

Table - 8 : Histochemical responses for Carbohydrates in the testis of *P. indicus*.

HISTOCHEMICAL TESTS	EPITHELIAL TISSUE	SPERMATOGONIAL CELLS		SPERMATOCYTES	SPERMATIDS	SPERMATOZOA	NURSE CELLS
		CYTOPLASM	NUCLEUS	NUCLEUS	NUCLEUS		
Schiff Alone	-	-	-	-	-	+	±
Periodic Acid Schiff (PAS)	+++	-	+	++	+++	+++	++
Deamination	++	-	+	++	+++	+++	+
Acetylation	-	-	-	-	-	±	-
Deacetylation	+++	-	+	++	+++	+++	++
Delipidation	+++	-	+	++	+++	+++	++
Diastase digestion	++	-	±	++	++	++	+
Best's Carmine Test	-	-	++	+++	+++	+++	+
Diastase digestion	-	-	+	+	+	+	±
Alcian blue C-E-C Method							
0.1 M	±	±	-	+	±	±	±
0.2 M	-	-	-	+	++	+	+
0.6 M	-	±	-	±	±	-	-
0.8 M	-	-	-	-	-	-	-
1.0 M	-	-	-	-	-	-	-
Alcian Blue - PAS (AB-PAS)	++(M)	-	+(M)	++(M)	+(M)	+++(B) ++(M) Acrosome	++(M)

M - Magenta. B - Blue.

Table - 9 : Histochemical responses for proteins and nucleic acids in the testis of P. indicus.

HISTOCHEMICAL TESTS	EPITHELIAL TISSUE	SPERMATOGONIAL CELLS		SPERMATOCYTES	SPERMATIDS	SPERMATOZOA	NURSE CELLS
		CYTOPLASM	NUCLEUS	NUCLEUS	NUCLEUS		
Mercuric Bromophenol Blue	+++	+	+++	++	++	+	++
Aq.Bromophenol Blue	+	-	+++	+++	++	++	++
Deamination	-	-	++	++	+	-	+
Aq.Toluidine Blue	-	-	+++	+++	++	+	++
Methylation	-	-	-	-	-	-	-
Ninhydrin Schiff Test	-	-	++	+	+	++	-
Deamination	-	-	-	-	-	-	-
Ferric Ferricyanide Test	+	+++	+	++(PN)	++(PN)	+	-
Mercaptide	-	+	-	+	+	+	-
Performic Acid Alcian Blue	±	±	-	-	-	-	±
Alcian Blue Alone	-	-	-	-	-	-	-
Millon's Test	-	-	++	+	+	-	-
Iodination	-	-	-	-	-	-	-
DMAB-Nitrite Test	-	±	+	±	±	-	-
Formaldehyde	-	-	-	-	-	-	-
Methyl Green Pyronin	+(R)	+(R)	++(G)	++(G)	+++(G)	+++(G)	+(G)
10% Perchloric	+(R)	-	+(G)	+(G)	++(G)	++(G)	+(G)
Fuelgen reaction	-	-	+	++	+++	+	+++
Alkaline Fast green	-	-	+	++	+++	+	++

PN - Perinuclear, R - Red, G - Green.

Table - 10 : Histochemical responses for lipids in the testis of P. indicus

HISTOCHEMICAL TESTS	EPITHELIAL TISSUE	SPERMATOGONIAL CELLS		SPERMATOCYTES	SPERMATIDS	SPERMATOZOA	NURSE CELLS
		CYTOPLASM	NUCLEUS	NUCLEUS	NUCLEUS		
Sudan Black B Test	±	-	+	+	++	++	±
Delipidation	-	-	-	-	-	-	-
Nile Blue method	±	-	+(B)	++(B)	++(B)	+++(B)	+(B)
Delipidation	-	-	-	-	-	++	-
Baker's Acid Hematein	±	-	±	±	±	+	+
Pyridine extraction	-	-	-	-	±	-	-
Oil Red O method	±	-	+	+	+	+	±
Delipidation	-	-	-	-	-	-	-
UV Schiff Reaction	-	-	+	+	+	+	±
non UV	-	-	+	±	+	±	±

B - Blue.

to the high arginine and lysine content in them. Maximum DNA content as indicated by fuelgen positivity was observed in the spermatid and spermatozoa stage (fig. 34). Nurse cells were observed to be strongly toluidine blue positive due to the presence of acidic proteins.

All the spermatogenic developmental stages were poor in lipids (Table 10). The spermatozoa were moderately sudanophilic and weakly positive to tests for acidic, neutral and phospholipids. In general the spermatozoa of P. indicus had a remarkable abundance of polysaccharides and basic proteins like arginine and lysine.

5. Histology of the testicular ducts and formation of spermatophores

Histological investigations on the entire testicular duct revealed the detailed mechanism of spermatophore formation in P.indicus. This process was found to take place inside the vas deferens ducts with the aid of the glandular epithelial cells lining them. The histological structure of the proximal, mid and distal vas deferens and the terminal ampoule is described below.

5.1 Proximal vas deferens (PVD)

The PVD was observed to be a short slender tube leaving the posterior margin of the main axis of the testis (fig.1B). The duct which had an outer connective tissue sheath (fig.35), measured 1 - 1.5 mm in diameter. Thick (350 μ) circular muscle fibres were seen lining the inner wall of the duct. No glandular cells were observed in this portion of the deferent ducts. Sperm cells were rarely noticed and most of the time the lumen was empty. It appeared from the histological structure that the PVD's main function was to transport the mature sperms from the testis to the MVD through peristaltic movements.

5.2 **Mid vas deferens (MVD)**

The PVD further dilates to form the blind pouch of the MVD (fig.1B) which in turn continues anteriorly as the ascending limb. The blind pouch functions as a storage site for the sperms. No specialized glandular epithelium was observed in this duct (fig.41), instead a muscular lining was present. The MVD at this point measured 4-5 mm in diameter.

The ascending limb of the MVD was divided internally into two unequal ducts by a connective tissue septum (fig.36). The larger of the ducts, viz., the sperm duct

measured 2-3 mm in diameter, through which the sperm mass passes for the deposition of spermatophoric layers (figs. 36 and 38). The sperm duct was lined with glandular epithelial cells which secrete the spermatophore matrix and the spermatophore layers (fig.37). The glandular cells were observed to have a mean diameter of 13.4 $\pm$ 2.3 μ. The cytoplasm was seen as granular and extremely vacuolated. A single round vesicular nucleus with an eccentric nucleoli was observed in the secretory cells. These cells had a basophilic secretion which was deposited around the sperm mass as spermatophore layer I (figs. 36 and 37). In order to provide more surface area for the glandular secretory activity, a large typhlosole with blood vessels was observed in the sperm duct (fig.39). Cross sections of the descending limb revealed that after the formation of the spermatophore layer I, the sperm mass was arranged in pouches within the matrix (fig.40). The smaller wing duct of the ascending limb of MVD measured 0.5 mm in diameter. The wing duct secretory cells were identical to that found in the sperm duct, and are involved in the secretion of the wing of the spermatophore. The septum which divided the two ducts although complete initially (fig.38) was open at one end in the descending limb (fig.36). This facilitates the wing to be attached to the main body of the spermatophore. A smaller typhlosole was observed in the wing duct to aid in

the secretion of the amorphous wing (figs.38 and 42). The descending limb of the MVD gets constricted and becomes narrow to continue as the DVD.

5.3 Distal vas deferens (DVD)

This long slender tube proceeding to the terminal ampoule measured approximately 0.5 mm in diameter. In transverse sections circular muscle fibres were evident beneath the outer connective tissue sheath (fig.43). The inner wall of the DVD was lined with secretory glandular epithelial cells, similar in structure to that found in the MVD. The secretion of the outer spermatophore layer II takes place in this duct with the help of epithelial cells (fig.44). Highly convoluted typhlosoles in addition to smaller typhlosoles were noticed in the lumen of the duct (fig.43), to facilitate the deposition of layer II in the narrow DVD. Layer II was deeply acid fuchsin positive with Mallory's triple stain.

5.4 The terminal ampoule (TA)

The DVD dilates to form the terminal ampoule or the ejaculatory duct which was found embedded in the coxal muscles of the 5th pereiopod. The final and complete

moulding of the spermatophore takes place in this ampoule. Fig.45 shows the completely formed amorphous wing in the TA. A very thick outer layer of circular muscles and an inner layer of longitudinal muscles formed the walls of the terminal ampoule, the inside of which was lined with secretory epithelial cells. (fig.46 and 53). While the muscular walls of the TA help in the forceful ejaculation of the spermatophore, the secretion of the glandular epithelial cells formed the adhesive globules required to cement and join the parts of the spermatophores ejaculated from the TA on either side.

The formation of the speratophore and the role played by each region of the vas deferns is summarized as given below.

Region of Vas Deferens		**Role played in spermatophore formation**
1. Proximal vas deferens	-	Transport of sperms from testis to MVD by peristaltic movements
2. Mid vas deferens	-	
Blind pouch	-	Accumulation and storage of sperms.
Ascending & descending limbs.	-	

Sperm duct	-	Secretion of the spermatophore layer I (Sph.I) and sperm matrix. Packing of sperm mass into lobules.
Wing duct	-	Secretion of the amorphous wing of the spermatophore.
3. Distal vas deferens	-	Secretion of the spermatophore layer II (Sph.II).
4. Terminal Ampoule	-	Secretion of the adhesive globules, final moulding of the spermatophore and ejaculation.

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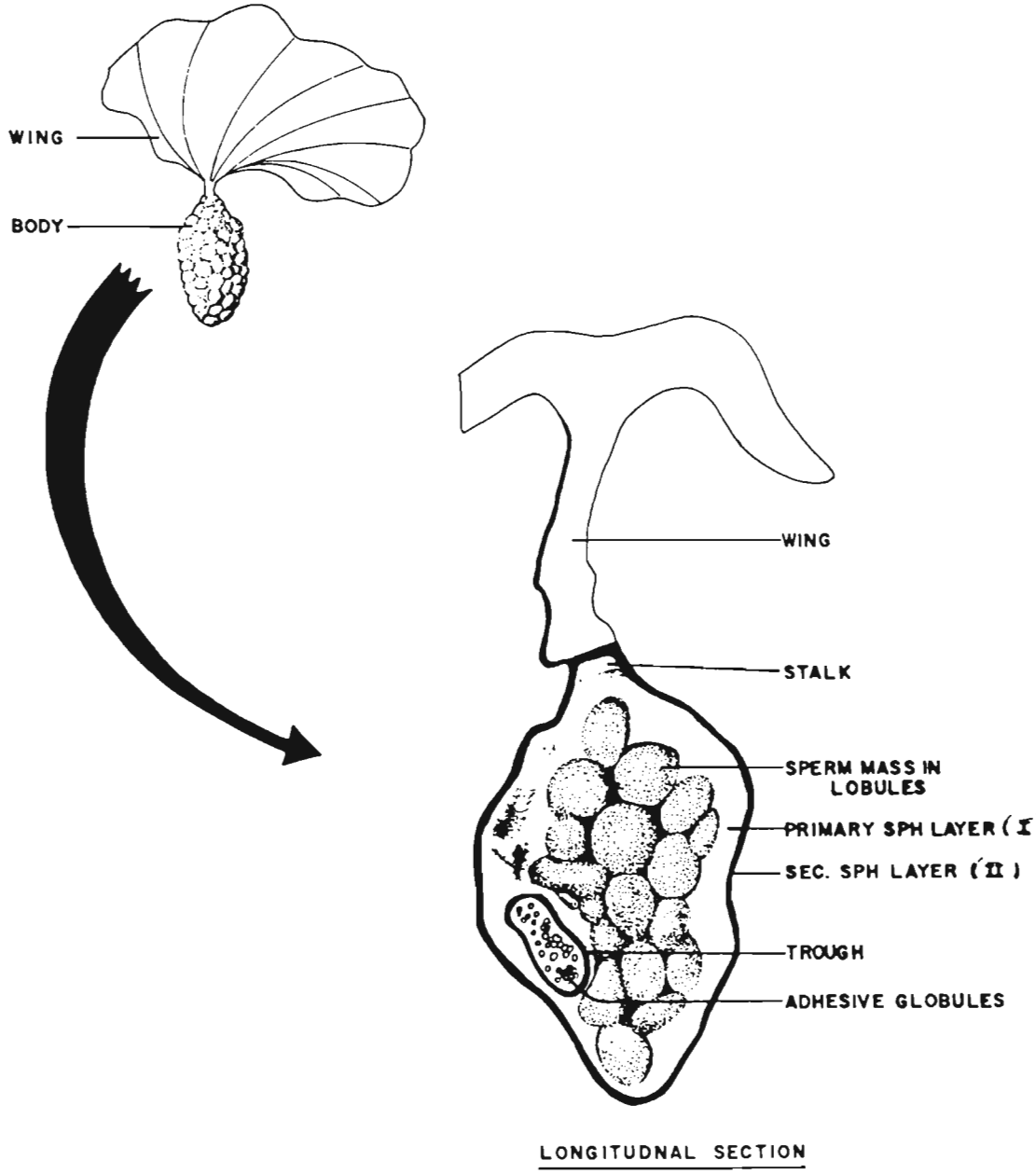
6. Structure and chemical composition of Spermatophore

A freshly extruded spermatophore of P. indicus was found to be roughly oval in shape and measured approximately 3.5 - 4.0 mm in length and 2.0 - 2.5 mm in breadth. Externally the body of the spermatophore which had a dirty cream colour was thrown into folds resembling a bag full of grapes (fig. 29). The translucent parachute like wing was found to be 9 mm long 9-10 mm wide and was connected to the body by means of a short stalk (fig. 29).

Longitudinal sections of the spermatophore stained with Mallory's triple stain revealed the complex structure inside. The sperm mass was embedded in an aniline blue positive matrix in distinct oval lobules which measured

FIG. 29 STRUCTURE OF EXTRUDED SPERMATOPHORE



301.9 ± 71.9 μ in diameter (fig.51). The spermatophore layer I showed deep aniline blue positivity and was observed to be of variable thickness (fig.52). The spermatophore layer II which is the outer bounding layer was 21.3 μ thick and deeply acid fuchsin positive (fig.52). On the lateral edge of the body of the spermatophore a sheathed trough was present in which the adhesive globules were seen (fig.29). The sheath was also observed to be acid fuchsin positive and identical to layer II (fig. 49 and 52). Adhesive globules which were spherical in outline and aniline blue positive had a mean diameter of 7.8 μ (fig.50). The stout stalk attaching the wing to the body was observed as an outward extension of spermatophore layer I (fig. 48). The wing had an amorphous structure and was weakly stained (fig. 47).

Ultrastructural studies showed that the moderately electron lucent spermatophore layer I was composed of a homogenous flocculent material (fig. 56). In contrast the spermatophore layer II was structurally electron dense and composed of small filaments which were arranged compactly.

6.1 Histochemical reactions of the spermatophore

The histochemical tests applied to the spermatophore

Fig.36 Transverse section of the ascending limb of the MVD showing the septum (ST) which divides the wing duct (WD) and the sperm duct (SD). S - sperm. Haematoxylin and Eosin. x50.

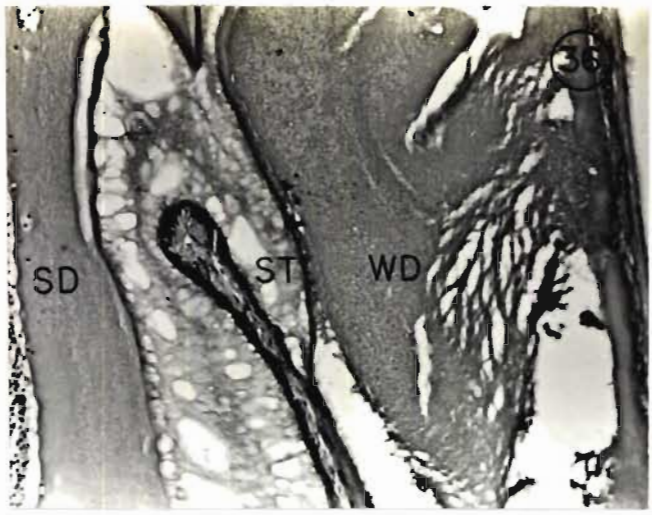
Fig.37 Photomicrographic detail of the sperm duct. The wall of the duct is lined with glandular epithelial cells (EC) which secrete the spermatophore matrix (SM). S - sperm. Haematoxylin and Eosin. x100.

Fig.38 Photomicrographic detail of the wing duct (WD) with a typhlosole (TS) and amporphous secretion (AS). Haematoxylin and Eosin. x50.

Fig.39 Photomicrograph of the typhlosole (TS) in the sperm duct with well developed blood vessels (BV). EC - epithelial cells, S - sperm. Haematoxylin and Eosin. x50.

Fig.40 Cross section of the descending limb of the MVD revealing the arrangement of sperm (S) in pouches and the primary spermatophore layer (PSL). Haematoxylin and Eosin. x50.

Fig.41 Transverse section of the blind pouch which functions as a storage site for the sperms (S). A muscular wall (M) is apparent. Haematoxylin and Eosin. x200.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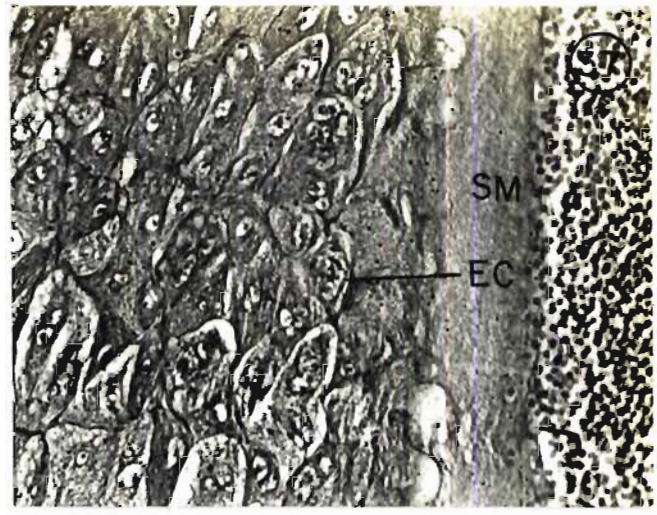
SD
ST
WD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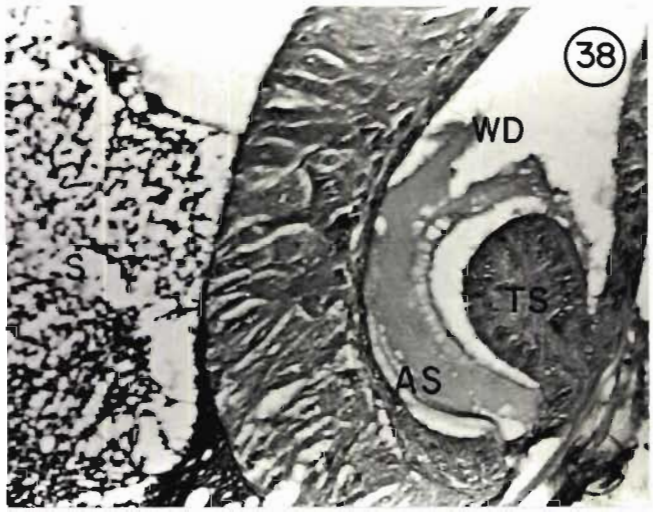
EC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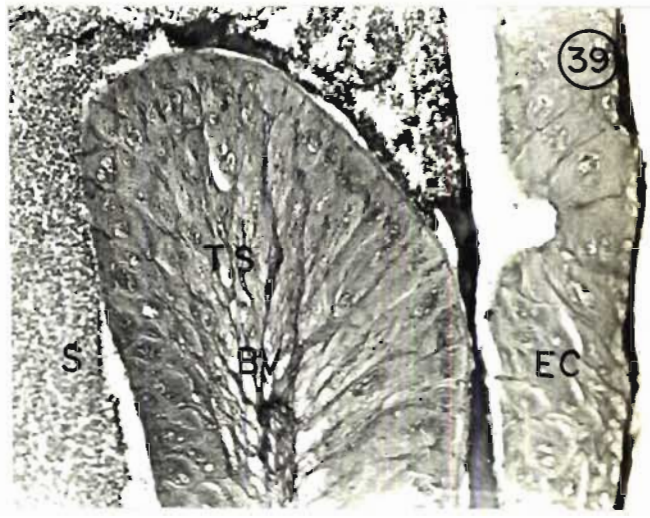
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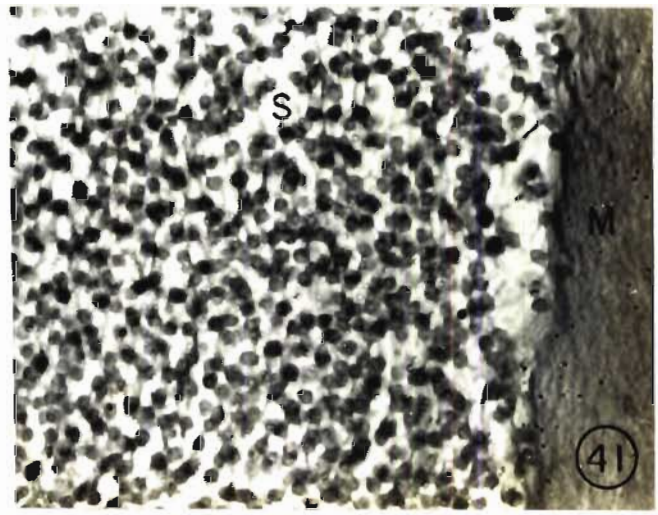

S
M
41

Fig.42 The second typhlosole (TS) in the wing duct (WD) with the amorphous wing (W) in the descending limb of MVD. Haematoxylin and Eosin. x100.

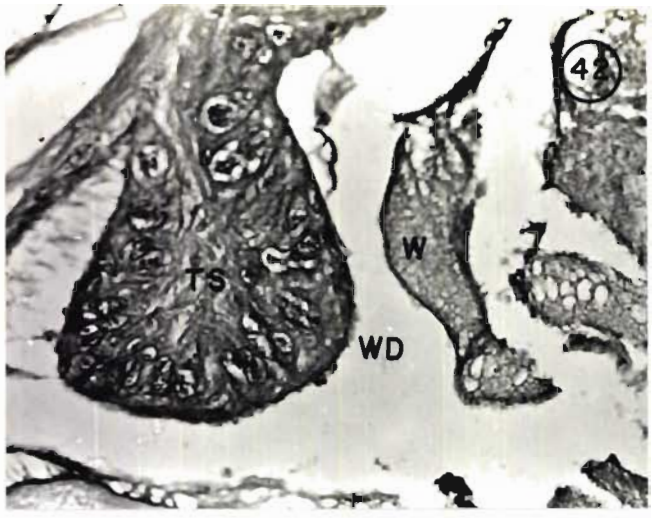
Fig.43 Cross section of the DVD showing the connective tissue sheath (CT) and the circular muscle lining (CMF). The epithelial cells are thrown into a highly convoluted typhlosole (TS). The formation of the secondary spermatophore layer (SSL) is also seen. Mallory's triple stain. x50.

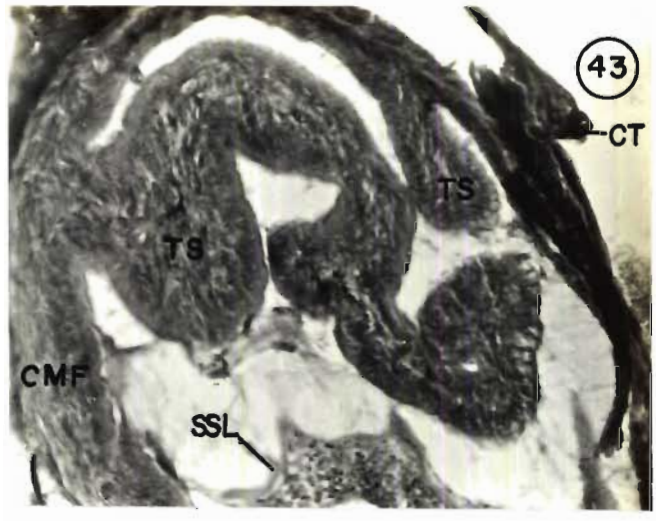
Fig.44 The formation of the acid fuchsin positive secondary spermatophore layer (SSL) in the DVD. S - sperms. Mallory's triple stain. x50.

Fig.45 Photomicrograph of the fully formed, lightly stained amorphous wing (W) in the terminal ampoule. Mallory's triple stain. x50.

Fig.46 Transverse section of the wall of the terminal ampoule showing the outer circular muscle fibres (CMF) and inner logitudinal muscle fibre (LMF). The secretary epithelial cells secrete the adhesive globules (AG). Mallory's triple stain. x50.

Fig.47 Longitudinal section of the parachute-like amorphous wing (W). Mallory's triple stain. x50.


42
TS
W
WD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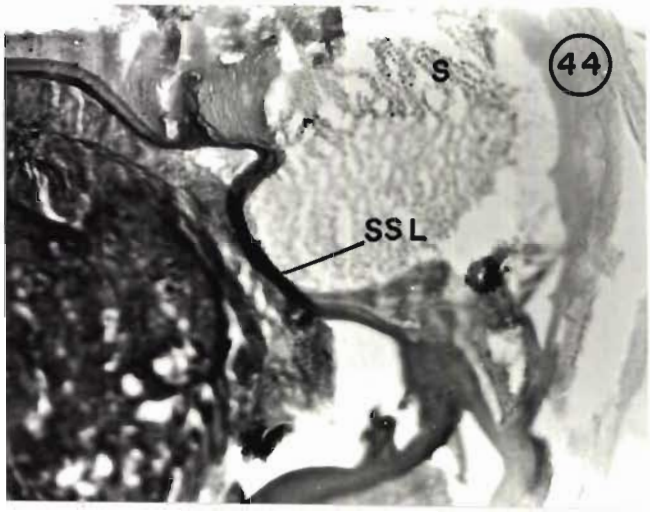
CT
TS
TS
CMF
SSL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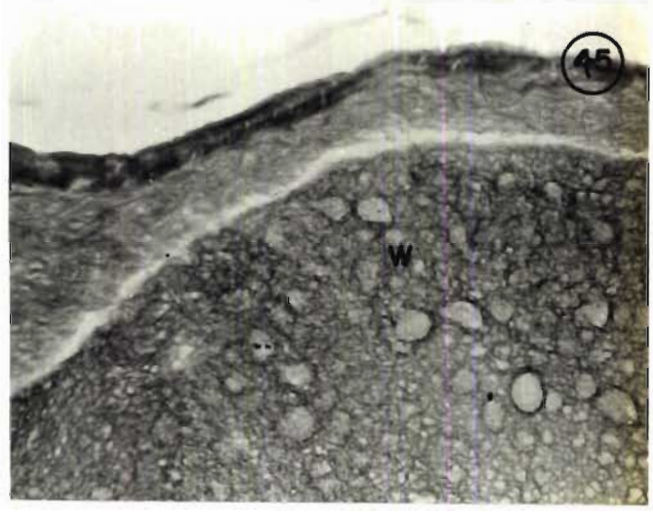
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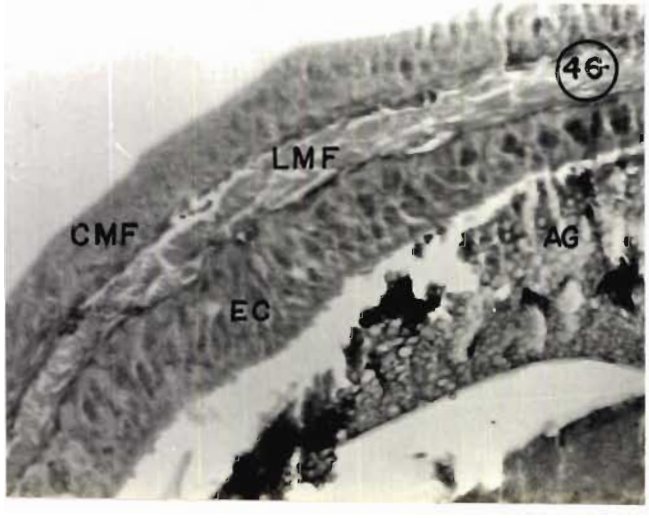
W


46
LMF
CMF
AG
EC

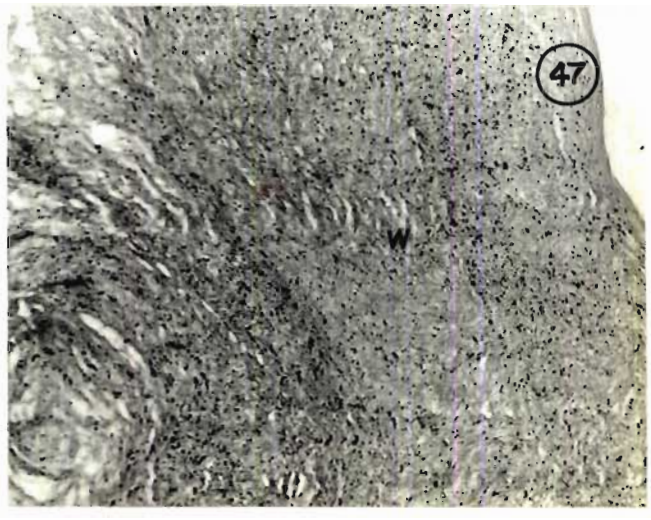
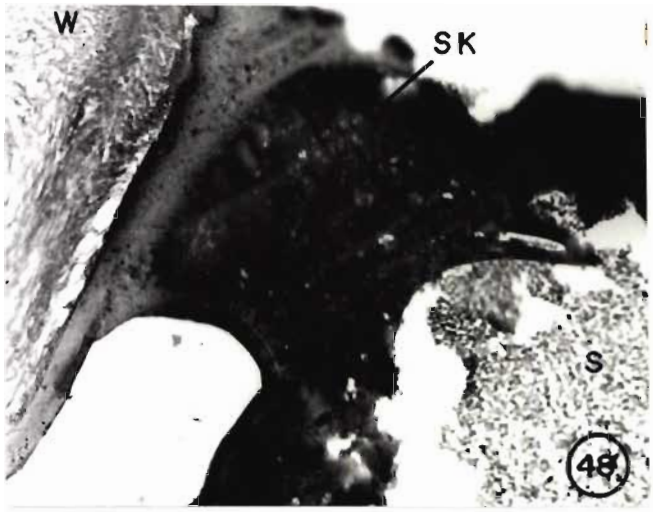

47
W

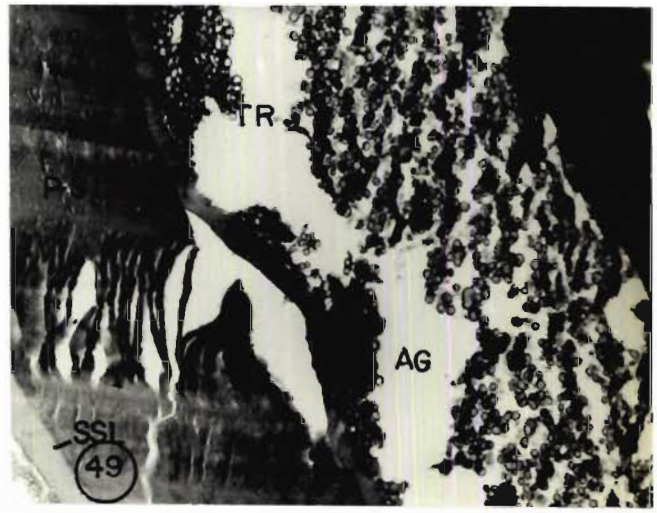
Fig.48 Light micrograph of the stalk (SK) which attaches the body of the spermatophore (S) to the wing (W). Mallory's triple stain. x50.

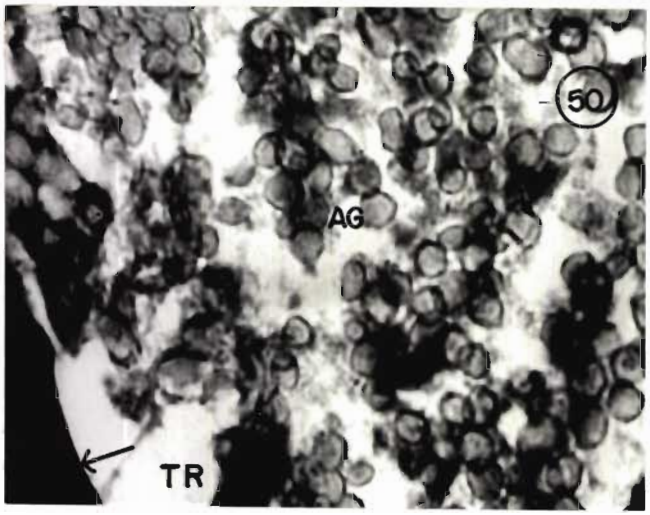
Fig.49 Photomicrograph of the wall of the spermatophore showing the secondary spermatophore layer (SSL) and primary spermatophore layer (PSL). A trough (TR) with adhesive globules is also seen (AG). Mallory's triple stain. x50.

Fig.50 Detail of the adhesive globules (AG) in the trough (TR). The TR was also covered by a sheath (arrow head). Mallory's triple stain. x200.

Fig.51 Photomicrograph of the sperm mass in the sperm matrix (SM). The 'Y' shaped acrosome (AC) is clearly seen. Mallory's triple stain. x200.


W
SK
S
48


TR
AG
SSL
49


50
AG
TR

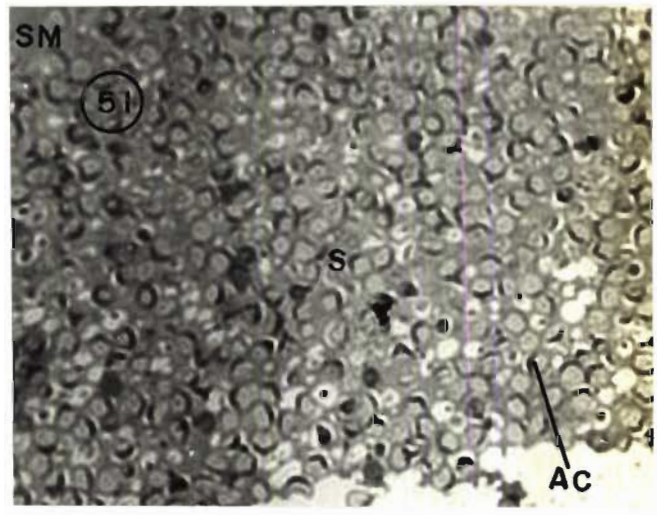
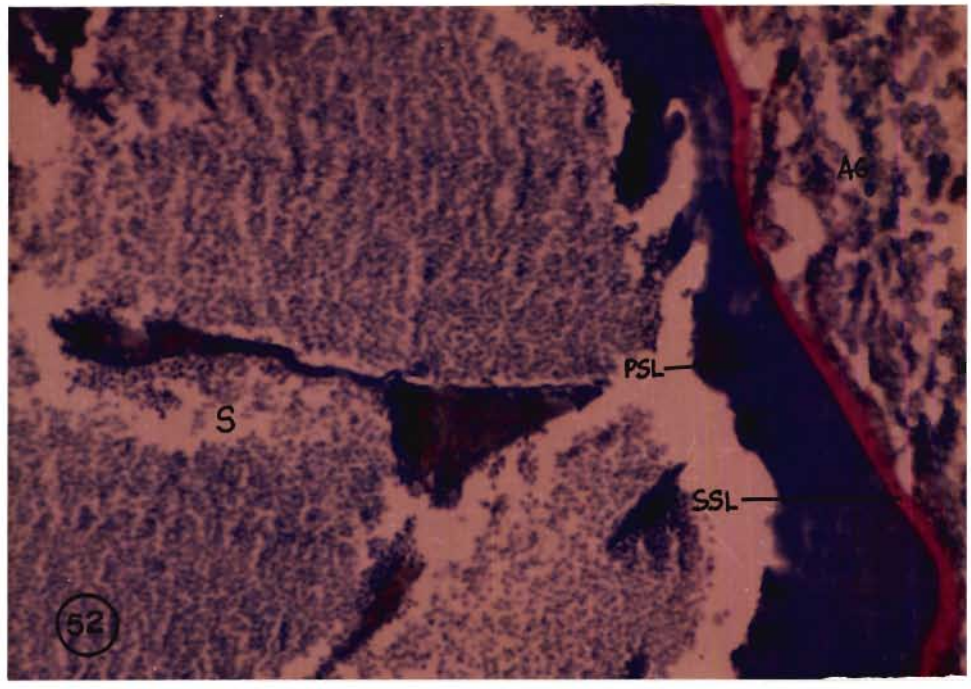

SM
51
S
AC

Fig.52 Mallory's triple stained spermatophore showing primary (PSL) and secondary (SSL) spermatophore layer. Sperms (S) in lobules and adhesive globules (AG) are also seen. x50.

Fig.53 Cross section of the TA showing circular muscle fibre (CMF), longitudinal muscle fibre (LMF) and glandular epithelial cells (EC). The wing (W) is seen in the lumen. Mallory's triple stain. x50.


AG
PSL
S
SSL
52

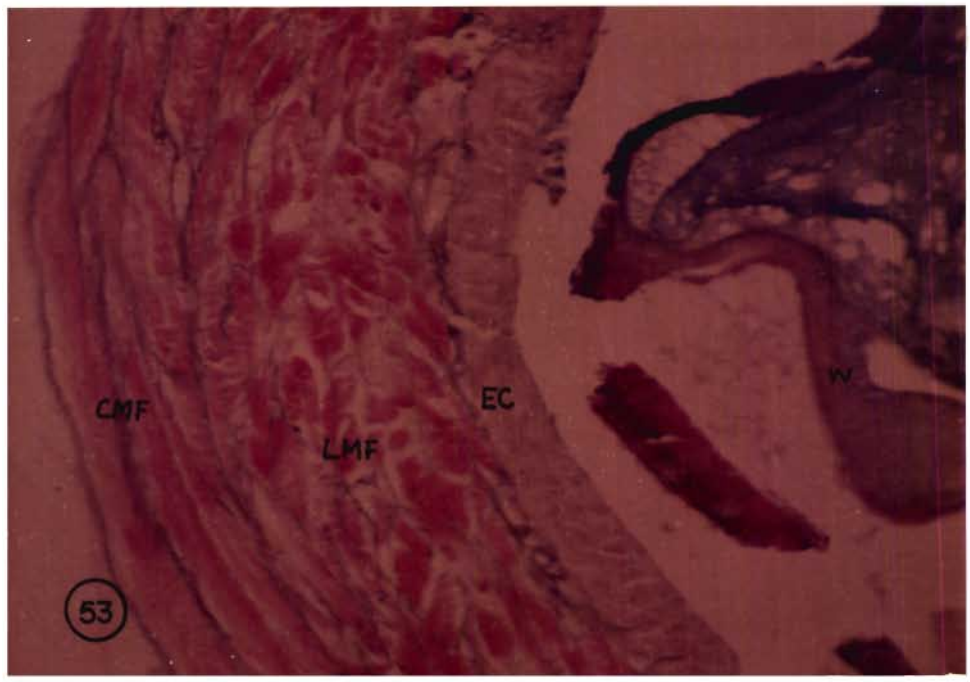
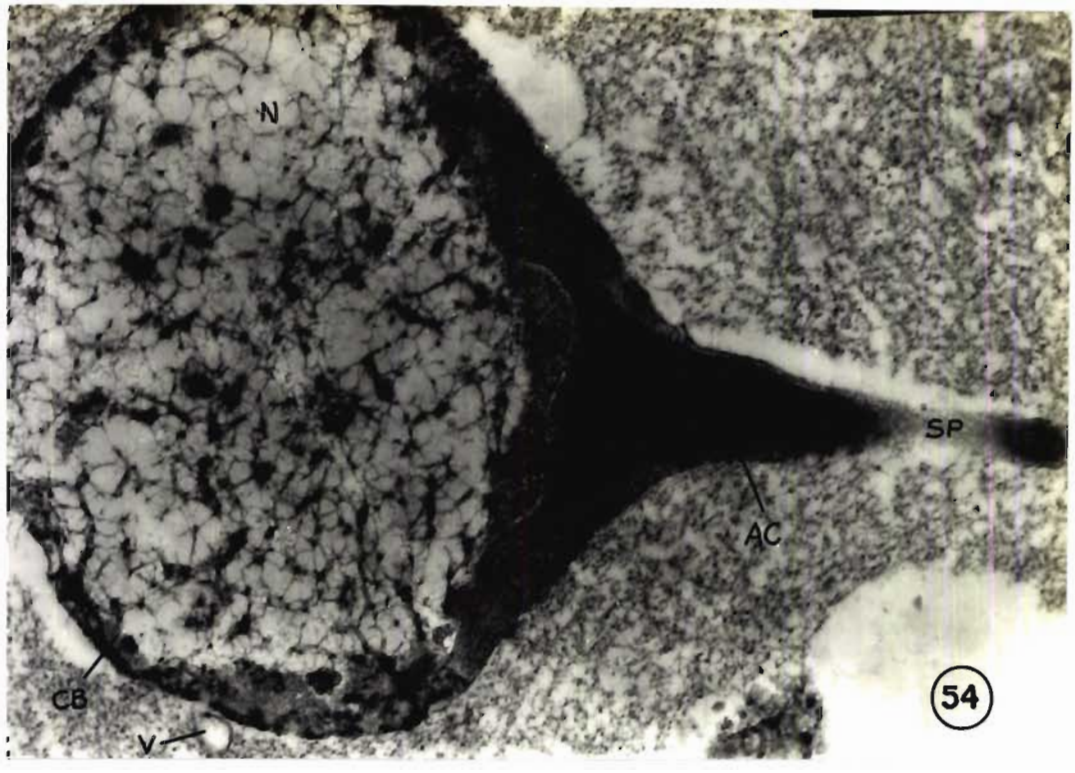
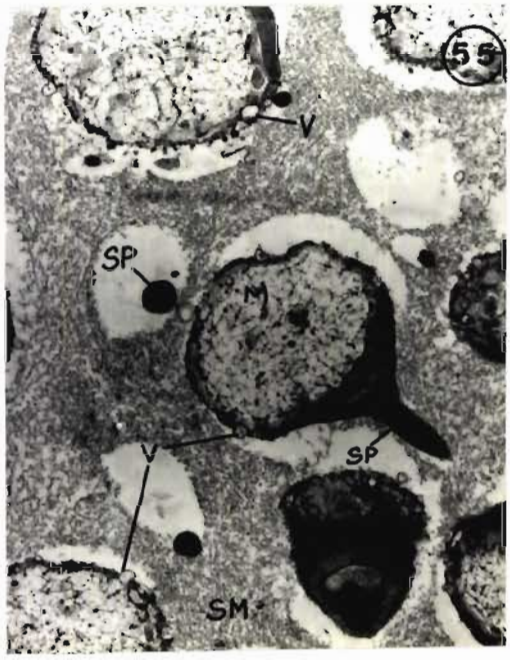

CMF
LMF
EC
W
53

Fig.54 Electronmicrograph of the mature sperm in the spermatophore. N - nucleus, CB - cytoplasmic band, V - vesicles, LM - latticed matrix, SP - spike. x9000.

Fig.55 Electronmicrograph of the mature sperms in the spermatophore matrix (SM). N - nucleus, SP - spike, V - vesicle. x4000.

Fig.56 Fine structure of the primary (PSL) and secondary (SSL) spermatophore layers. Note the difference in densities. x2700.


N
SP
AC
CB
V
54


55
V
SP
M
V
SP
SM


56

revealed the chemical nature of the spermatophore layers as well as the sperm matrix and wing.

Both the spermatophore layers were PAS positive, the outer Sph layer II being intensely so (Table 11). The positivity was due to the presence of 1,2 glycol groups as revealed by the acetylation + PAS test. The outer bounding layer was also strongly carmine positive whereas the inner spermatophore layer I was carmine negative. The outer wall of the spermatophore was mainly composed of neutral mucopolysaccharides as evident by the magenta reaction with the AB-PAS combination. Both sulphated and carboxylated mucosubstances were demonstrated particularly in the matrix by the alcianophilia at low molar concentrations. The wing did not respond to any of the carbohydrate tests applied. The adhesive globules were intensely PAS positive, but acetylation failed to block them completely, suggesting their possible conjugation with proteins. Further, these globules were partially diastase resistant and delipidation reduced the PAS positivity suggesting also their lipid nature.

Sulphur containing amino acids (-SH and -S-S groups) were the only proteins detected in abundance in both the

Table - 11 : Histochemical responses for Carbohydrates in the extruded spermatophore (SPH) of *P. indicus*.

HISTOCHEMICAL TESTS	SPH LAYER I	SPH LAYER II	SPH MATRIX	SPH WING	ADHESIVE GLOBULES
Schiff alone	-	-	-	-	-
Periodic Acid Schiff (PAS)	+	+++	++	-	+++
Deamination	+	++	+	-	++
Acetylation	-	-	-	-	++
Deacetylation	+	+++	++	-	+++
Delipidation	+	+++	++	-	++
Diastase digestion	±	++	+	-	+
Best's Carmine Test	-	++	+	-	+++
Diastase digestion	-	-	-	-	++
Alcian Blue C-E-C Method					
0.1 M	+++	++	+	±	-
0.2 M	++	+	+	-	-
0.6 M	-	-	-	-	-
0.8 M	-	-	-	-	-
1.0 M	-	-	±	-	-
Alcian Blue - PAS (AB-PAS)	+(M)	++(M)	+(B)	±(B)	+(M)

M - Magenta, B - Blue.

Table - 12 : Histochemical responses for proteins and nucleic acids in the extruded spermatophore (SPH) of P. indicus.

HISTOCHEMICAL TESTS	SPH LAYER I	SPH LAYER II	SPH MATRIX	SPH WING	ADHESIVE GLOBULES
Mercuric Bromophenol Blue	+	+	+	+(PP)	+
Aq. Bromophenol Blue	-	-	-	+	-
Deamination	-	-	-	-	-
Aq. Toluidine Blue	-	-	-	-	-
Methylation	-	-	-	-	-
Ninhydrin Schiff Test	±	-	-	-	+++
Deamination	-	-	-	-	-
Ferric Ferricyanide Test	+++	++	++	+	++(PP)
Mercaptide	+	+	+	-	+
Performic Acid Alcian Blue	+++	++	++	-	-
Alcian Blue alone	-	-	-	-	-
Millon's Test	±	±	-	-	-
Iodination	-	-	-	-	-
DMAB - Nitrite Test	-	-	-	-	-
Formaldehyde	-	-	-	-	-
Methyl Green Pyronin	±(R)	+++(R)	++(R)	-	+++(R)
10% Perchloric	-	++(R)	+(R)	-	+++(R)
Fuelgen Reaction	-	-	-	-	-

PP - Peripheral, R - Red.

Table - 13: Histochemical responses for lipids in the extruded spermatophore (SPH) of P. indicus.

HISTOCHEMICAL TESTS	SPH LAYER I	SPH LAYER II	SPH MATRIX	SPH WING	ADHESIVE GLOBULES
Sudan Black B Test	-	-	+	-	±
Delipidation	-	-	-	-	-
Nile Blue Method	±	-	+	-	+++(B)
Delipidation	-	-	+	-	+++
Baker's Acid Hematein	+++	+++	+	-	+++(PP)
Pyridine extraction	+	+	±	-	+
Oil Red O Method	±	-	±	-	-
Delipidation	-	-	-	-	-
UV Schiff reaction	±	±	±	-	+
non UV	±	±	±	-	+

B - Blue, PP - Peripheral.

spermatophore layers and matrix (Table 12). The outer layer alone was weakly positive to Millon's test indicating the presence of small quantity of tyrosine. The wing was weakly positive to tests for basic proteins. An accumulation of amino groups in the adhesive globules was demonstrated by the Ninhydrin Schiff test. Spermatophore layers were deeply acid hematein positive indicating the strong presence of phospholipids (Table 13). Phospholipids were also indicated in the adhesive globules.

DISCUSSION

In P.indicus the present investigations revealed that the ovaries are paired, partly fused, bilaterally symmetrical bodies, extending from the cardiac region to the telson. These observations are remarkably similar to that made by King (1948) in P. setiferus. Similar observations on the morphology of the penaeid ovaries have been made by Cummins (1961), Tuma (1967), Rao (1968) and Duronslet et al (1975). Evidently the general structure of the female reproductive system in penaeids are homologous with little inter-specific variation. In females five maturity stages have been recognized based on ovarian size, appearance, colour, oocyte diameter and GSI in the present study.

Observations on maturity stages in most crustacean groups are not consistent, however, in penaeids the maturity stages generally fall into five groups - immature, early maturing, late maturing, mature and spent with little modifications (Tuma, 1967; Rao, 1968; Brown and Patlan, 1974; Duronslet et al., 1975; Aquacop, 1977 and Primavera, 1984).

Two protective layers were observed to encompass the ovary in the present study. In P. setiferus, King (1948) also observed a similar arrangement with an outer layer of pavement epithelium and an inner layer of connective tissue. Further in P. indicus the connective tissue layer was observed to be invaded with blood capillaries, ostensibly for the transport of nutrients. There is wide variation in the placement of the germinal zone in the ovary among crustaceans (Adiyodi and Subramoniam, 1983). In P. indicus it was observed to be consistently ventro-median in position similar to that seen in P.setiferus by King (1948) and in P.japonicus by Yano (1988). The germinal zone was not described by Duronslet et al. (1975) in P. aztecus.

As in other crustaceans, the process of oogenesis in P. indicus too was completed by the involvement of two main

processes. The first was the proliferative phase wherein the primary oogonial cells multiplied to form secondary oogonial cells. The second was the differentiative phase, when immature oocytes developed from meiotic division of the secondary oogonial cells, accumulates yolk and develops into the mature oocytes. The germinal zone was observed to be present in ovaries in all maturity stages indicating that the ovary is active throughout the reproductive period of the female. Identical observations have been made in other crustaceans (Adiyodi and Subromoniam, 1983) and in the penaeid, P. japonicus (Yano, 1988). Descriptions of the oogonial cells in penaeids as well as other crustaceans are rare (Fyhn and Costlow, 1977). Histochemical studies showed that the oogonial cells of P. indicus were rich in RNA and protein but poor in carbohydrates. A corresponding situation was met with in the oogonial cells of Balanus amphitrite by Fyhn and Costlow (1977). Further, in P. indicus, oogonial cells were devoid of the follicle cell covering and were present in clusters.

The cytological changes observed during different maturity stages of the ovary in P. indicus bear close resemblance to that occuring in the oocytes of P. setiferus (King, 1948), P. stylifera (Shaikhmahmud and Tembe, 1958),

P. indicus (Subrahmanyam, 1965) and P. aztecus (Duronslet et al., 1975). In P. indicus during the early and previtellogenic phases the nuceloli were deeply RNA positive and was observed along the peripheral margin of the nuclear membrane. Further it was clear from the present study that considerable amount of this RNA material is extruded out into the perinuclear cytoplasm through nuclear pores. Such nuclear emissions are not uncommon in early vitellogenic oocytes in the autosynthetic phase of various crustaceans (Adiyodi and Subromoniam, 1983). The transfer of nuclear material to the ooplasm acts as a prelude to protein synthesis. In addition the presence of numerous free ribosomes, rough ER and mitochondria in the cytoplasm indicated that the ovary of P. indicus during Stage I and II had autosynthetic capabilities. The progressive development of an elaborate array of rough ER is found to be an important characteristic of the early vitellogenic oocytes of many crustaceans like the mole crab, Emerita analoga, true crabs, Pachygrapsus crassipes, Cancer sp. and the lobsters Homarus and Panulirus, which are reported to rely predominantly on autosynthesis (Kessel, 1968 and Adiyodi and Subromoniam, 1983).

In P. indicus there was a decided shift in the mode of

yolk synthesis to extra-oocytic or heterosynthesis, when the oocytes advanced to the late vitellogenic phase. Evidence for this is derived from the presence of pinocytotic vesicles along the oolemmal margin. Further the cell organelles like ER and mitochondria become scarce in the ooplasm. In the spider crab Libnia emarginata, Hinsch and Cone (1969), have described a similar shift in emphasis during late vitellogenesis due to micropinocytotic absorption. Such a temporal separation of intraoocytic and extra-oocytic origin of yolk is well defined in Orchestia gammarella (Zerbib, 1977). The formation of yolk platelets in P. indicus is apparently identical to that observed in the Crayfish Cambarus (Beams and Kessel, 1963), Libnia emarginata (Hinsch and Cone, 1969) and Cancer pagurus (Eurenius, 1973).

Folliculogenesis or the investment of follicular cells around the oocytes, in P. indicus was initiated in previtellognic oocytes and completed by early vitellogenic phase. The flattening of the follicle cells with the growth in volume of the oocyte as observed in the present study was characteristic of the process. According to Charniaux-Cotton (1975) follicle cells facilitate vitellogenic activity by aiding in the uptake of yolk protein from external sources.

Apparently in P. indicus too, the follicle cells function in a similar manner. Added evidence for this phenomenon was the observation of pinocytotic vesicles along the oolemma in late vitellogenic phases. Evidence for the protein synthetic ability in follicle cells was obtained by the presence of rough ER and mitochondria in their cytoplasm. In P. japonicus, the follicle cells were implicated as the possible cell type responsible for ovarian vitellogenin synthesis (Yano and Chinzei, 1987). An ultrastructural study on the follicle cells of the amphipod Orchestia gammarella by Rateau and Zerbib (1978) also drew similar conclusions.

Follicle cells displayed decreasing PAS positivity with the progress in maturity. The positivity was maximum in spent follicle cells. This indicated that apart from protein, follicle cells may be involved in synthesis and mobilization of carbohydrate substances into the ooplasm for incorporation as the carbohydrate moeity of the yolk. Follicle cells of P. indicus were exceptionally rich in AMP in all stages of maturity. One of the well documented functions of AMP in vertebrates is that of allowing a ready diffusion of dissolved substances between cells and capillaries (Hohnke and Scheer, 1970). Therefore it is probable that the follicle cells may facilitate an exogenous

supply of yolk material as suggested by Varadarajan and Subramoniam (1980) in the case of the anomuran crab C. clibanarius.

A striking feature of the mature oocyte in P. indicus was the appearance of rod-like cortical bodies in the cortex of the oocytes. Termed variously as marginal bodies (Subrahmanyam, 1965), cortical rods (Duronslet et al., 1975), peripheral bodies (King, 1948 and Rao, 1968), jelly substance (Hudinaga, 1942) and cortical crypts (Yano, 1988), these are unique to the genus Penaeus. They do not occur in the ovaries of Parapenaeopsis stylifera (Shaikhmahmud and Tembe, 1958). In a study on the post-vitellogenic changes in P. aztecus, Clark et al. (1980) demonstrated that the cortical bodies are responsible for the jelly layer which surrounds the egg during early development. The feather-like substructure observed in the cortical body of P. indicus obviously aids in the formation of the jelly layer. Histochemically the cortical bodies were rich in sulphur containing amino acids (-SH and -S-S groups) and sulphated and carboxylated AMP. According to Meyer (1947), non-sulphated AMP controls the permeation of water (through gel forming properties) and protects the egg against bacterial attack. Thus the AMP of the jelly layer may have these

roles plus the conventional role of cell construction during morphogenesis. The presence of disulphide and sulphydryl bonds may perform a role in aiding cytokinesis in penaeid eggs. In the sea urchin it has been suggested that thiol-disulphide (-SH, -S-S) interaction on proteins may account for the contraction of the equatorial ring during cleavage (Sakai, 1960).

The histochemical reactions of the developing oocytes in P. indicus are mostly in agreement with descriptions in other crustaceans (Fautrez-Firlefyn, 1957; Raven, 1961; Bonina, 1974; Fyhn and Costlow, 1977 and Varadarajan and Subramoniam, 1980). The fully formed yolk in P. indicus was observed to be a glycolipoprotein complex, as in most other crustaceans. The present investigation indicated that proteins (tryptophanyl, aromatic and amino end groups) were the first to be formed in the yolk. Thiol-disulphide end groups were incorporated into the yolk platelets subsequently from without. The follicle cells were positive to -S-S and -SH groups prior to the mature stage, thereby suggesting that cystine and cysteine may be derived from these encompassing cells. The carbohydrate moeity of the yolk complex was the next to be formed and lipids the last. Carbohydrate substances were observed to be mainly PAS

positives and AMPs. The PAS reaction was diastase and acetylation resistant during late vitellogenic stages indicating their conjugation with proteins and lipids (glycoproteins and glycolipids).

Sudanophilic lipid bodies were apparent in the ooplasm of P. indicus from the early vitellogenic phase itself. Only the Oil Red O positive neutral lipids were formed later. In P. indicus there was no evidence to support an endogenous origin of lipid yolk. It is most likely that the bulk of the lipid is transported to the oocyte from the rich reserves of the hepatopancreas. This organ showed fluctuation in lipid content concomitant with vitellogenesis (see chapter III). Such a relationship was also obtained by Anilkumar (1980) in the crab Paratelphusa hydrodromous. The observation of deepening colour of the ovary in P. indicus as oogenesis advances suggests an entry of carotenoids from an extra-oocytic source, probably from the hepatopancreas. Ceccaldi and Martin (1969) showed in Carcinus maenas that carotenoid pigments are concentrated from the heatopancreas into the hemolymph during vitellogenesis. In C. clibanarius, Varadarajan and Subramoniam (1980) have discussed the possibility of lipoprotein from an extraovarian source being linked to carotenoid pigments, to

serve as carriers facilitating entry of lipoproteins into oocytes.

Thus, the caroteno-glycolipoprotein yolk in P. indicus is synthesized initially through autosynthetic means,and later the emphasis shifts to hetrosynthetic accumulation of yolk materials. The latter process appears to contribute the majority of the yolk in this species.

The male reproductive system of P. indicus had a pair of laterally lobate testes which lay dorsoventral to the hepatopancreas. Identical observations were made by King (1948) in P. setiferus, Cummins (1961) in P. duorarum, Tuma (1967) in P. merguensis and Rao (1968) in P. indicus. Unlike female penaeids, males provide no grossly visible clues as to the physiological state of the testes. Therefore, very few workers have described any well defined maturity stages in males. Using the small variation in opacity and size of the testes with size of the animal, Subrahmanyam (1965) had described five maturity stages in P. indicus. In the present study however, no such distinction was made as the histological investigaions showed that adult males had all the spermatogenic stages in each testicular acini.

The process of spermatogenesis observed in P. indicus is similar to that reported in other crustaceans (Pochon-Masson, 1983). A general feature of the process was the reduction in cytoplasmic volume and condensation of the chromatin matter as spermatogenesis progressed through cellular stages like spermatogonia, spermatocytes and spermatids to form the spermatozoa. Only few histological studies have been made on the testis of penaeids (King, 1948 and Subrahmanyam, 1965) and their results bear close resemblance to that observed in the present investigation. However, Subrahmanyam (1965) describing the morphology of the testis of P. indicus mistakenly reported the thick mid vas deferens as a tubular portion of the testis. In the present study, the histological structure of the mid vas deferens revealed that this duct was chiefly responsible for the packaging of the mature sperms into the spermatophore.

Fine structure studies on the sperm in P. indicus, showed that it possessed a spike which is unlike a flagellum and therefore was non-motile. Observations on the sperm in P. indicus, bear resemblance to that reported in Sicyonia ingentis by Kleve et al. (1980) and P. setiferus by Lu et al (1973). Surprisingly no organelles were observed in P.

indicus sperm in the present investigation. This may be due to the fact that among Malacostraca itself, the decapod spermatozoon shows considerable modification and loss of organelles (Adiyodi, 1985). The degeneration of such organelles as mitochondria, golgi complex and centrioles and an elaboration of the membrane system have been reported in the spermatozoon of Procambarus clarkii by Moses (1961). The penaeid sperm differs even from the pleocyematan sperm in the absence of radiating nuclear arms. The present observations indicate that the sperm in P. indicus belongs to the altered vesicular type, which is different from the other flagellate and non-flagellate gametes in crustaceans (Pochon-Masson, 1983).

The cytochemical study on spermatogenesis in P. indicus revealed an abundance of polysaccharides and basic proteins like arginine and lysine in the spermatozoa. The spermatogonial cells and spermatocytes have an intense metabolism as revealed by the presence of RNA granules and protein in their cytoplasm. The increase in intensity of fuelgen positivity in the nucleus with the advancement of spermatogenesis is probably due to the condensation of the chromatin matter rather than a real increase in DNA content. The amount of RNA and proteins decreased in spermatids and

spermatozoa, a phenomenon apparently common in Crustacea (Descamps, 1969 and Pochon-Masson, 1983). Usually, gamete DNA is linked to basic proteins (Pochon-Masson, 1983) and this therefore explains the significant presence of nuclear histones in P. indicus sperm as observed in the present study. In the spermatozoa of the Norway lobster and Emerita analoga, Vaughn et al. (1969) reported the basic proteins to be free and enriched with arginine and lysine respectively. The acrosome whose origin has been frequently debated in the Crustacea (Pochon-Masson, 1983), was always found to be PAS positive in P. indicus.

In P. indicus, spermatozoa are stored in the thelycum of the female during the short period extending between copulation and ovulation. Transmoult retention of sperm for long periods is common among crustaceans (Pochon-Masson, 1983). Histochemical investigations indicate that glycogen and glycoproteins are the prinicpal reserve nutrient material for the spermatozoa in P. indicus. Ascorbic acid and high carbohydrate content in the cirripede gamete has been reported by Barnes and Finlayson (1962) to be used as reserve material.

The vas deferens in P. indicus serves not only to

transport the sperm from the testis to the outside, but also to package them into spermatophores. The protective envelope of the spermatophore was secreted by the epithelial secretory cells on the wall of the vas deferens facing the lumen. Similar functions for the vas deferens exists in other decapods like P. kerathurus (Malek and Bawab, 1974 a and b); Macrobrachium rosenbergii (Chow et al., 1982); A. symnista and E. asiatica (Subramoniam, 1984) and P. homarus (Radha and Subramoniam, 1985). Typhlosoles, composed of columnar cells, observed in the lumen of MVD and DVD of P. indicus served to increase the surface area of the glandular epithelial cells. The presence of rich blood supply to the typhlosoles indicated the high metabolic rate of these cells. Similar typhlosoles were observed in the vasa deferentia of the lobster P. homarus (Berry and Heydorn, 1970 and Radha and Subramoniam, 1985) and E. asiatica (Subramoniam, 1984). Clearly demarked functions were attributed to each segment of the vas deferens for spermatophore formation in P. indicus. These bore close resemblance to that observed in P. kerathurus by Malek and Bawab (1974). Most workers have investigated spermatophores of crustaceans by using material teased out from the distal vas deferens (Malek and Bawab, 1974; Chow et al., 1982; Subramoniam, 1984 and Radha and Subramoniam, 1985). The

technique of electroejaculation of spermatophores is a recent one and has greatly helped in understanding the structure of the fully formed spermatophore (Kooda-Cisco and Talbot, 1982). The fully formed extruded spermatophore of P. indicus has been subjected to detailed study for the first time. The presence of a parachute like wing is apparently unique to penaeids as it was not observed in other crustaceans like lobsters (Kooda-Cisco and Talbot, 1982 and Radha and Subramoniam, 1985). This wing seemingly helps in anchoring the spermatophore inside the thelycum of the female after which they dissolve in the surrounding sea water.

Two acellular layers were seen to encompass the spermatophore in P. indicus as opposed to three in the lobster H. americanus observed by Kooda-Cisco and Talbot (1982). The inner layer of amorphous material and the outer layer of parallel fibrils observed in the spermatophores of P. indicus were strikingly identical to that observed in H. americanus. An intermediate layer, as observed by Kooda-Cisco and Talbot (1982) in H. americanus was lacking in P. indicus. In H. americanus adhesive properties were ascribed to the outer bounding layer, since this forms a permenant bond between the female cuticle and the spermatophore. Due

to the similarity in ultrastructure of the outer bounding layer of Homarus and the spermatophore layer II of P. indicus, a parallel function could be attributed to the latter also.

Mucopolysaccharides were found to be the principal components of the spermatophore layers of P. indicus. Based on a biochemical investigation Sasikala and Subramoniam (1987) reported the predominance of AMP like chondroitin sulfate and hyaluronic acid in the spermatophore of P. indicus. The present study revealed that the outer bounding layer or spermatophore layer II is composed of a mucoprotein, with cystine and cysteine being the main protein components. The spermatophore layer I apparently consisted of a neutral mucopolysaccharide with large amounts of glycogen. Both these layers also possessed sulphated and carboxylated AMP. Such a mucopolysaccharide heterogeneity has been reported in Crustacea by Subramoniam (1984) in the anomuran crabs A. symnista and E. asiatica. The gel forming antimicrobial properties of AMPs are well known (Itow and Sekiguchi, 1984) and hence these spermatophore layers may have an adhesive and protective role. The spermatophore matrix in P. indicus was composed purely of AMPs as indicated by the AB-PAS combination. In E. asiatica and A.

symnista, Subramoniam (1984) reported the sperm mass substance or sperm matrix to be an AMP, and the gelatinous cord to be a neutral mucopolysaccharide. The glycogen content observed presently in the spermatphore layer I probably has a nutritive function during the short storage in the female thelycum. However, Subramoniam (1984) found only meagre quantities of glycogen in these layers.

The presence of tyrosine-rich proteins was taken as an indirect evidence for phenolic tanning in the spermatophore of P. trisulcatus by Malek and Bawab (1971). Subramoniam (1984) also detected tyrosyl groups and the enzyme phenolase in anomurans to support this view. However in P. indicus, such an evidence is wanting. Perhaps tropical penaeids do not need to have a hardened spermatophore, since the duration between moults is comparatively short. With each moult, the impregnated thelycum is lost and fresh copulation is necessary for a renewed supply of sperms.

S U M M A R Y

1. The process and events leading to the maturation of the ovary and testis in P. indicus has been investigated using histological, electron microscopic and histochemical methods. Further the process of the development of the

spermatophore in the deferent ducts of the male has also been studied.

2. The female reproductive system was observed to consist of an ovary with 3 lobes, paired oviducts and externally a single ventral thelycum. Ovarian maturation was accompanied by distinct colour changes as well as increase in volume of the ovary and diameter of the oocytes and nuclei. Based on these factors and the changes in GSI five different maturity stages were identified, viz., immature, early maturing, late maturing, mature and spent recovering (stages - I to V). The oocytes were found to develop and accumulate yolk in a graded manner and based on the changes evident in the cytoplasm and nucleus of the oocytes, the complete development of the oocyte was classified into five different phases, viz., pre-vitellogenic, early vitellogenic, late vitellogenic, vitellogenic and spent.

3. The germinal zone consisting of primary and secondary oogonial cells was observed in prawns in all stages of maturity as a thin band along the innermost layer of the ventral ovarian wall.

4. Pre-vitellogenic oocytes had a basophilic cytoplasm and vesicular nucleus with 5-10 darkly stained nucleoli. The deeply RNA positive nucleolar material was observed to be extruded out into the cytoplasm through nuclear pores.

5. Early vitellogenic oocytes had a less basophilic, but more granular cytoplasm due to the formation of the primry yolk vesicles. The investment of the follicle cells around the oocytes was completed during this phase. Numerous free ribosomes and rough endoplasmic reticulum were seen in the cytoplasm. Oocytes in spent animals also displayed similar characteristics.

6. During the late vitellogenic phase the cytoplasm was acidophilic and granular due to the formation of yolk platelets. Yolk platelets were formed by the aggregation of electron dense flocculent granules in the yolk vesicles. The follicle cells were flattened around the oocytes and along the oolemmal wall numerous micropinocytotic vesicles were observed for the uptake of yolk material from outside.

7. Vitellogenic oocytes were characterized by the presence of elongated cortical bodies with a feathery matrix and

abundance of cystine. The whole ooplasm was filled with mature yolk platelets and the nucleus was devoid of any nucleoli.

8. Histochemical investigations showed that the yolk in P. indicus was a glycolipoprotein complex. Yolk formation takes place by the selective deposition and sequestration of organic material from both within (autosynthesis) and without (heterosynthesis), with the emphasis on the former during the initial phase of vitellogenesis. From late vitellogenic phase onwards extraoocytic sequestration of organic material, especially lipids was prevalent.

9. The male reproductive system in P. indicus was found to consist of internal organs viz., paired vas deferens and terminal ampoules and external organs viz., a petasma and a pair of appendix masculina. The testis was composed of a mass of minute convoluted seminiferous tubules or acini in which sperm cells were seen. Spermatogenesis which involved the progressive reduction of cytoplasm and condensation of chromatin was observed to be initiated from the peripheral germinal zone from where spermatogonia were transformed to spermatocytes and spermatids and finally into the spermatozoa.

10. Ultrastructurally, the sperm of P.indicus was composed of a spherical main body (3 μ diameter) that was partially encompassed by a morphologically diverse cap region containing the acrosomal complex, from which extends a single spike (5-7 μ long).

11. Histochemical studies on the testis revealed that in general, the spermatozoa had a remarkable abundance of polysaccharides like glycogen and basic proteins like arginine and lysine. The glycogen content was presumed to aid in the transmoult maintenance of the spermatozoa in the thelycum of the female.

12. The histological investigations on the entire testicular duct revealed that the formation of the spermatophore took place with the aid of the glandular epithelial cells lining the duct. The ducts were divided into 4 distinct portions, the proximal vas deferens, mid vas deferens, distal vas deferens and terminal ampoule and the functonal morphology of each portion in relation to the formation of the spermatophore was investigated.

13. The electro-ejaculated spermatophore was found to be oval in shape and dirty cream in colour with the surface

thrown into folds resembling a bag full of grapes. A translucent parachute like wing was found at the apical end of the main body. Sperms were packed inside the spermatophore in lobules and were encommpassed by two structurally diverse spermatophoric layers. Cytochemical studies revealed that the spermatophore layers were mainly composed of acid mucopolysaccharides and sulphur containing amino acids.

CHAPTER III

MORPHOLOGY OF THE NEUROENDOCRINE SYSTEM AND ITS CONTROL ON REPRODUCTION

INTRODUCTION

Reproduction in crustaceans is perceived to be hormonally controlled (Highnam and Hill, 1977). Knowledge about the control of reproduction by neuroendocrine hormones is necessary to successfully manipulate the life cycle of the commerically important prawns in acquaculture. The development of the classical extirpation - replacement technique has made it possible to differentiate many areas and sites where neuroendocrine hormones are being produced in crustaceans. Neuroendocrine centres include those areas in the central nervous system having morphologically specialized neurons (neurosecretory cells) which perform a secretory function. These neuroendocrine organs are invariably associated with the blood-vascular system which are termed as neurohaemal organs.

Studies on crustacean neuroendocrinology has its beginnings in the latter half of 1920's. Initially, much of the interest was focused on the neuroendocrine organs located in the eyestalk of crustaceans. The classical work of Panouse (1943) revealed that the removal of the eyestalk of the prawn Leander serratus resulted in precocious maturation of the ovary. Since then many workers have established that the physiological activity of reproduction in crustaceans is under the control of the neurosecretory

cells of the X-organ in the eyestalk (Adiyodi and Adiyodi, 1970). Subsequently many studies were made to elucidate the morphological details of the various neuroendocrine areas in crustaceans like eyestalk, brain and thoracic ganglia (Enami, 1951; Bliss, 1951; Aoto and Nishida, 1956; Carlisle, 1959; Gomez, 1965; Lake, 1970; Hisano, 1974; Diwan and Nagabhushanam, 1975; Van herp et al., 1977; Bellon-Humbert et al., 1981; Nanda and Ghosh, 1985 and Nagabhushanam et al., 1986).

The concept of neurosecretion and neurosecretory cells was enhanced with the classification of these modified neuronal cells based on cytomorphological differences in different decapod crustaceans by Durand (1956), Matsumoto (1958), Bliss (1966), Lake (1970), Nakamura (1974) and Diwan and Nagabhushanam (1975). The spatial distribution or mapping of the neurosecretory cells within the ganglia has been studied by Lake (1970) in Paragrapsus gaimardii, Hisano (1974) in Palaemon paucidens and Nagabhushanam et al. (1986) in Parapenaeopsis stylifera. There are however only few studies on the secretory behaviour of the neurosecretory cells in crustaceans (Enami, 1951; Matsumoto, 1958; and Williams et al.1980). Histochemical studies on the neurosecretory cell products were carried out in the crabs Carcinus maenas,Telmessus cheiragonus and P.gaimardii by Rehm (1959), Miyawaki (1960) and Lake (1970) respectively to

derive the chemical nature of the hormones involved. Ultra-structural investigations on the neurosecretory cells in the diverse groups of crustaceans has helped in identifying the primary substance responsible for the control of various physiological events as electron dense neurosecretory granules (Fingerman and Aoto, 1959; Bunt and Ashby, 1967; Shivers, 1967; Smith, 1974; Hisano, 1976; Strolenberg et al., 1977; and Bellon-Humbert et al., 1981).

In the course of these fast developments considerable confusion arose in the terminology of different structures and groups of neurosecretory cells. The work of Carlisle and Knowles (1959), Passano (1960), Gabe (1966), Adiyodi and Adiyodi (1970) and Fingerman (1970) have consolidated the present state of knowledge in crustacean endocrinology. Nagabhushanam etal.(1980),Cook and Sullivan (1982), Newcomb et al. (1985), Quackenbush (1986) and Fingerman (1987) have recently reviewed the advances made in this field of science particularly in the light of the results obtained by the use of modern technique like electron microscopy, high pressure liquid chromatography, radioimmunoassay, immunocytochemistry and immuno-electrophoresis.

A survey of the literature revealed that investigations on penaeid neurosecretion is extremely limited. The

earliest report on the neuroendocrine organs of a penaeid prawn is that of Dall (1965) who described the X-organ sinus gland complex of the Australian school prawn Metapenaeus sp. in relation to its moulting. Neurosecretion in the Japanese 'Kuruma Shrimp' Penaeus japonicus has ben studied by Nakamura (1974). Among the Indian penaeids, Metapenaeus monoceros (Madhyastha and Ranganekar, 1976, P.monodon (Nanda and Ghosh, 1985) and Parapenaeopsis stylifera (Nagabhushnam et al., 1986) have been studied briefly with regard to neurosecretion. However none of these authors have studied these structures in relation to reproduction.

Reproduction and moulting are interlinked in decapod Crustacea and consequently, so are their respective hormone systems. This hormonally controlled mutual antagonism has been excellently reviewed by Adiyodi and Adiyodi (1970). Most crustaceans can be placed into one of three groups based on the organization of these two processes (Adiyodi and Subramoniam, 1983; Charniaux-Cotton, 1985). Crabs and lobsters fit into type 1 where reproduction takes place during the long intermoult period. Isopods, amphipods and some shrimps fit into Type 2 where gonadal and somatic growth occur simultaneously. Type 3 includes the rapidly moulting cirripedes where reproduction may require several

moult cycles.

Removal of one or both the stalked eyes of decapods have been one of the most widely used experimental techniques in the study of crustacean hormones. Properly done, the operation removed the entire X-organ sinus gland complex and with it the neurosecretory tissues that influence reproductive physiology, moulting, metabolic and heart rate, sugar and protein metabolism, water balance and pigmentation (Newcomb, 1983). Paradoxically, several of the reproductive hormones are only postulated, direct chemical evidence of their existence is lacking (Aiken and Waddy, 1980).

The gonad inhibting hormone (GIH) elaborated from the neurosecretory cells in the eyestalk was demonstrated in crustaceans by eyestalk ablation experiments showing stimulation of vitellogenesis in Pleocyemata (Brown and Jones, 1949; Demeusy and Veillet, 1952; Gomez, 1965; Rangenekar and Deshmukh, 1968; Cheung, 1969; Nagabhushanam and Diwan, 1974 and Bomirski and Klek, 1974) and in Dendrobranchiata (Panouse,1943; Carlisle, 1953; Aoto and Nishida, 1956; Klek-Kawinska and Bomirski, 1975; Nagabhushnam and Kulkarni, 1980 and Quackenbush and Herrnkind, 1981). Similarly implantation of the cerebral or thoracic ganglia or their extracts stimulating gonadal

growth has been shown by several workers ina number of crustaceans (Otsu, 1963; Gomez and Nayar, 1965; Hinsch and Bennett, 1979; Rao et al., 1981 and Joshi, 1989). The moult inhibting hormone (MIH) also elaborated from the eyestalk has been reported to assist in reproduction by suppressing the moulting process by Adiyodi and Adiyodi (1970).

In recent times the aquaculture of penaeids has received an added impetus by the application of the eyestalk ablation technique to beget precocious maturation and spawning. It was in 1970 that maturation was first observed in ablated Penaeus duorarum (Caillouet, 1972), almost 30 years after Panouse (1943) first obtained precocious maturation in Palaemon serratus. Since 1970, studies on induced maturation by eyestalk ablation in penaeids has multiplied (Arnstein and Beard, 1975; Alikunhi et al., 1975; Aquacop, 1975; Muthu and Laxminarayana 1977; Halder, 1978; Lumare, 1979; and Emmerson, 1980). Presently around 14 penaeidi species have been matured and spawned in captivity using this technique (Primavera, 1984).

Inspite of the almost routine use of the technique of eyestalk ablation in penaeid prawn hatcheries throughout the world, very little is known about the morphological and

physiological changes that occur in the neuroendocrine system operating under these circumstances. Morphological and anatomical details of the neuroendocrine system of a species is an essential prerequisite for proper understanding of the complex hormone induced changes associated with reproduction. However among penaeids information on these aspets is extremely poor. In Decapoda itself, only few attempts have been made to correlate changes in the gonad with those in the neuroendocrine organs (Matsumoto, 1958; Perryman, 1969; Kulkarni and Nagabhushanam, 1980; Babu et al., 1980; Rao et al., 1981; Deecaraman and Subramoniam, 1983 and Joshi,1989). Such studies have added significance, especially in penaeids, as hatcheries are becoming increasingly dependant on endocrine manipulations to achieve their production targets.

Therefore, in the present investigation the neuroendocrine centres in the eyestalk, brain, subesophageal, thoracic and abdominal ganglia of P. indicus has been studied in detail using histological and histochemical techniques. Electron microscopic methods have been used to investigate the fine structure of the neurosecretory cells. Attempts have been made to correlate the structural changes in the neurosecretory cells in relation to the different phases of gonadal naturation. Eyestalk ablation experiments

were performed to study the neuroendocrine factors of the eyestalks concerned with the regulation and control of gonadal development. In addition, the relationship between gonadal maturity and the moult cycle has also been examined.

MATERIALS AND METHODS

1. Collection of animals

Live adults of P. indicus in different maturity stages used in the present study were collected from the Arabian sea off Cochin by trawling as described earlier in Chapter II. On rare occassions, when prawns could not be obtained from trawl operations, they were collected from the perennial prawn farms of Edavanakad village in Vypeen island near Cochin. The prawns thus obtained were carefully transported to the laboratory in 10 L capacity plastic seed transportation bags.

2. Dissection and Fixation of tissues

Female animals were segregated according to the five

maturity stages described in Chapter II (Stages I-V). Dissections of the prawns were carried out in crustacean saline (Smith and Ratcliffe, 1980) between 1600 and 1800 h to avoid the interference of circadian changes on neurosecretion. The gonadal tissues were carefully excised out and fixed in Bouin's fluid for routine histological studies to assess the maturity stage. Then the gross morphological observation of the nervous system was made under a stereoscopic dissection microscope and the various neuroendocrine centres like eyestalk, supraesophageal, tritocerebral, subesophageal, thoracic and abdominal ganglia were excised and fixed in Bouin's fluid for 24 - 48 h for histological studies. Routine histochemical investigations of the ganglia were carried out with materials fixed in 10 % neutral buffered formalin for 24 h. Lipid histochemical tests were performed with either fresh frozen ganglia or with those fixed in Baker's formol-calcium for 24 hours.

3. Processing and Sectioning

Bouin fixed tissues were washed over night in running tap water to remove the excess picric acid and dehydrated using an alcohol series (30% to 100% alcohol). After dehydration the tissues were cleared in methyl benzoate. The tissues were further transferred to a mixture of wax

shavings and benzene and kept overnight for cold impregnation. Subsequently the solvent was evaporated by keeping the tissue in an oven at 58°C. They were then transferred to fresh paraffin wax (Paraffin wax with Ceresin, BDH, 58 - 60°C) and kept for 2 hours each in two changes. Tissue blocks were prepared using paper boats or glass petri dishes after proper orientation.

Approximately 6-8 μ thick sections were cut using a rotary microtome (Weswox make) and serial sections were affixed on clean glass slides by using Mayer's egg albumin. The ribbon of sections were individually flattened by adding a drop of distilled water and then placing them on a slide warmer. The excess fluid was then drained off and the slide was allowed to dry.

4. Staining

Sections were hydrated through a down series of alcohol grades after initial dewaxing in two changes of xylene. Routine staining for gross observations were done using Harris or Heidenhain's hematoxylin with 1% aqueous eosin as the counter stain. Blueing was done in tap water or 1% lithium carbonate. Eosin stained sections were washed

repeatedly in 95% alcohol to remove the excess stain and were further dehydrated in two changes of absolute alcohol. After clearing in 2 changes of xylene the slides were mounted using DPX or Canada balsam of neutral pH and these sections were examined under the microscope.

Visualisation of neurosecretory cells and neuroendocrine structures in P. indicus was made by using specific staining techniques. Chrome-hematoxylin, basic fuchsin and victoria blue stains were used for staining neurosecretory structures because of their affinity to acidic groups appearing after the oxidation of the neurosecretory material with oxidising agents such as performic acid and potassium permanganate (Lake, 1970). The staining methods used were (a) Gomori's chrome-hematoxylin phloxine method (Bargman 1949, modification) (b) Gomori's paraldehyde fuchsin method (Kurup, 1972 modification), (c) Mallory's triple stain (Mallory, 1944), (d) Heidenhain's Azan technique (Pearse, 1968) and (e) Performic acid victoria blue method (Dogra and Tandan, 1964 modification). The Victoria blue method was used for in situ observation of neurosecretory cells and axonal pathways. Whole ganglia fixed in 10% formol-saline were oxidised in performic acid until they became transparent and then stained in a victoria blue resorcin lake for 12 - 18 hours, dehydrated, cleared in cederwood oil and mounted

in Canada balsam.

5. Histochemical tests

Histochemical tests for the detection of protein, carbohydrate and nucleic acid were performed with ganglia fixed in 10% neutral buffered formalin for 24-48 h. The tests applied for proteins were mercuric bromophenol blue (Mazia et al., 1953) for general proteins, ninhydrin schiff test (Pearse, 1968) accompanied by deamination as control for amino (-NH2) groups, ferric-ferricyanide method (Adam and Sloper,1956) with mercaptide block as control for sulphydryl (-SH) groups, and performic acid-alcian blue method (Adams and Sloper, 1956) with alcian blue alone without oxidation as control for disulphide (-S-S) groups. The periodic acid schiff (PAS) test (McManus and Mowry, 1960) with diastase digestion was employed for detecting carbohydrates in general and glycogen in particular. Mucopolysaccharides were detected using the alcian blue-PAS test (McManus and Mowry, 1960). RNA and DNA were detected by using the methyl green pyronin method of Pearse (1968). Treatment with 10% perchloric acid proved satisfactory as the control for nucleic acids.

Detections of lipid in the neurosecretory cells was carried out with either fresh tissues or with those fixed in Baker's formol-calcium for 24 h. Blocks were prepared and cut at -20°C using an American Opticals-Histostat freezing microtome. Sections (10 μ thick) were stained in saturated sudan black B in 70% ethanol according to Pearse (1968). Chloroform-methanol delipidation was resorted to as control. Such histochemical tests were carried out in the eyestalk, brain and thoracic ganglia from immature, mature and spent animals. Standard notations were used to indicate the intensity of reactions.

Histological and histochemical preparations were examined under a monocular research microscope with upto 1500x magnification. Morphometric measurements of cells and other neurosecretory structures were carried out using a calibrated ERMA ocular micrometer. Photomicrographs were taken using an OLYMPUS universal research microscope (PM 10AD photomicrographic system) with Ilford FP4 (125 ASA) black and white film. Colour photographs were taken using the same system with Kodak colour film (100 ASA).

6. Ultrastructural Studies

Ultrastructural investigations on the neurosecretory

material were performed on the X-organ sinus gland complex of the eyestalk. The eyestalks fixed in 4% gluteraldehyde were processed as described in Chapter II. Uranyl acetate and lead citrate stained ultra thin sections were observed in a JEOL-JEM 100CX II Electron microscope at 80 KV. Photographs were taken using Agfa plate negatives.

7. Quantitative Study of NSC phases in different maturity stages

The neurosecretory cells in the eyestalk and other ganglionic centres (Supraesophageal, Subesophageal and thoracic ganglia) of animals in different maturity stages (Stage I-V) were staged according to their secretory phases. The secretory phases were Quiescent (Q), Vacuolar (V) and Secretory (S). The number of cells, irrespective of the type, in each phase in all the ganglia were recorded in each maturity stage. For each stage cell counts from three animals were made and the average percentages of the cells found were recorded as given below:

$$\%\ \text{of cells in Q,V and S phases} = \frac{\text{No of cells in Q/V/S phases} \times 100}{\text{Total no. of cells counted.}}$$

8. Eyestalk ablation and eyestalk extract administration experiments

To study the role of eyestalk neurosecretory hormones on reproduction,eyestalk ablation and eyestalk extract injection experiments were conducted at CMFRI's Marine Prawn Hatchery Laboratory (MPHL) at Narakkal, 30 km from Cochin in Vypeen island. Experiments were conducted during the summer months (March to May) of the year.

Female P.indicus above 140 mm TL obtained from the ponds in the hatchery complex were used in the study after acclimatization to laboratory conditions for 48 h. Animals were maintained in 3 feet diameter collapsible plastic pools (Plasticrafts corporation, Bombay) having a capacity of 0.25 tonnes. Stored and settled seawater was pumped into the pools through a 60 micron nylobolt cloth. The salinity of the seawater used ranged from 28 to 36 ppt, temperature from 27 C to 30 C and pH between 8.0 and 8.2. The pH was regulated within this range as and when necessary by the addition of anhydrous sodium carbonate (Muthu et al., 1984). Aeration (two air-stones per pool) was provided from an air-grid supplying oil-free compressed air from a rotary air-blower. Prawns were fed ad libitum with fresh or frozen clam (Meretrix spp.) meat. The uneaten food and faecal

matter was siphoned out and 50% of the water was replaced with fresh seawater daily.

In order to find the effect of eyestalk ablation and administration of an extract of the eyestalk on ovarian maturation, 40 immature females of P.indicus in intermoult and early premoult stages were selected and divided into 4 experimental groups (I,II,III and IV) of 10 animals each.

In group I, prawns were maintained without any treatment for a period of 10 days and used as the intact control. In group II, animals were subjected to unilateral ablation of the right or left eyestalk. Both the eyestalks of the prawns were removed in group III (bilateral eyestalk ablation). Eyestalk surgery was performed with the help of an electrocautery apparatus (Du-Caut make). For group III, after removal of one eye, the animals were returned to the pool, and the remaining eye was ablated only after 24 h. Experimental animals were examined daily for signs of gonadal development. The development of the ovary could be clearly seen through the transparent dorsal cuticle and the formation of the triangle shaped ovary in the first abdominal somite was taken as an indication of full maturity. The number of days taken for attaining ovarian maturation by each prawn was recorded as the latency period.

Animals were sacrificed on reaching full maturation or after 10 days whichever was earlier. The moult stage was recorded and the gonads of each prawn was weighed for the determination of the gonado-somatic index (GSI) as given below:

$$GSI = \frac{\text{wet weight of ovary} \times 100}{\text{wet weight of animal}}$$

Further the different parts of the CNS like eyestalk, brain, subesophageal and thoracic ganglia and the gonads were preserved in Bouin's fluid for histological preparations. The ova diameter was recorded using an ocular micrometer.

In group IV unilateral eyestalk surgery was performed for all 10 prawns and simulataneously these prawns were administered with an aqueous extract of fresh eyestalks. Eyestalk extract in the ratio of 2 eyestalks/0.2 ml was prepared by macerating fresh eyestalks (after removing the ommatidia and cuticle) in cold crustacean saline. The extracts were then centrifuged at 3000 rpm for 10 minutes and the supernatent was used for the injections. Each of the animals was injected once with 0.2 ml of the extract using a hypodermic syringe. After an experimental period of 10 days, all the animals were sacrificed and their GSI and ova diameter were determined as described previously.

The activity of GIH was also determined and expressed as the percentage degree of ovarian inhibition (Bomirski et al.,1981).

$$\text{Degree of ovarian inhibition} = \frac{(GSI^{A} - GSI^{E}) \times 100}{(GSI^{A} - GSI^{C})}$$

Where GSI^{A} is the gonad index of unilaterally ablated animals, GSI^{E} is the gonad index of eyestalk extract treated animals and GSI^{C} the gonad index of intact control.

9. Effect of CNS extract administration

To assess the impact of neurosecretory principles contained in the brain, subesophageal and thoracic ganglia of P. indicus, immature females were administered an aqueous extract of these ganglia. Experimental conditions and source of animals were similar to that described above. Twenty immature females in intermoult or early premoult stages were collected and divided into 2 experimental groups of 10 animals each.

Prawns in group I were injected with 0.2 ml of crustacean saline and was treated as the control. Each animal in group II was administered 0.2 ml of the ganglionic

extract (I CNS equivalent) through the first abdominal somite using a hypodermic syringe. Prawns were injected only once to avoid excessive handling stress. An aqueous extract of the brain, subesophageal and thoracic ganglia of one prawn was taken as I CNS equivalent. Freshly dissected ganglia from mature female P.indicus were macerated in cold crustacean saline and centrifuged at 3000 rpm for 10 min. The supernatent thus obtained was used for injections.

Prawns were monitored daily for signs of gonadal development. After an experimental period of 10 days, all animals were sacrificed and their GSI and ova diameter were recorded as described earlier.

10. **Statistical analyses**

The significance of the differences between the mean values obtained for the different experimental groups were compared and tested with Student's t test (Snedecor and Cochran, 1968).

RESULTS

1. General morphology of the nervous system

P.indicus has a typical dendrobranchiate crustacean nervous system which is characteristic in having a ventral nerve cord with a pair of ganglia corresponding to each embryonic somite. The different neuroendocrine centres identified in P.indicus on the basis of the present study and their relative position in the nervous system are depicted in Fig.1. Essentially, the nervous system consists of a supraesophageal ganglion (Brain or Cerebral ganglion), from the dorsal surface of which arises a pair of stout nerves, the optic nerves, ending in the optic ganglia (eyestalks). Posteriorly the supraesophageal ganglion is joined to the subesophageal ganglion by a pair of tritocerebral connectives. Midway through the tritocerebral connectives are a pair of tritocerebral ganglia connected to each other by a thin tritocerebral commissure.

The ventral nerve cord continues posteriorly from the subsesophageal ganglion via dual connectives to the five thoracic ganglia, each corresponding to the five thoraco-

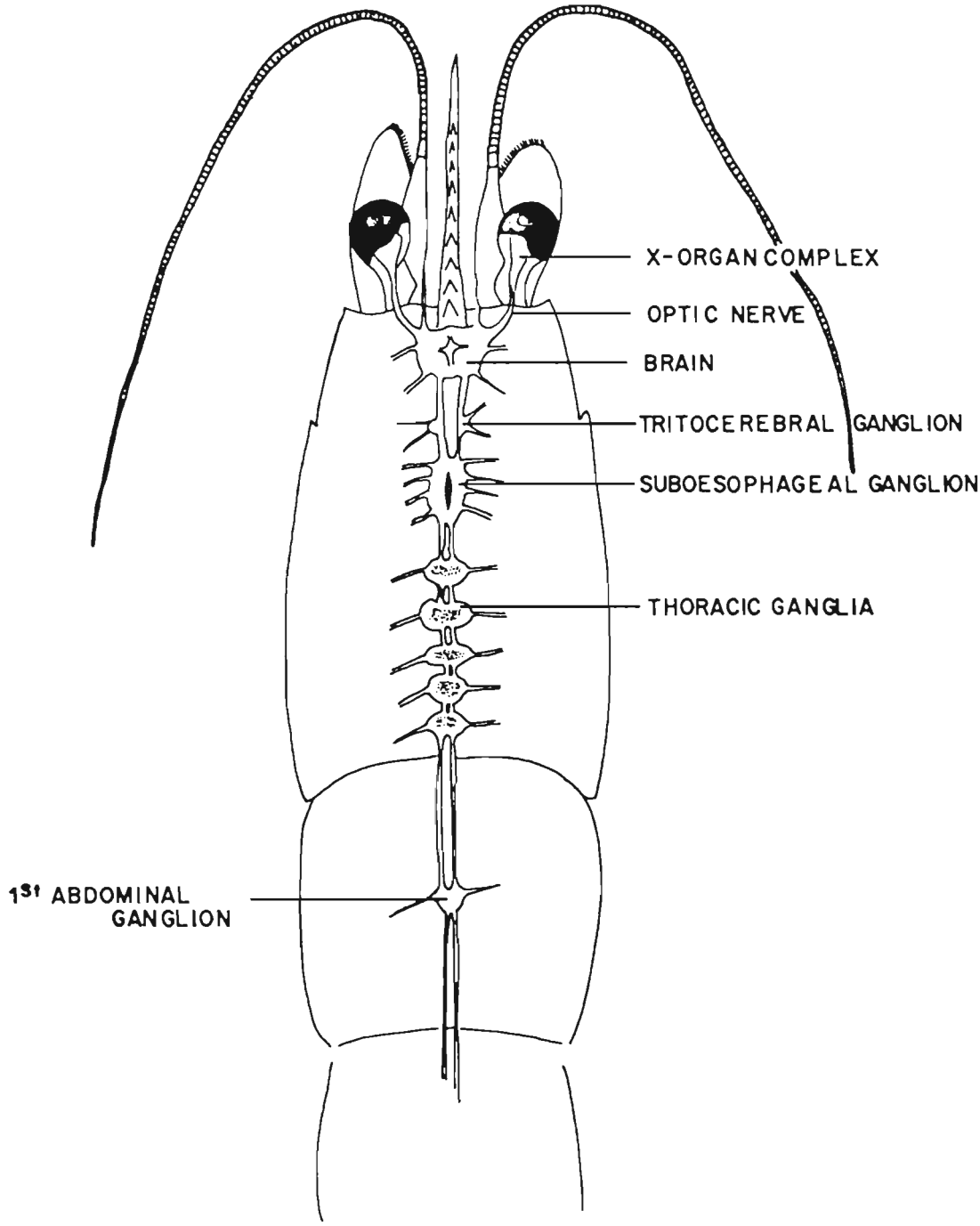


FIG. 1 NEUROENDOCRINE CENTRES IN *P. INDICUS*

pods. The connectives between individual thoracic ganglia are short. The six abdominal somites are each represented by an adbominal ganglion. The abdominal ganglia too are connected to each other by dual connectives, the distance between two ganglia being comparatively longer than that of the thoracic ganglia.

The nerve cord was composed of bundles of longitudinally running nerve fibres packed closely together. The ganglionic masses as well as the nerve cords were covered protectively by a connective tissue sheath (ca, 5 μ thick), which stained blue with Mallory's triple stain (Fig.4). Externally, many randomly distributed, dark (blue-green) chromatophores were observed, especially on the dorsal surface of the ganglia and nerve cord.

2. Neurosecretory cells - character and types

Microscopic observations of the serial sections of the optic, supraesophageal, tritocerebral, subesophageal, thoracic and abdominal ganglia stained with different staining techniques like chrome hematoxylin phloxine (CHP), paraldehyde fuchsin (PAF), azan, and Mallory's triple stain (MTP) revealed the presence of different neurosecretory cell

groups in all the ganglia. No neurosecretory cells (NCSs) were noticed in the nerve cord. Histologically the NSCs in P.indicus were different from the multitude of non-neurosecretory neurons present in the ganglia and were characterized by the presence of large nuclei, abundant cytoplasm and conspicously staining granules in their perikarya (fig. 3,4 and 5). Further, they exhibted a distinct cyclic variation in their secretory activity.

All the neurosecretory cells observed were unipolar (i.e, with a single axon) and nondendritic. Nevertheless, the NSCs in P.indicus were found to differ significantly in their size, shape, secretory activity and tinctorial affinity to the different staining techniques that were used in the present study. On the basis of these characters, the neurosecretory cells were classified arbitrarily into four morphological types, viz., Giant neurons (GN), A-cells, B-cells and C-cells (Fig 2 and Table I and II). Basically all the NSCs in a ganglia were found in groups mainly in the peripheral regions.

2.1. **NSC Type I - Giant Neuron - GN**

Giant Neurons were the largest of the NSCs observed in P. indicus. They were found distributed in limited numbers

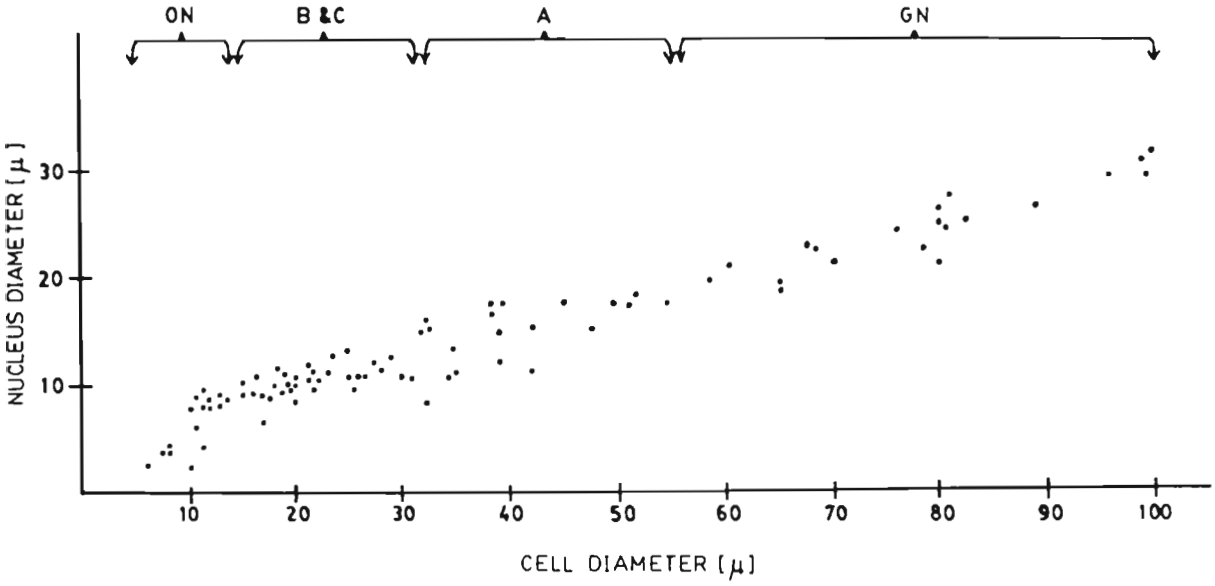


FIG:2 Scatter diagram of Cell-Nucleus dimensions in NSCs of P. indicus, ON - Non-neurosecretory neurones GN, A, B and C - NSC types.

Table - 1 : Cell and nucleus diameters and distribution of NSC in P. indicus.

Cell type	Cell diameter (u ± SD)	Nucleus diameter (u ± SD)	Nucleus cytoplasm Ratio - Range	Distribution
GN Cells (Giant Neuron)	75 ± 12	27 ± 5	0.3 - 0.4	Supraesophageal Subesophageal Thoracic Tritocerebral ganglia.
A Cells (Large Oval Cells)	40 ± 7	15 ± 6	0.3 - 0.5	Eyestalk, CNS
B Cells (Small Oval Cells)	23 ± 4	10 ± 3	0.4 - 0.6	Eyestalk, CNS
C Cells (Pyriform Cells)	15 ± 4	9 ± 3	0.4 - 0.7	Eyestalk, CNS (except tritocerebral)
Non-neurosecretory neurons	10 ± 3	7 ± 2	0.7 - 1.0	All parts of CNS

Table 2 : Tinctorial response of Neurosecretory cells to different staining techniques in P.indicus

Cells	Technique	Neurosecretory material	Nucleus	Nucleoli
Giant Neuron 'GN'	PAF	Deep purple	Pale white	Purple
	AZAN	Reddish blue	Pale Red	Red
	CHP	Greyish purple	White	Red
	Mallory's triple	Deep purple	Off white	Red
Large Oval cells 'A'	PAF	Bluish purple	Off white	Purple
	AZAN	Dark purple	Pale Red	Red
	CHP	Purple	Pale blue	Purple
	Mallory's triple	Reddish purple	Off white	Pale red
Small Oval cells 'B'	PAF	Yellowish purple	White	Pale purple
	AZAN	Purple	Pale red	Red
	CHP	Violet	Light blue	Red
	Mallory's triple	Deep purple	Off white	Red
Pyriform cells 'C'	PAF	Purple	White	Purple
	AZAN	Magenta	Pale Red	Red
	CHP	Purple	Pale Blue	Brown
	Mallory's triple	Bluish purple	Yellowish white	Red

in all the ganglia except the optic ganglia. Generally GN cells were oval to polygonal in shape (fig.3) and their mean diameter observed was 75.0 ± 12.0 μ. The GN cells possessed a centrally placed vesicular nucleus about 27 μ in diameter with the nucleus-cytoplasm ratio ranging from 0.3 to 0.4 (Table 1). Nucleoli were found dispersed between the chromatin material in the karyoplasm (fig.6) and their number ranged from 8 to 10. Pericellular capillary network in the form of a plexus was also observed in the neuropile surrounding these cells (fig.3).

Irrespective of minor differences in cellular dimensions, all the GN cells were rich in cytoplasm, in which significant vacuole formation was seen. Perinuclear vacuoles of shapes ranging from round and oval to horseshoe were observed in the GN perikarya (fig.3 and 16). These vacuoles were found filled with darkly stained basophilic granules or were sometimes empty with feebly stained matrix (fig.3 and 16). GN cells were found to be unipolar with the stout axons traceable for a short distance (fig. 12). Rarely, axoplasmic streaming was also observed in these cells (fig.20).

The staining response of these cells to CHP, PAF, Azan

and MTP are given in Table 2. With the four staining techniques applied, the secretory granules in the perikarya stained as bluish purple to reddish purple. The ground substance of the cytoplasm stained pale orange with the PAF and pale red with CHP. With the Mallory's triple stain the ground substance of the cytoplasm was palely fuchsinophilic and the secretory granules were deep purple. The neurosecretory material stained uniformally as redish purple with Azan.

The reactions of GNs to the histochemical tests are given in table 3. The GN cells were, in general, rich in protein and carbohydrate and moderately so in lipids and nucleic acids. The perikarya showed intense positive reaction to mercuric bromophenol blue (fig.14) indicating an abundance of proteinaceous material. The intense positive reaction to the performic acid - alcian blue test indicated that the principal amino acid present was cystine (-S-S group). The cytoplasm also stained positively with the Ninhydrin-Schiff test and ferric - ferricyanide test revealing the presence of moderate amounts of amino and sulfydryl (-SH) groups. The cytoplasm showed strong positive reaction to the PAS test which was wholly removed by diastase digestion, thereby indicating that the cytoplasm has a carbohydrate moiety composed mainly of glycogen. This

Table - 3 : Histochemical responses of neurosecretory cells in P. indicus

Tests applied	Giant neuron			A - cell			B - cell			C - cell		
	Cy	Nu	Nul	Cy	Nu	Nul	Cy	Nu	Nul	Cy	Nu	Nul
1. Mercuric Bromophenol Blue test	+++	±	++	++	+	++	+++	+	++	+	±	++
2. Ninhydrin-schiff test	+	-	+	±	-	+	+	-	+	+	-	+
Deamination control	-	-	-	-	-	-	-	-	-	-	-	-
3. Ferric-ferricyanide test	±	±	+	+	±	+	+	±	+	+	±	++
Mercaptide control	-	-	-	-	-	-	-	-	-	-	-	-
4. Performic acid - alcian Blue test	+++	-	±	+	-	+	++	±	+	+	±	+
Alcian blue alone-control	±	-	-	-	-	-	-	-	-	-	-	-
5. Periodic acid schiff test	++	-	±	+++	-	+	++	-	±	+	-	+
Diastase digestion control	-	-	-	+	-	-	+	-	-	±	-	-
6. Sudan Black B test	±	-	-	+	-	+	++	-	+	++	-	+
Delipidation control	-	-	-	-	-	-	-	-	-	-	-	-
7. Methyl green pyronin test	±(R)	+(G)	±(R)	±(R)	+(G)	±(R)	±(R)	+(G)	+(R)	±(R)	+(G)	±(R)
10% perchloric control	-	-	-	-	-	-	-	-	-	-	-	-
8. Alcian blue PAS test	++(M)	-	+(B)	+(M)	-	+(B)	++(M)	-	+(B)	No data		

Key:- Cy = Cytoplasm, Nu = Nucleus, Nul = Nucleolus, R = Pale Red, G = Green, M = Magenta, B = Blue, - = Negative reaction, ± = Very mild positive reaction of doubtful nature; + = Positive reaction; ++ = Strong positive reaction; +++ = Intense positive reaction.

was further substantiated by the magenta reaction to the AB-PAS test which is indicative of neutral mucopolysaccharides such as glycogen.

The GN perikarya showed only very mild postivity to lipids and RNA. The methyl green pyronin test revealed small quantity of distinct pyroninophilic RNA granules and methyl green positive DNA material in the karyoplasm. The nucleus generally, showed faint reactions with all the methods applied. The nucleoli stained only very lightly with pyronin of the methyl green pyronin test but stained strongly with the ninhydrin-Schiff and ferric ferricyanide test revealing and presence of amino and sulfydryl end groups. In the nucleoli the presence of acid mucopoly-saccharides was revealed by its positivity to the alcian blue fraction of the AB-PAS test. The sudan black B test was negative in both nucleus and nucleoli showing absence of lipids.

2.2 **NSC Type II - A-Cells**

These NSCs were found distributed in all the ganglia examined. These cells were present in very few numbers in the optic ganglion of the eyestalk. The other ganglia of

the CNS have comparatively more number of such cells.

A-cells ranged from oval to polygonal in shape (fig.4) and were comparatively smaller than GN cells with a mean size of 40.0 $\pm$ 7.0 μ (range - 30 to 50 μ). They possessed a centrally placed vesicular nucleus measuring 15.0 $\pm$ 6.0 μ (Table 1). The number of nucleoli range from 2 to 4 and were found dispersed in the karyoplasm amongst the chromatin material. A-cells had considerable cytoplasm as evidenced by the nucleous-cytoplasm ratio which ranged from 0.3 to 0.5 as shown in table 1. The constitution of the cytoplasm varied from homogenous to dense and coarse, depending on the secretory phase of the cell. Vacuoles were noticed confined to the peripheral regions of the cell. Peripheral vacuoles were sometimes observed to coalesce and form a halo like contiguous ring around the cytoplasm (fig.11).

Glial cell investments were observed around A- cells. The soma of the glial cells being submicroscopic could not be seen, however, the fuchsinophilic nuclei of the glial cells (mean size 2.0 μ) were distinctly visible in a single layer around the NSCs. In the optic, cerebral, subesophageal and thoracic ganglia pericellular capillary plexus were also observed circumscribing the cells (fig.4 and 11).

The tinctorial responses of A- cells to the neurosecretion specific stains is depicted in table 2. Similar to the GN cells, the neurosecretory granules in the A- cell cytoplasm also stained in various shades of purple with PAF, CHP, Azan and Mallory's triple stain (fig.9, 10, 11 and 12). With the PAF technique the ground substance was positive to orange G, the nucleus whitish and the nucleoli was bluish violet. Nuclei were pale blue with CHP technique and the nucleoli purple. Ground substance as well as capillary plexus were distinctly phloxinophilic. With Mallory's triple the ground substance was acid fuchsin positive and with Azan it was palely azocarmine positive. Nuclear and nucleolar staining responses are also given in table 2.

The perikarya revealed considerable amount of proteins as evinced by the strong positivity to the mercuric bromophenol blue test (table 3). However, A- cells were only moderately postive to tests for -S-S group and -SH group and for -NH2 group the reaction was of doubtful nature. Intense positive reaction was observed with the PAS test in the cytoplasm. This positivity however, was diastase resistant and the cytoplasm remained moderately positive to the PAS test revealing the presence of 1,2 glycol groups. With the AB-PAS the magenta reaction of the cytoplasm disclosed its

glycogen content. The cytoplasm was only moderately rich in lipid and was uniformly poor in RNA.

The nucleus showed only moderate reaction to tests for protein and DNA and was PAS negative. The nucleoli showed intense positive reaction to tests for general proteins and amino acid end groups like amino, sulphydryl and disulfide. Nucleoli were also moderately PAS and sudan black B positive. Only meagre amountsof nucleolar RNA was detected. Glial cell nuclei were intensely positive to protein (fig.14).

2.3. **NSC Type III - B-Cells**

B cells were ubiquitous in NSC groups of P.indicus, although their number varied from ganglia to ganglia. Their shape varied from oval to polygonal, but were considerably smaller than A cells (mean size 23 $\pm$ 4 μ, table 1) with relatively less cytoplasm (nucleus cytoplasm ratio 0.4 to 0.6). The cells had a single round vesicular nuclei and were uninucleolated (fig.5 and 6). Nucleoli were promient, centrally placed and measured approximately 3 - 4 μ in diameter. Unlike GN and A- cells, chromatin material was not distinguished in the B- cell karyoplasm.

The cells were invariably found in groups, each group having 10 to 20 cells (fig. 6 and 7). Extracellular capillary plexus and glial cell investments around these cells were also observed frequently. As in other NSC, the cytoplasmic constitution ranged from homogenous to coarse granular. Significantly, vacuole number was by far the maximum in these cells, with the cytoplasm frequently being full of vacuoles interspersed with the secretory granules giving it a frothy appearance (fig. 23). The axons were rarely seen in these cells (fig.5). However, it is presumed that all these cells are unipolar.

The tinctorial response of the cells to the neurosecretion sepecific stains is given in table 2. The NSM stained yellowish purple with PAF (fig.9) and the cytoplasmic ground substance was distinctly orange G positive. Bluish and reddish purple colour was observed in the cytoplasm with CHP, Azan and Mallory's triple stain. The nucleus did not show much affinity to any of the stains and remained whitish or pale blue or red. The nucleoli stained conspicously red with Azan, CHP and Mallory's triple and pale purple with PAF.

The perikarya recorded an intense positive reaction to

general proteins (table 3). All the amino acids tested were present in considerable quantity (fig.13). The cytoplasm was strongly PAS positive and this positivity was partially diastase resistant. A strong magenta reaction with the AB-PAS test confirmed the presence of glycogen. The perikarya also revealed small quantities of pyroninophilic and sudan black B positive granules indicating thereby, the presence of RNA and lipids in meagre amounts. The nucleus was moderately positive to bromophenol blue and methyl green revealing the presence of proteins and DNA. The nucleoli was positive to tests for amino, sulfydryl and disulfide amino acids. Although the PAS positivity was of doubtful nature, the mild blue reaction observed with AB-PAS indicated the presence of acid mucopolysaccharide (AMP) in the nucleoli. The nucleoli were also mildly positive to tests for lipids and RNA.

2.4. NSC Type IV - C-Cells

Type IV, C cells were the smallest neurosecretory cells observed and were found in very limited numbers in all the ganglia except the tritocerebral ganglion. The cells were characterized by their pyriform or conical shape and the mean size was 15 $\pm$ 4 μ (Table 1). The narrow ends of the

cells were invariably continued as axons, C-cells were uninucleated with a single conspicuous nucleoli (fig.5 and 6). The vesicular nucleus had a mean size of 9.0 $\pm$ 3.0 μ. Round nucleoli were centrally placed and exceptionally large having a diameter of 2.0 - 3.0 μ. As in B- cells chromatin matter in the cells was not clearly distinguishable. The nucleus cytoplasm ratio was rather high ranging from 0.4 to 0.7 (see Table 1). The cells were invariably found in groups in close association with B-cells (Fig.6), though their numbers were few. Constitution of the cytoplasm was similar to that of the B-cells ranging from fine to coarse granular. Further, the cells exhibted intense vacuolar and secretory activity in conjunction with its secretory cycle (fig.23). Peripheral glial cell covering and extracellular capillary plexus were also observed.

As indicated in Table 2, the perikarya of these cells were found to be positive to all the neurosecretion specific stains. Excepting the curious magenta reaction with Azan, the NSM in the perikarya stained uniformally as different shades of purple. Nuclear and nucelolar staining reactions are also given in the table.

The results of the histochemical reactions for these

cells are given in Table 3. The cytoplasm displayed only moderate positivity to all classes of proteins and carbohydrates. Nevertheless strong positivity to sudan black B stainable lipid was noticed. Similar to the other NSCs, the cytoplasm was only very mildly positive to RNA. The nucleus was positive only to methyl green indicating the presence of DNA. The conspicuously large nucleoli displayed strong positivity to proteins, especially -SH groups. Positivity to the ferric-ferricyanide test was wholly removed by mercaptide blocking. The nucleoli was also positive to the PAS test and tests for lipids and RNA.

2.5. Non-Neurosecretory Neurons

These very small nerve cells were found distributed freely in all the ganglia examined. The non-neurosecretory neurons showed a mean size of 10.0 $\pm$ 3.0 μ and were characterized by their poor cytoplasm, the boundaries of which were extremely difficult to detect with light microscopy (fig. 7 and 8). Neurons had a prominent nucleus of average size 7.0 $\pm$ 2.0 μ which did not take up any stain. However, the round, vesicular and unitary nucleoli were darkly stained and hypertrophied with an average diameter of 5 μ.

Fig. 3 Photomicrograph of GN cell in the subesophageal ganglion. Note the vacuolated (V) cytoplasm as well as axon (AX). Capillary plexus (CP) in the pericellular region. Mallory's triple stain. x100.

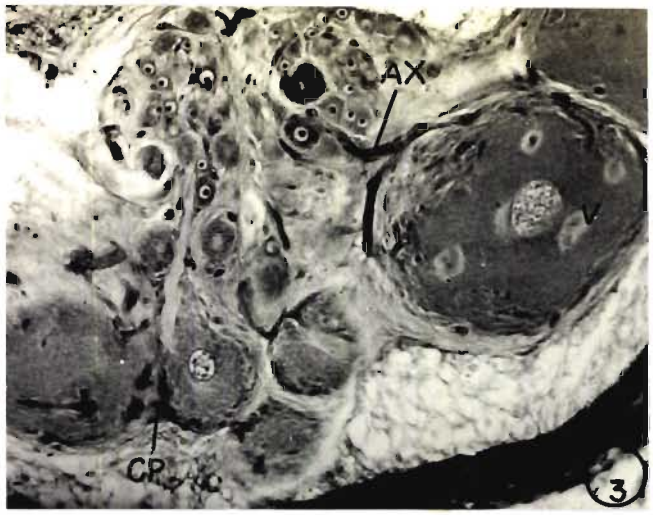
Fig. 4 Photomicrograph of A cells in the supraesophageal ganglion showing glial cells (GC) and capillary plexus (CP) circumscribing them. CT - connective tissue sheath. Mallory's triple stain. x200.

Fig. 5 Photomicrograph of oval B cells and pyriform C cells in the supraesophageal ganglion. GC - glial cells, CP - capillary plexus, AX - axon. Paraldehyde fuchsin. x400.

Fig. 6 Photomicrograph of a dorsomedian group of NSC in the subesophageal ganglion with B and C type cells N - neuropile. Mallory's triple. x100.

Fig. 7 Photomicrograph showing the relationship between NSC and non neurosecretory neurons in the brain. CP - capillary plexus, CT - connective tissue sheath, NN - non neurosecretory neurons. Mallory's triple. x100.

Fig. 8 Photomicrograph showing detail of the non neurosecretory neurons. Note cells with conspicous nucleoli but indistinct cell boundries. Arrows indicate nuclei with mitotic figures and incomplete karyokinesis. Mallory's triple. x400.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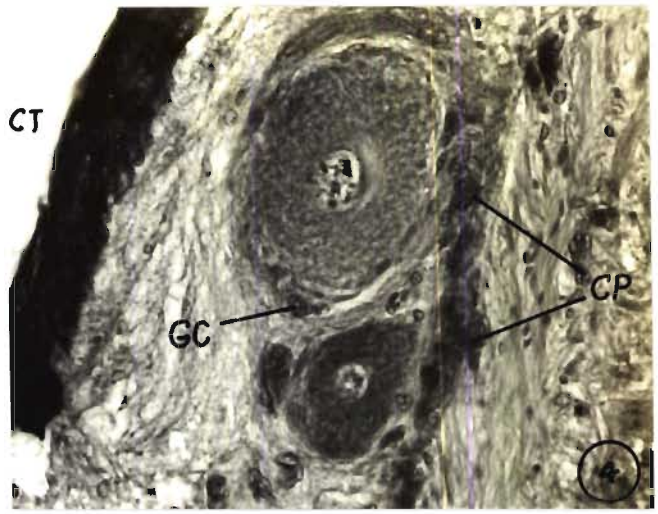
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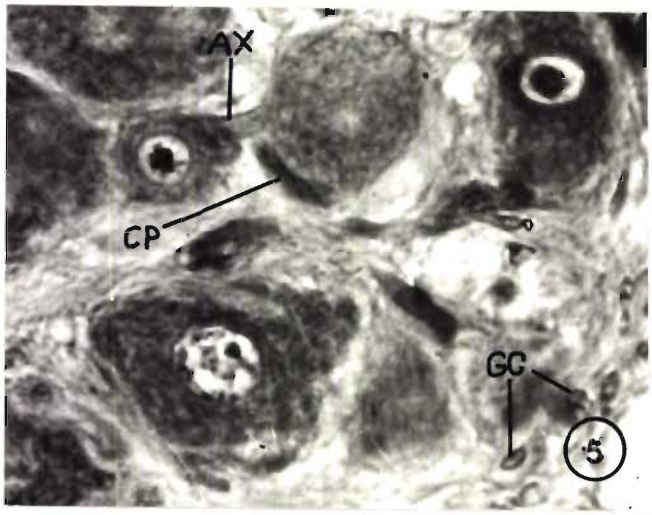
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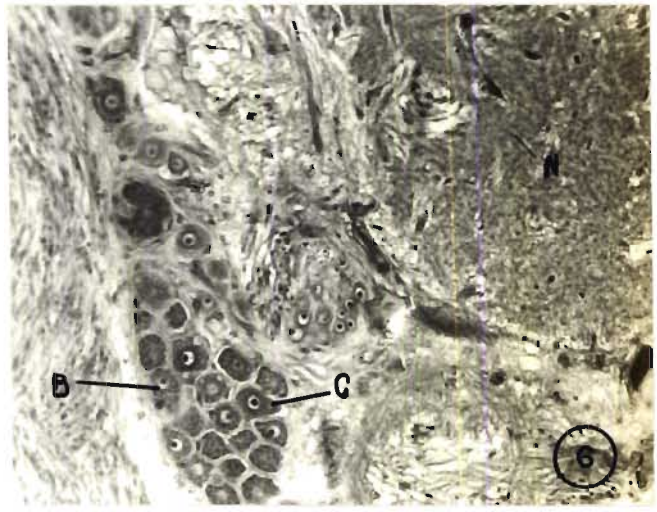
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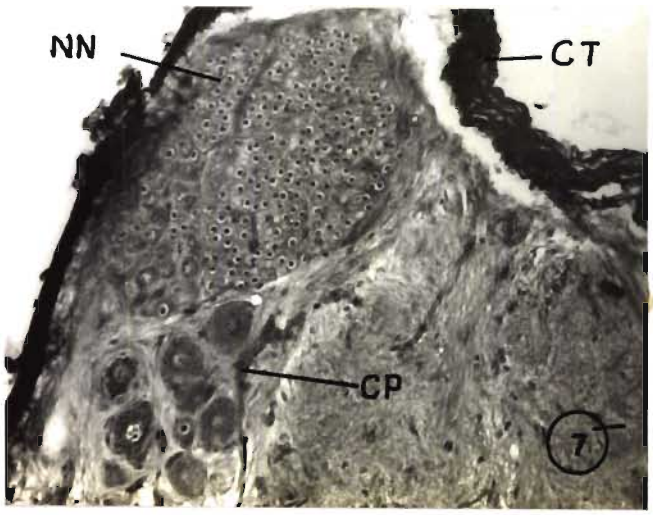
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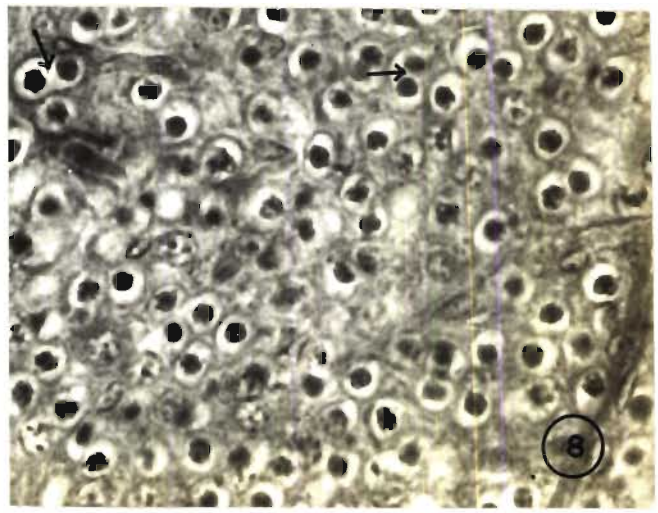
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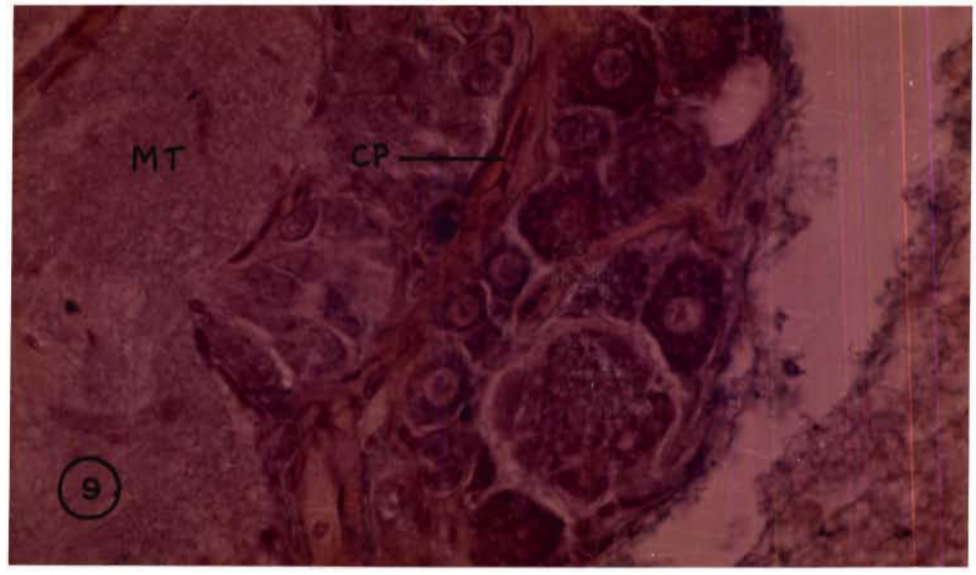
Fig. 9 The paraldehyde fuchsin (PAF) reaction in the MTGXO-2 of the optic ganglia. Note the Orange G positive capillary plexus (CP). MT - medulla terminalis. x200.

Fig.10 The chrome hematoxylin phloxine (CHP) reaction in the MEGXO of the optic ganglia. Note the phloxinophilic capillaries (CP). ME - medulla externa. x200.

Fig.11 The Mallory's triple (MTP) positivity in the MTGXO-1 of the optic ganglia. MT - medulla terminalis, CP - capillary plexus, AX - axons, V - vacuole. x200.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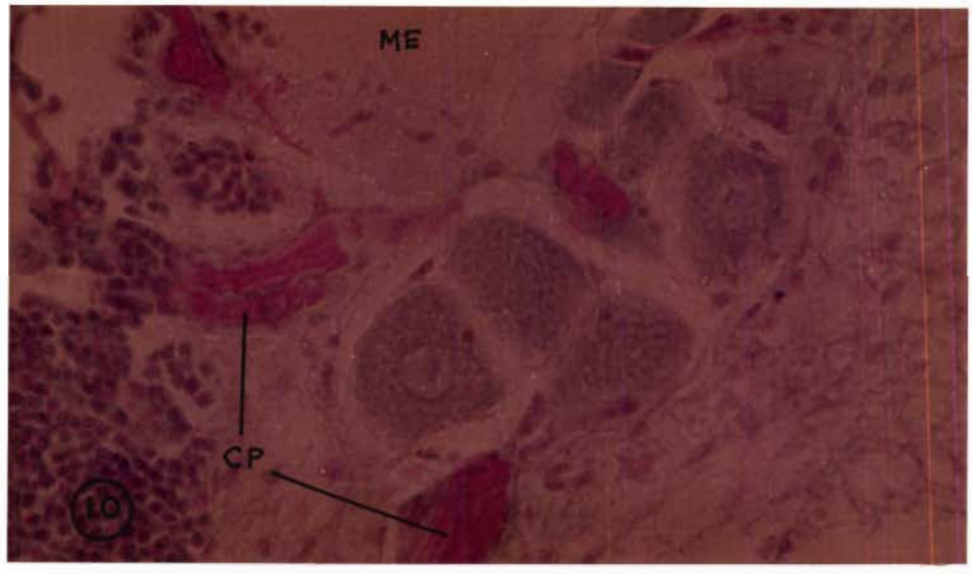
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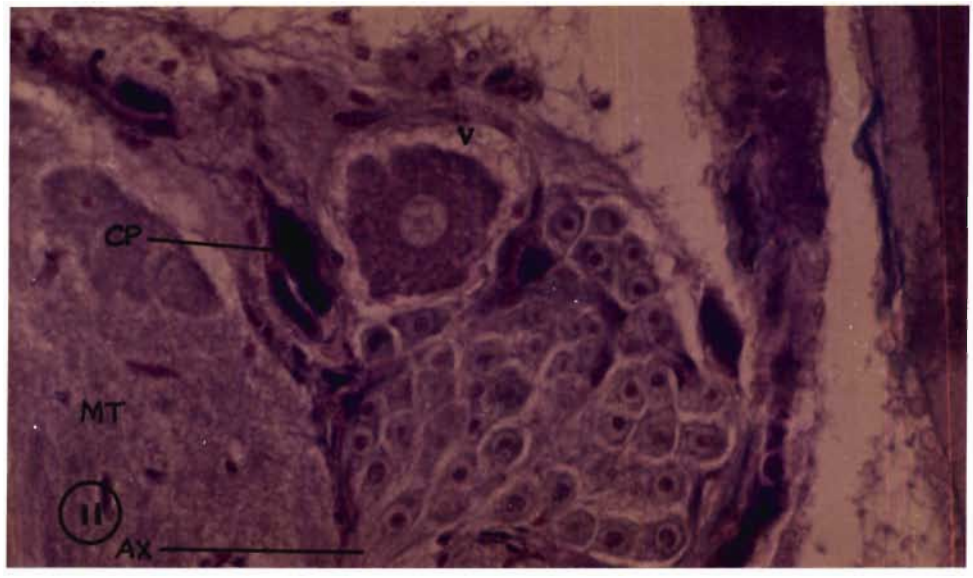
V
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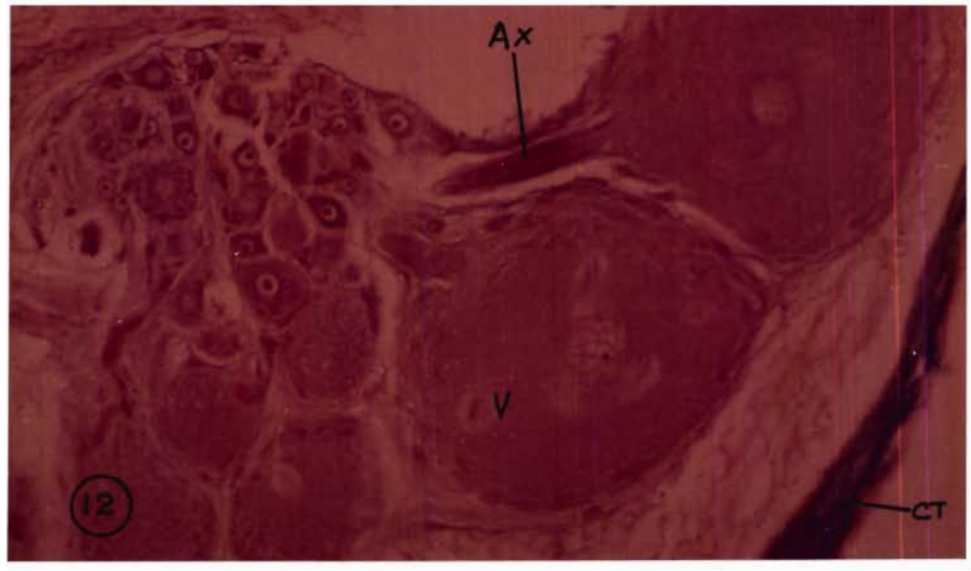
AX

Fig.12 The Haedenhain' Azan reaction in the NSC of the subesophageal ganglion. Note the deeply azocarmine positive axon (AX). V - vacuole, CT - connective tissue sheath. x100.

Fig.13 The performic acid alcian blue (PFAB) reaction in the NSC of the thoracic ganglion. Mark the deeply alcian blue positive axon (AX) and neurosecretory material. x100.

Fig.14 NSCs displaying strong positivity to general proteins (Mercuric bromophenol blue test). Strongly positive glial cells (GC) and capillary plexus are also visible. x100.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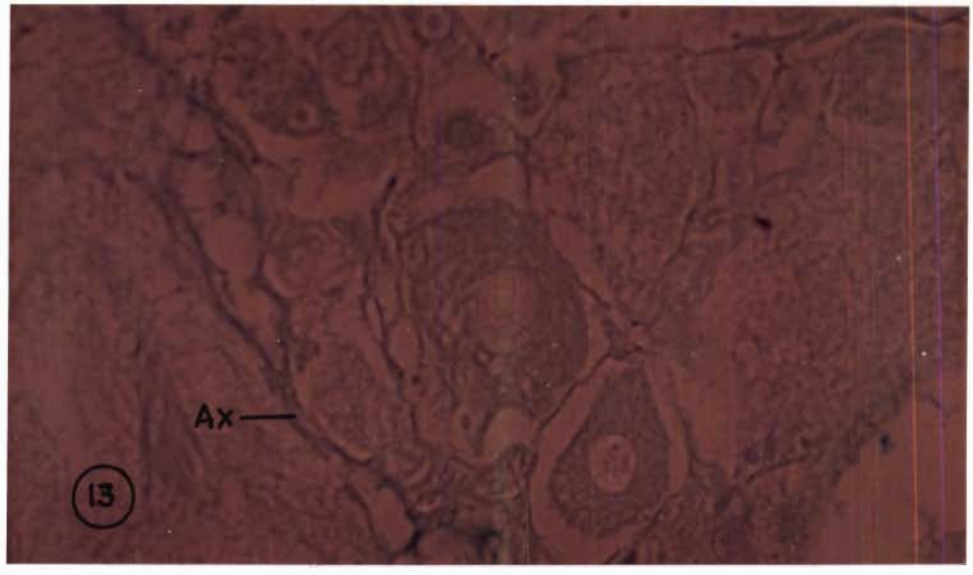
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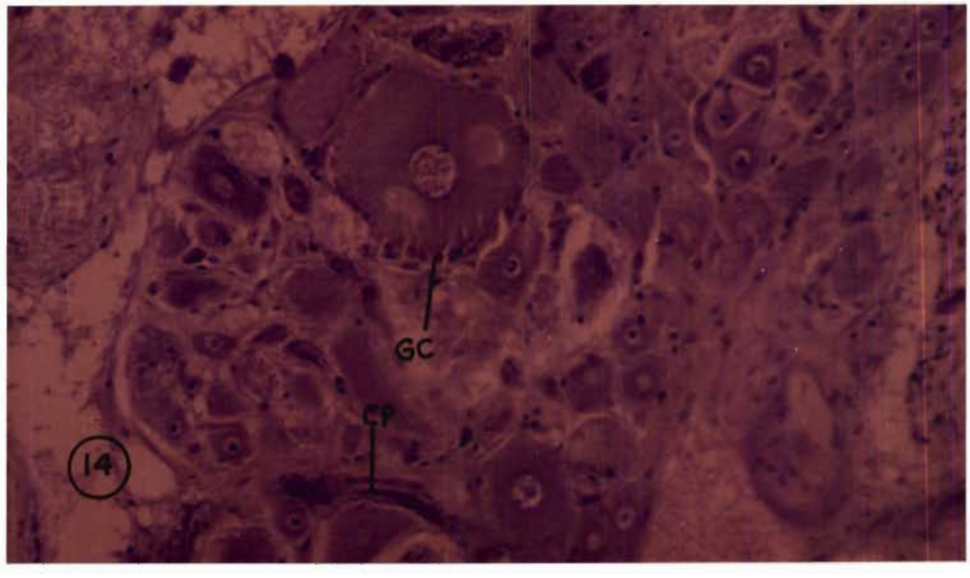
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These cells were considered non-neurosecretory because of the lack of stainable cytoplasm and the absence of definitive changes in their perikarya sugestive of secretory activity. Non-neurosecretory neurons were most often found in large numbers in a group, especially in the supraesophageal ganglion (fig.7). Mitotic phases like metaphase, anaphase and karyokinesis in particular were frequently observed among these cell groups (fig.8).

3. Neurosecretory cells and their secretory cycle

Distinct cyclic changes were observed in the perikarya of the NSCs, in concurrence with the synthesis of neurosecretory material. Among the different cell types there was no uniformity in the cyclic changes. Accordingly, two morphological types of secretory cycles were seen; one common to GN and A-cells and the other to B-and C-cells. However, the basic pattern of the secretory cycles were esentially the same, involving three secretory phases. The secretory phases were identified based on the appearance of secretory granules in the cytoplasm, vacuolization and involvement of extracellular glial cells and capillary plexus. The secretory phases, as observed in the present study, were arbitrarily classified into three categories viz, the quiescent phase, vacuolar phase and the secretory

phase.

3.1. Quiescent phase (Q)

This is the resting or inactive phase of the NSCs, wherein synthesis of secretory material is not apparent. Consequently in all the cell types the cytoplasm remained homogenous and lightly stained without any clumping (fig. 15 and 21). The perikarya in GN, A-, B- and C- cells showed only background stain levels with PAF,CHP,Azan and Mallory's triple stain. This phase is further characterized by the total absence of vacuoles and stainable granules in the cytoplasm.

Though glial cells were observed to circumscribe the NSCs in this phase, their number and size were minimal (fig.15). Infrequent occurrence of capillary plexus were also observed around the NSC groups, however they were poorly stained with all the staining techniques applied. In GN and A-cells nuclear and nucleolar characteristics remained essentially the same and in B- and C-cells, the nucleoli were centrally located in the karyoplasm as seen in fig.21.

3.2. Vacuolar phase (V)

The NSCs in this phase were characterized by the presence of vacuoles in their perikarya. Vacuoles in the GN and A-cells varied widely in their size and shape. In contrast, the B and C cell vacuoles had fairly uniform size and shape.

In the GN and A-cells the vacuole size ranged from 10 to 30 u in diameter and interestingly their shape varied from round to oval to a curious horseshoe shape (fig. 16). Further the vacuoles in these cells were perinuclear in distribution. On occassions when the horseshoe shaped vacuoles were observed, they were found to almost encompass the nucleus and also sometimes were in contact with the nuclear membrane (fig.16). Vacuoles which were filled with a lightly staining matrix were also membrane bound, although on most occassions these membranes were difficult to detect and cytoplasmic material appeared to be diffusing (fig.16). The cytoplasm of GN and A-cells during this phase was distinctly granular and darkly stained. Also characteristic of this phase in these cells was the hypertrophy and apparent hyperactivity of the glial cell nuclei around the periphery of these cells (fig.16). Glial cell processes were not distinct, but presumably they encompass the whole

cell. Pericellular capillary plexus were also seen.

In B- and C-cells, the membrane bound vacuoles seen are of fairly uniform size and shape. Their diameter ranged from 10-15 μ and shape remained constantly round or oval. Further in B- and C-cells vacuolization was peripheral in disposition and apparently in origin too (fig.22). Strangely enough, individual vacuoles tended to merge or coalesce to form a peripheral vacuolar ring round the narrow band of darkly stained perinuclear cytoplasm (fig.22). This unique vacuolar configuration tended to make the cytoplasm appear seemingly empty. The darkly stained perinuclear cytoplasm was however, conspicuously granular. Hypertrophied glial cell nuclei (average size 2.0 μ) were observed around the pericellular margin of B- and C-cells (fig.22) together with capillary plexus. The prominent nucleoli of B- and C-cells in this phase underwent a perceptible migration to an eccentric position in the nucleus and sometimes were observed to be in close contact with the nuclear membrane (fig.22).

3.3 Secretory phase (S)

In this final phase of the secretory cycle the NSCs are

apparently ready for the release of NSM. Characteristic of this phase in all the NSC types was the increasingly darkly stained and granular cytoplasm which was interspersed with vacuoles. The vacuoles were observed to have within them distinct PAF and CHP positive granula (fig.17 and 23).

In the GN and A-cells the vacuole size diminished and ranged form 5 - 10 μ in diameter. Moreover, they underwent a peripheral migration from their previous perinuclear position. Consequently in this phase the vacuoles were predominantly seen near the outer cell membrane with darkly stained granular material within them (fig.17 and 18). Also evident were the hypertrophied glial cell nuclei (2-3 μ in diameter) distributed randomly around the cell. Frequently the outer boundaries of the cytoplasm became intensely granulated and clumped presenting a broken appearence (fig.18 and 19). Capillary network adjacent to GN-and A-cell boundries was also seen (fig.19). GN and A-cell axons, during this phase were intensely stained (fig.12) with CHP, Azan and PAF and on occasions axoplasmic steaming was also observed (fig.20).

The perikarya of B-and C-cells showed remarkable changes in this phase. The breadth and span of the peripheral vacuolar ring became considerably reduced and was present

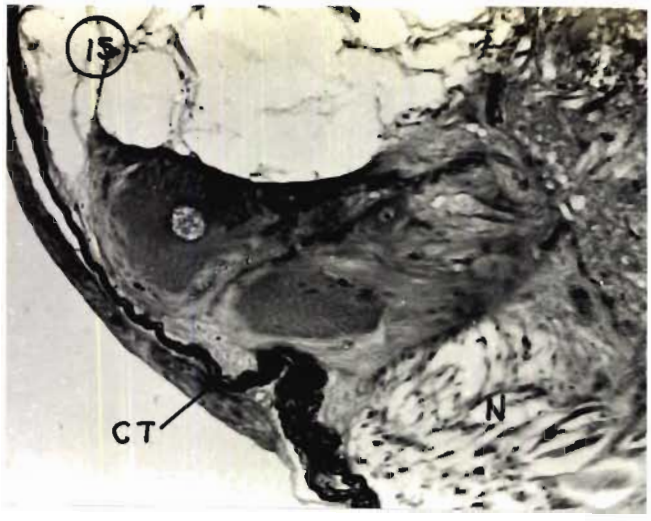
Fig.15 Abdominal ganglia A cells in the quiescent (Q) phase with homogenous and lightly stained cytoplasm. N - neuropile, CT - connective tissue sheath. MTP. x100.

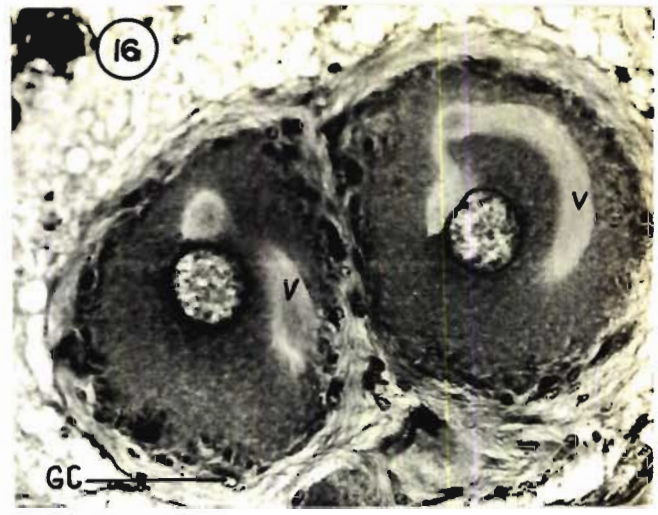
Fig.16 GN cells in the thoracic ganglion in the vacuolar (V) phase. Note the large perinulcear vacuoles (V) and the investment of glial cells (GC) around the NSC. MTP. x200.

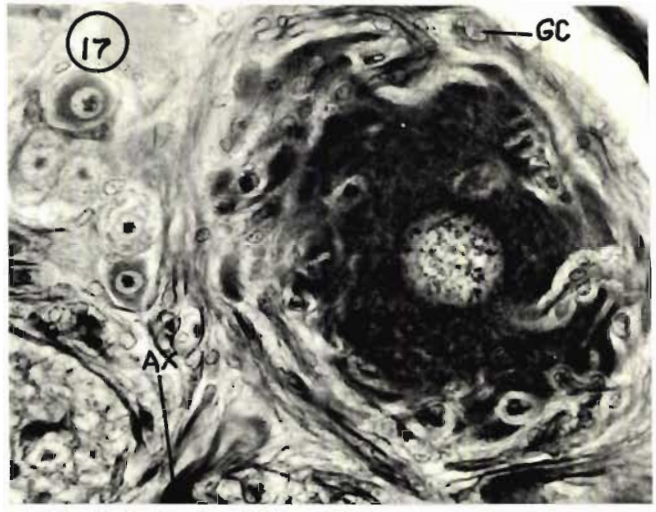
Fig.17 GN cell in its terminal Secretory (S) phase with peripheral vacuoles and hypertrophied neuroglia (GC). The axon (AX) is also deeply stained. x200.

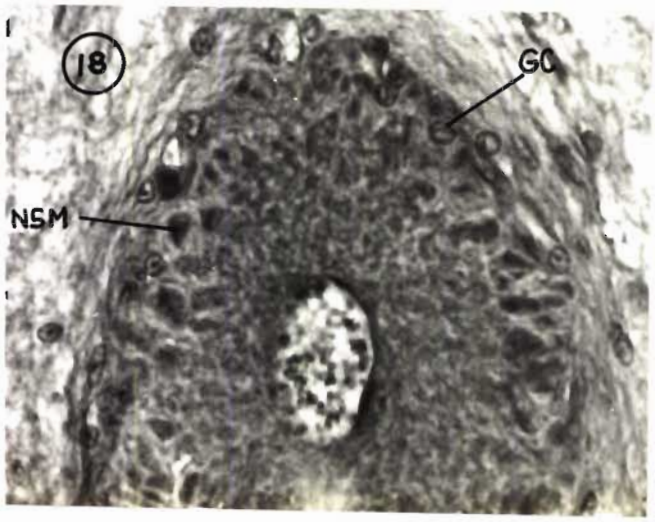
Fig.18 Detail of a Secretory phase A cell in the brain. Note the hypertrophied glial cells (GC) and the coarse nature of the cytoplasm with granular neurosecretory material (NSM). PAF x400.

Fig.19 A, B and C NSC in the subesophageal ganglion. A cell displays broken cytoplasm and pericellular capillary plexus (CP) in the secretory phase. MTP. x100.


15
CT
N


16
V
V
GC


17
GC
AX


18
GC
NSM

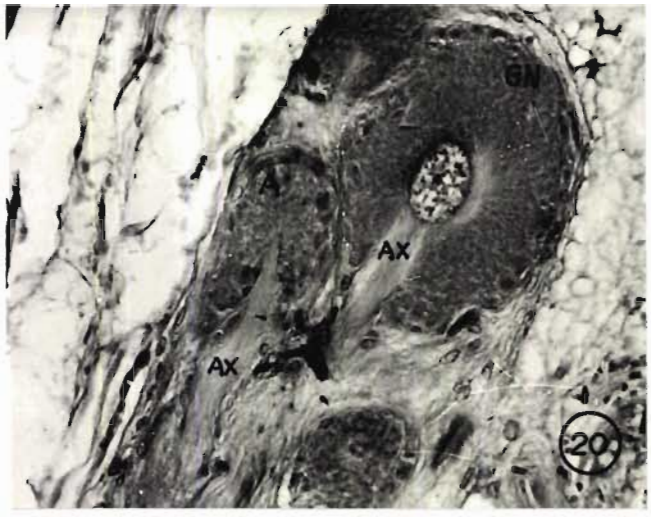

19
CP

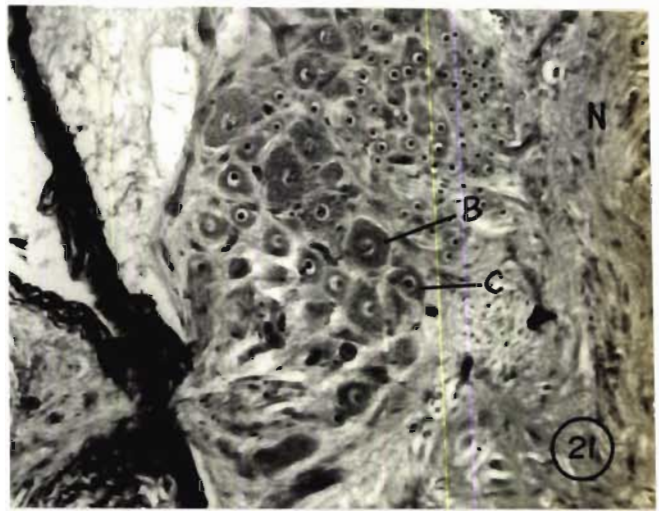
Fig.20 GN and A cells of the thoracic ganglion displaying axoplasmic streaming (AX) in the secretory phase. MTP. x100.

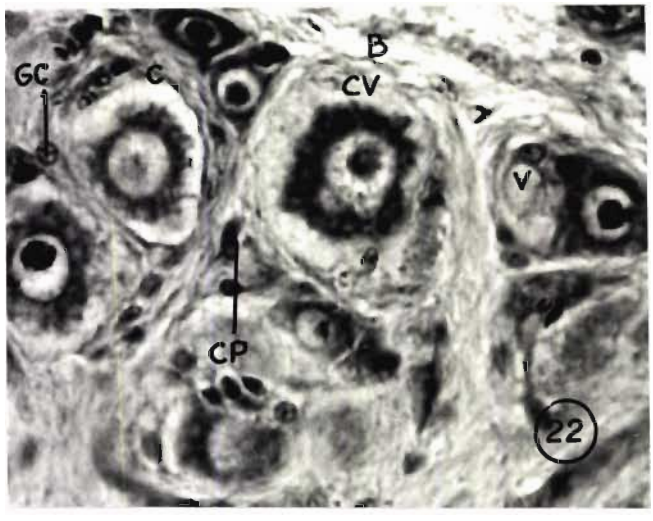
Fig.21 B and C cells in the cerebral ganglion in the quiescent phase with lightly stained and homogenous cytoplasm. N - neuropile. MTP stain. x100.

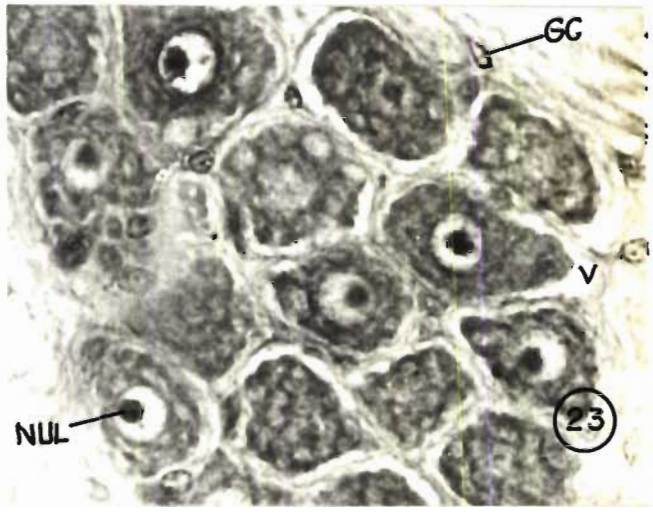
Fig.22 Vacuolar phase B and C cells of the cerebral ganglion. Vacuoles (V) originate in the peripheral region of the cell and later coalesce to form a pericellular vacuole (CV). Also note the glial cell (GC) investiture and capillary plexus (CP). PAF. x400.

Fig.23 Secretory phase B and C cells in the subesophageal ganglion. Note the extremely granular and frothy nature of the cytoplasm interspersed with vacuoles (V). The nucleolus (NUL) is eccentric in position and the glial cells (GC) are hypertrophied. PAF stain. X400.


GN
A
AX
AX
20


N
B
C
21


GC
C
B
CV
V
CP
22


GC
V
NUL
23

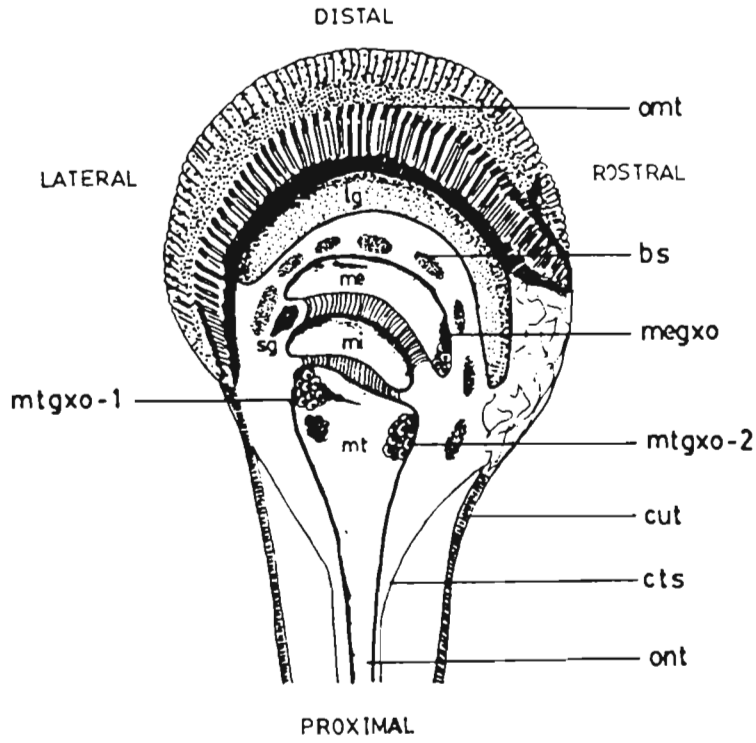
only as a thin covering around the cytoplasm (fig.23). Similar to GN-and A cells, the vacuole size also became diminished to approximately 5 u. The cytoplasm during this phase was frothy or foamy in appearance (fig.23) due to the random dispersion of small vacuoles and secretory granules. In the nucleus, the nucleolar eccentricity persisted and the latter was sometimes observed to be closely applied to the nuclear membrane (fig.23). Though glial activation was present, it was not to the magnitude of that observed in GN- and A-cells. Capillary network or plexus was also seen (fig.11) around B and C cells during this phase.

4. Eyestalk neuroendocrine complex and distribution of NSC

4.1. Gross morphology

Histological preparations of the eyestalk revealed that the eyestalk is composed of several important structures, the microanatomy of which is found to be rather complex. A collaged photomicrograph of the left eyestalk is depicted in Fig.25, in which the retina and exoskeleton has been removed. A schematic representation of the same is given in Fig.24. Standard eyestalk nomenclature (Gabe, 1966) has been used throughout.

FIG.24 SCHEMATIC REPRESENTATION OF THE IMPORTANT NEURAL STRUCTURES IN THE LEFT EYESTALK AS SEEN FROM THE DORSAL SIDE.



Key: omt - ommatidia, lg - lamina ganglionaris, bs - blood sinus, me - medulla externa, megxo - medulla externa ganglionic X-organ, sg - sinus gland, mi - medulla interna, mt - medulla terminalis, mtgxo-1 - medulla terminalis ganglionic X-organ one, mtgxo-2 - medulla terminalis ganglionic X-organ two, cut - cuticle, cts - connective tissued sheath and ont - optic nerve tract.

Fig.25 Collaged, photomicrograph of the left eyestalk. Longitudinal section. Key -

LG - lamina ganglionaris
ME - medulla externa
MEGXO - medulla externa ganglionic X-organ
MI - medulla interna
SG - sinus gland
MT - medulla terminalis
MTGXO-1 and MTGXO-2 - medulla terminalis ganglionic X-organ one and two
ON - optic nerve


LG
ME
SG
MI
MEGXO
MTGXO
MTGXO
MT
ON

Proximally, the eyestalks were covered externally by a thin exoskeleton cuticle (fig.24 and 29). Distally it was covered by the dark ommatidia. The dorsal surface of the eyestalks could be easily recognized by the presence of a distinct 'V' shape notch on the proximal periphery of the ommatidial cap. The same was absent on the ventral side. The central axis of the eyestalk consists of the optic ganglia enclosed by a thin connective tissue. Under a dissection microscope, after removal of the surrounding muscles, four distinct medullary lobes of the optic ganglion could be made out. Distally just below the ommatidia the lamina ganglionaris (LG) was situated, connected by bundles of axons to the first of these medullae: the medulla externa (ME), followed by the medulla interna (MI) and the medulla terminalis (MT). The latter was the smallest and the most proximal and connected with the brain via an optic nerve tract. The sinus gland was not visible as a bluish body under the dissection microscope as reported in most other species by earlier workers.

Histologically, longitudinal step-serial sections revealed the anatomy and complexity of the neural structures and distribution of NSC in the eyestalk. Apically below the ommatidial layer, the LG was present as an inverted cup

shaped lobe which extended like an umbrella over the other lobes (fig 29). In the right eyestalk the left proximal extremity of the LG extended farther than the one on the right side (fig 24 and 29). The LG was devoid of any neuro-secretory cells. Between the LG and the ME, a vascular layer was seen (fig 29 and 30). In dorsal sections (fig 29), the vascular organisation appeared as distinct finger like processes, extending from a main vascular trunk (fig 30) located ventrally. This vascular trunk was continued to the outside proximally, adjacent to the optic nerve tract. In an eyestalk 10 to 15 such finger like vascular processes (termed blood sinuses) were observed. The neurohaemal organ, viz the sinus gland was invariably found associated with one such vascular process (fig 31).

The ME was also an inverted cup shaped lobe immediately beneath the LG, the proximal extremities of which were inclined toward the rostral side (fig 24 and 29). The distal surface of the lobe was distinctly wavy or undulating and it was in these depressions that the NSCs were present. The MI was also similar in shape to the ME, though slightly smaller in size and devoid of NSC. Adjacent to and below the MI was the distinctly knob like MT, on the surface of which were present the principal NSC groups. In between the MI and MT on its dorso-lateral aspect a bundle of darkly

stained nerve fibres was seen (fig 32). This is apparently the optic chiasmata whose probable function is the transmission of visual impulses (Dr. Fingerman personal communication). The sensory pore X-organ (organ of Bellonci) was not observed in P.indicus.

4.2. Distribution of neurosecretory cells in the eyestalk

Histological preparations of the eyestalk revealed that the putative NSCs were found in three conspicuous groups and these were named on the basis of their location. Among the medullary lobes, the LG and MI did not show any NSCs.

The principal NSC groups were found on the apical surface of the MT. They were distributed in two distinct groups one each on each lateral side (fig 24). In a left eyestalk, the group on the left side was termed the medulla terminalis ganglonic X-organ-1 (MTGXO-1) and the NSC group on the right was termed the medulla terminalis ganglionic X-organ-2 (MTGXO-2). The MTGXO-1 (fig 35) was observed to be in two fractions but with cellular contiguity and bulged from the surface of the MT lobe. The MTGXO-1 was pyriform in shape with the tapering end continued as the axon terminals of all the NSCs in the group (fig 35 and 11).

Although the sinus gland was positioned immediately distal to the MGTXO-1, the axon terminals of this group were continued in the opposite direction, ie, toward the MTGXO-2. The cell types distribution is shown in fig 26 and 27. A,B and C-cells were commonly found in the MTGXO-1. B-cells were the most abundant with a few C-cells and one or two A-cells on its dorsal side.

The MTGXO-2 is also situated on the apical surface of the MT lobe, however its position is on the rostral side, diametrically opposite to that of the MTGXO-1 and the SG (fig 24). The number of NSCs and extent of the MTGXO-2 is considerably lesser than that of MTGXO-1. The MTGXO-2 also bulges from the surface of the MT lobe and is roughly ellipsoid in shape (fig 36). Neurosecretory axons were observed to leave the MTGXO-2 towards the distal end in histological sections. The distribution of NSCs in the MTGXO-2 is shown in figs 26 and 27. Type B and C cells were the most prominent among the cell types on the both dorsal and ventral aspects. A single large (45 - 50 μ diameter) A-cell was observed on the dorsal side of the MTGXO-2 (fig.36) The details of the A,B and C NSCs in their secretory phase are shown in fig 37.

Apart from the NSC groups seen in the MT lobe a single

NSC group was observed on the lateral surface of the ME on its rostral aspect (fig 24). This group termed the Medulla externa ganglionic X-organ (MEGXO) was diffused in nature. In longitudinal step-serial sections the MEGXO was the first X-organ to appear from both ventral and dorsal sides due to the umbrella like nature of the ME lobe. The NSCs in the MEGXO were frequently found interspersed with small neurons (fig 38). Further the apical and distal surface on the ME lobe was thrown into folds giving a distinct wavy appearence in sections and it was in these indentations that the NSCs of the MEGXO were found.

A schematic representation of the neurosecretory cell distribution is shown in figs 26 and 27. In the MEGXO also, B- and C-cells were the most common and they were found distributed on both the dorsal and ventral planes. Two or three A cells were also seen on the ventral side at the proximal extremity of the lobe (figs 10 and 38).

4.3. **Sinus gland and the X-organ-sinus gland tract**

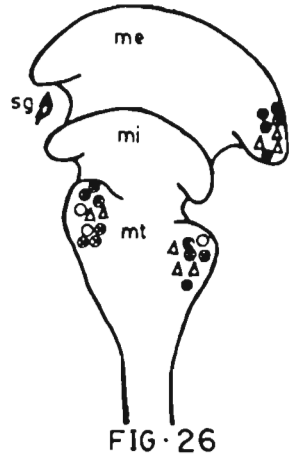
The principal neurohaemal organ of the eyestalk, viz., the sinus gland (SG), in P.indicus was always found associated with one of the finger like vascular processes

(external blood sinuses) seen around the medullary lobes. In a right eyestalk, the sinus gland was located laterally on the right side in the space between the ME and MI lobes (figs.24 and 33). The sinus gland was roughly elliptical in shape and flattened on a dorsoventral plane (fig. 34). In transverse sections the SG was observed to have an oval shape. The central part of the gland was occupied by the internal blood sinus. The swollen axonal endings containing the neurosecretory material was separated from the internal blood sinus by a thin basement membrane, which was stained dark purple with PAF. In adult animals (above 130mm TL) the gland was approximately 250 μ long and 100 μ wide.

As apparent in fig.34 neurosecretory granules and granular congregations were frequently seen in the sinus gland. However, the granules varied considerably in their size with a consistent gradient from the external to the internal surface of the gland. Larger granula, in the outer part of the gland were approximately 2 to 4 μ in diameter and they stained deep purple with PAF and CHP. Smaller purple granules 0.5 to 1.0 μ in diameter were located adjacent to the basement membrane and sometimes as fine granules within the internal blood sinus. No attempts were made to classify these granula according to size as they were observed commonly as aggregations. The swollen and

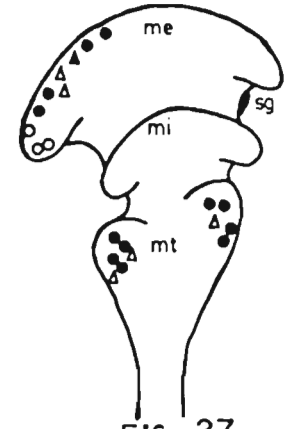
NSC MAPPING – EYESTALK

Dorsal



FIG·26

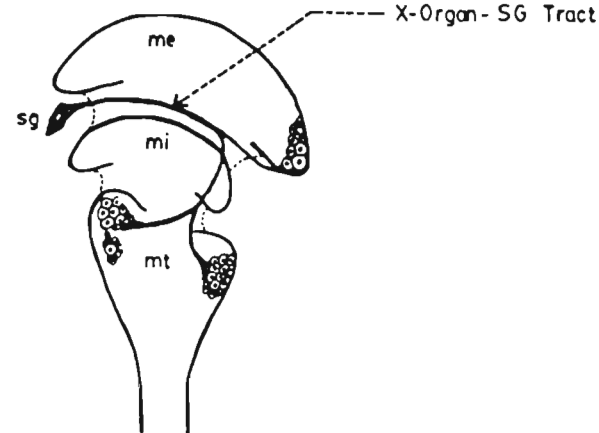
Ventral



FIG·27

me,mi,mt - medulla externa, interna and terminalis, sg- sinus gland
o -A Cell , ● -B Cell, Δ -C Cell

Axonal pathways



FIG·28

Fig.29 Extreme dorsal longitudinal section of the eyestalk showing the finger like vascular processes (VP) in the lamina ganglionaris (LG). ME - medulla externa, MI - medulla interna. Haematoxylin and eosin. x25.

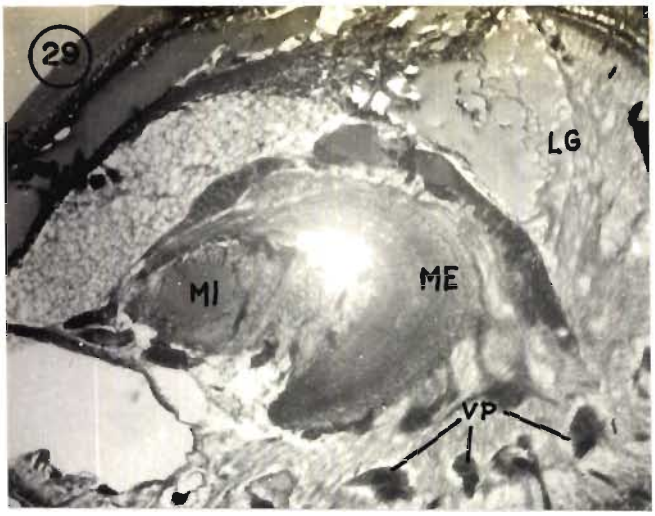
Fig.30 Ventral longitudinal section of the eyestalk showing the coalition of vascular processec (VP), LG - lamina ganglionaris, ME - medulla externa. Haematoxylin and Eosin. x50.

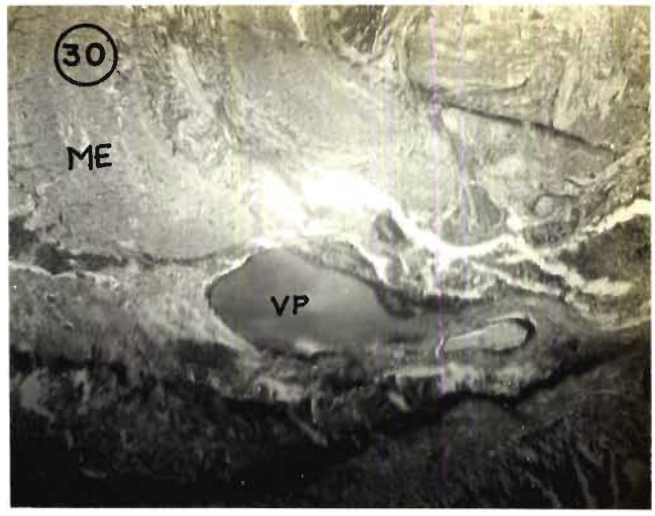
Fig.31 A large vascular process, called the external blood sinus (EBS), adjacent to the sinus gland (SG). PAF stain. x100.

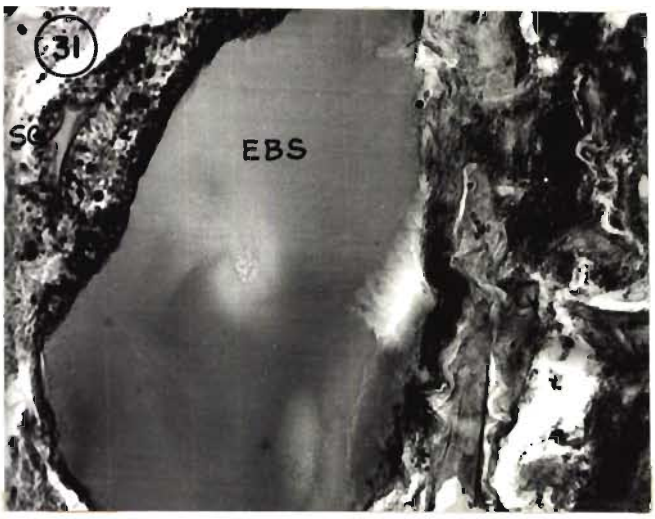
Fig.32 Photomicrograph showing the optic chiasmata (OC) immediately beneath the medulla interna (MI). MTP stain. x100.

Fig.33 Photomicrograph showing the combined neurosecretory axons (AX) approaching the sinus gland (SG), distal to the medulla interna (MI). MTP stain. x100.

Fig.34 Detail of the sinus gland adjacent to the external blood sinus (EBS). Note the abundance of neurosecretory granules (G), bulbous axonal endings (AX) and the internal blood sinus (IBS). PAF stain. x400.


29
LG
MI
ME
VP


30
ME
VP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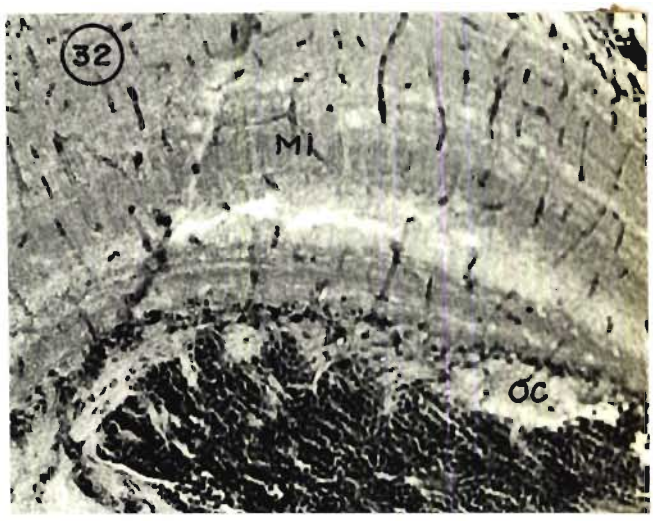
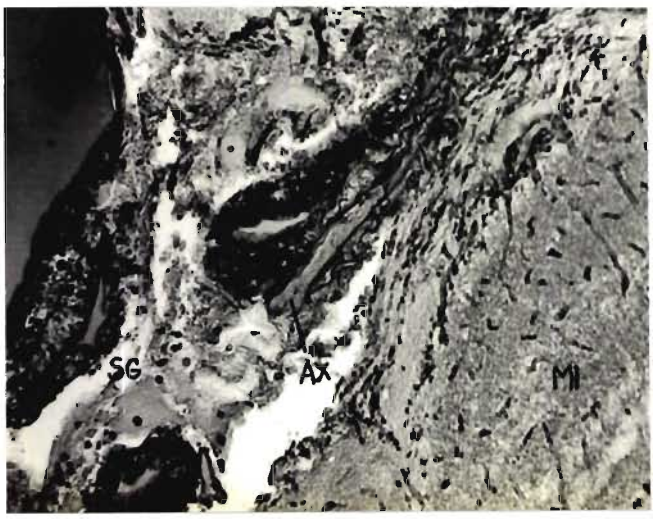
EBS


32
MI
OC


SG
AX
MI

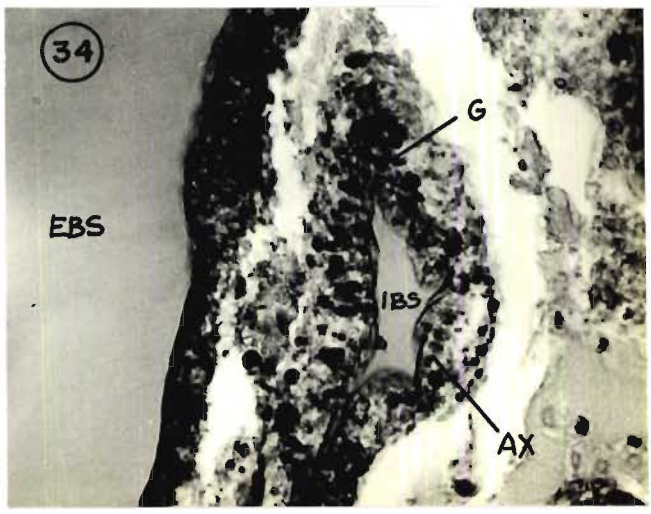
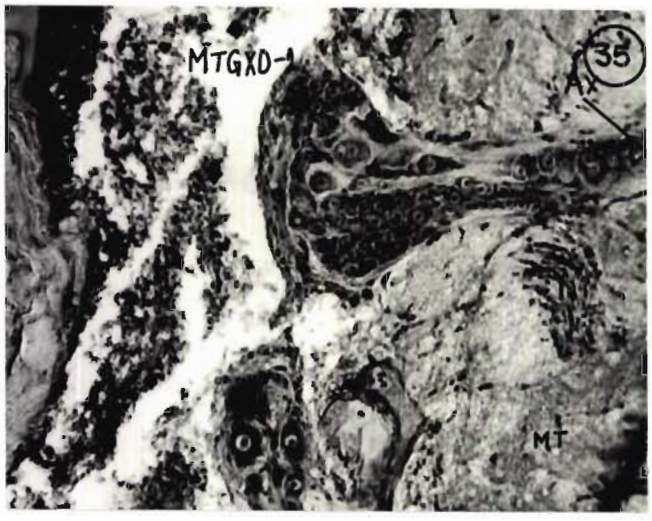

34
G
EBS
IBS
AX

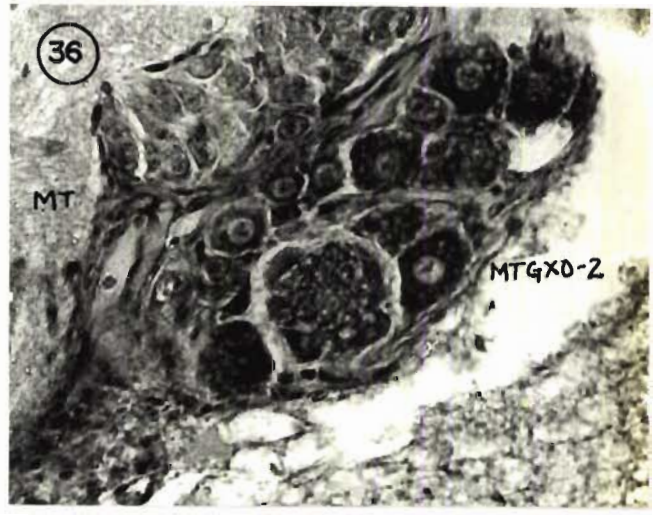
Fig.35 Photomicrograph of the MTGXO-1 on the distal surface of the medulla terminalis (MT) in two parts with cellular contiguity. Note the combined axons leaving (AX) the gland. MTP stain. x100.

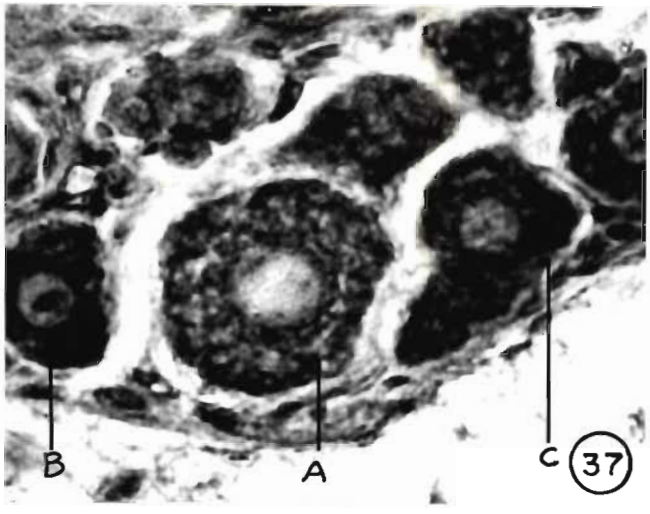
Fig.36 The MTGXO-2 on the surface of the medulla terminalis (MT) lobe. MTP stain. x200.

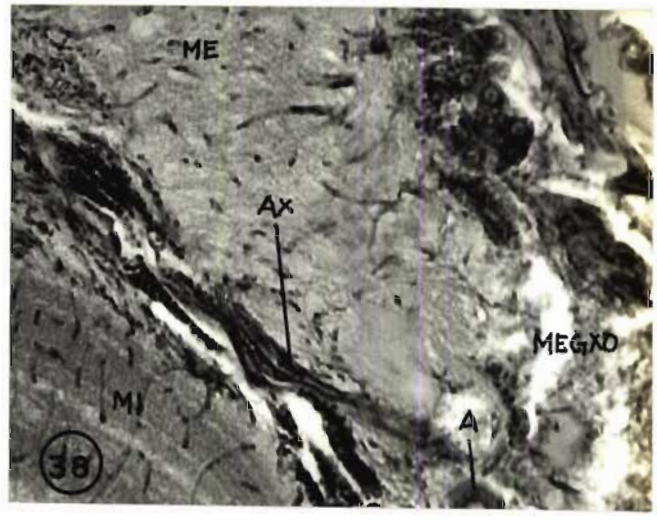
Fig.37 Detail of the MTGXO-2 showing a single large A cell as well as B and C cells. PAF stain. x400.

Fig.38 The MEGXO on the surface of the ME lobe. Note the small A cell at the tip of the lobe. The axons (AX) leaving the gland can be seen inbetween the ME and MI lobes. CHP stain. x100.


MTGXD-1
35
AX
MT


36
MT
MTGXD-2


B
A
C
37


ME
AX
MEGXD
MI
A
38

bulbous axonal endings measured approximately 6 to 7 μ in diameter.

The nervous tract from the X-organs to the SG called the X-organ-sinus gland tract was observed in histological preparations rather infrequently and never in its entirety (figs. 35 and 38). However what was effectively deduced from histological preparations was confirmed with the victoria blue technique and is shown schematically in fig.28.

The X-organ-SG tract near the SG (fig.33) was approximately 10 to 12 μ in thickness. The axons from the MTGXO-1 and 2 were apparently joined together ventrally, above the MTGXO-2 and beneath the optic chiasma. The axons from the MEGXO proceeded towards the posterior edge of the ME and MI lobes and joined with those from the MTGXO-1 and 2. From this juncture, the axons from all the three X-organs proceeded dorsolaterally towards the SG.

5. Distribution and mapping of NSCs in the CNS

5.1. Supraesophageal ganglion

The NSC groups in the cerebral ganglion were found to be distributed in the peripheral regions immediately underneath the connective tissue sheath. Totally 11 NSC groups were

identified on both the dorsal and ventrtal aspects of the supraesophageal ganglion. The dorsal side was observed to be richer in NSCs though there were only 5 NSC groups whereas the ventral side had fewer NSCs with 6 NSC groups (fig.45 and 46). With the exception of a centrally placed dorsal group, all the other NSC groups were found to be located in the shoulder region formed by the nerve branches leaving the main body of the ganglion.

Anteriorly on the ganglion's dorsal side, a pair of NSC groups, termed the anterior dorsal group were seen. This group lies at the junction between the origin of the antennary nerve and the optic nerve (fig.45). Type A- and B-cells were commonly observed in the anterior dorsal group, with the former in very few numbers. A single central dorsal group was present exactly in the middle of the ganglion at the point where the right and left optic lobes converge. This group was the largest ganglionic cell group in the brain with a large number of GN cells and a few A- and B-cells (fig.41). The posterior dorsal group was found at the point of origin of the circumesophageal connectives proceeding posteriorly and was the smallest of the NSC groups in the brain. The group comprised of 4 to 6 A- and B-cells lying in close apposition (fig.39).

Fig.39 Photomicrograph of the posterior dorsal group of NSC in the supraesophageal ganglion. Note the superficial and peripheral position of the group adjacent to the connective tissue sheath (CT). CP - capillary plexus. MTP stain. x100.

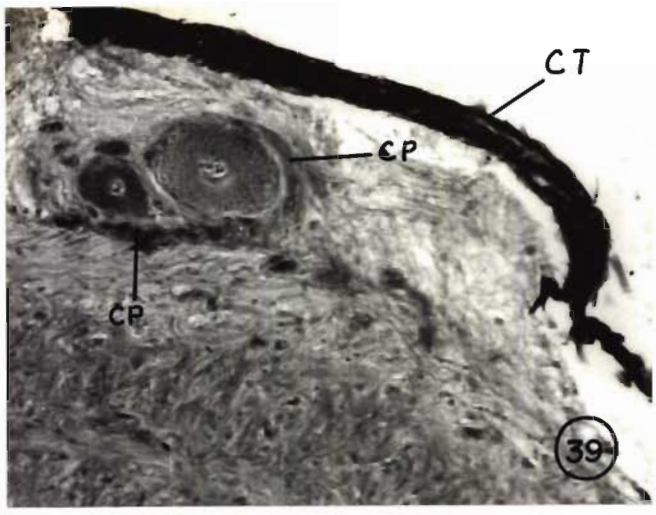
Fig.40 The median ventral group of the brain with B and C cells. CP - capillary plexus, GC - glial cells. MTP stain. x200.

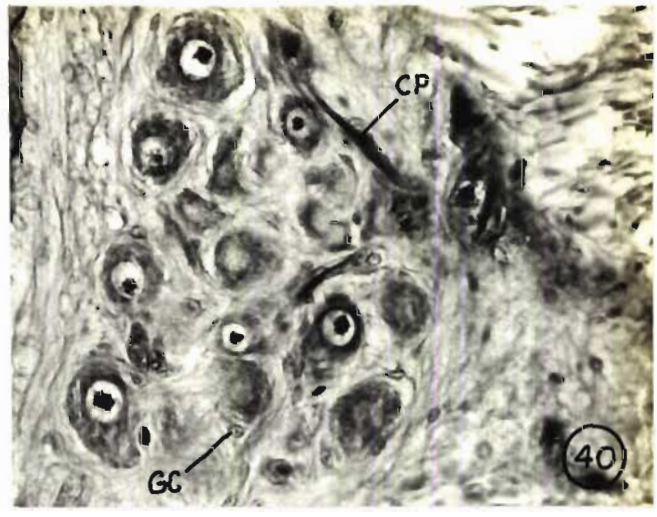
Fig.41 The central dorsal group of the supraesophageal ganglion with GN cell at its periphery. Note the broken nature of the cytoplasm and the release of neurosecretory material through the axon. MTP stain. x100.

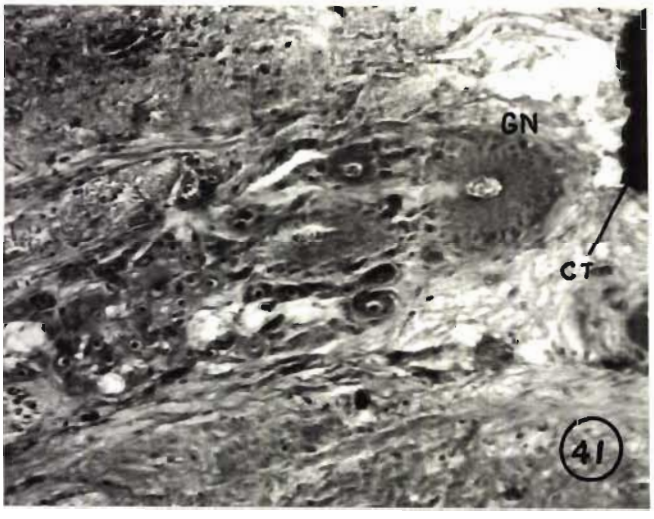
Fig.42 In situ demonstration of the neurosecretory (NSC) soma and axons (AX) in the supraesophageal ganglion using the Victoria Blue technique. x100.

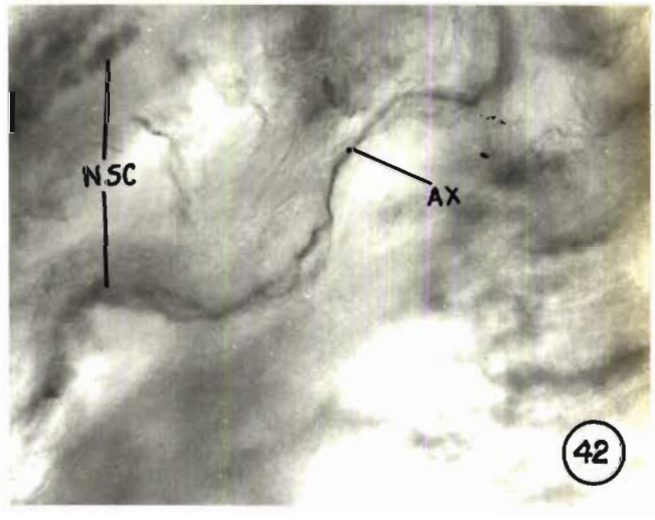
Fig.43 Photomicrograph of the tritocerebral ganglion at the level where it is attached to the nerve cord (NC). Note the A cells and the nerve branches (NB) leaving the ganglion. CHP stain. x50.

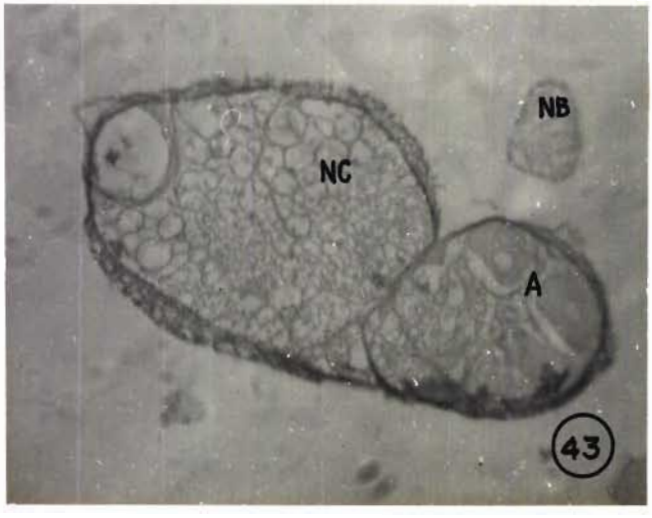
Fig.44 Detail of the tritocerebral gangion (TG) with an exceptionally large and active GN cell. NC - nerve cord. MTP stain. x100.

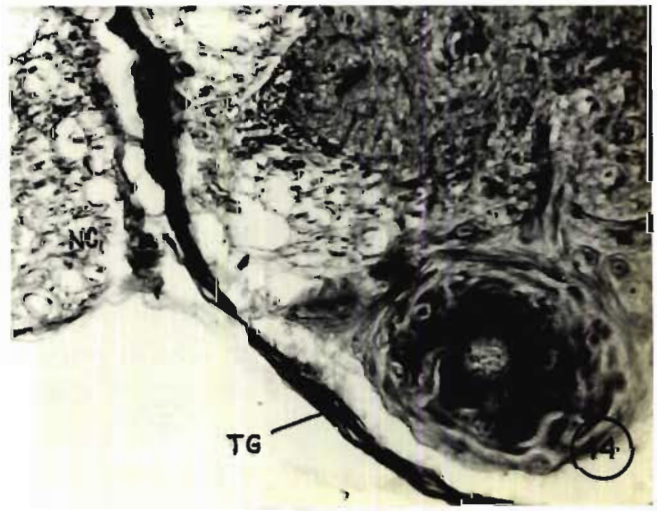

CT
CP
CP
39


CP
GC
40


GN
CT
41


NSC
AX
42


NB
NC
A
43


NC
TG
44

NSC MAPPING - Supraesophageal Ganglion (Brain)

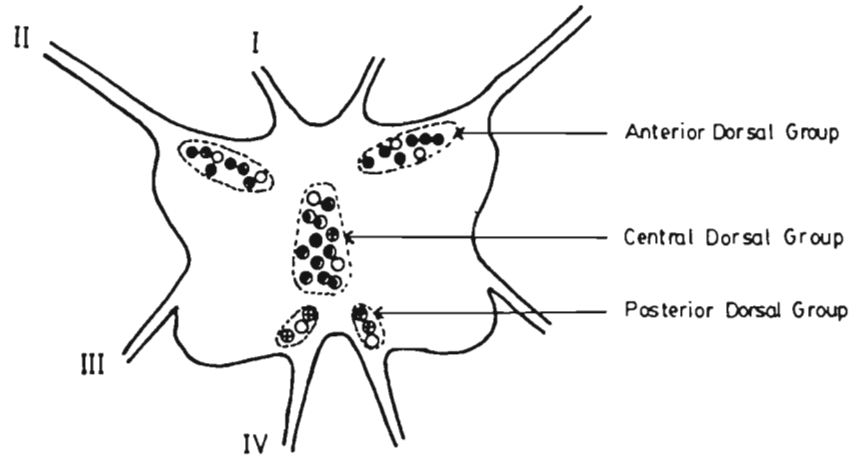


Fig.45- DORSAL

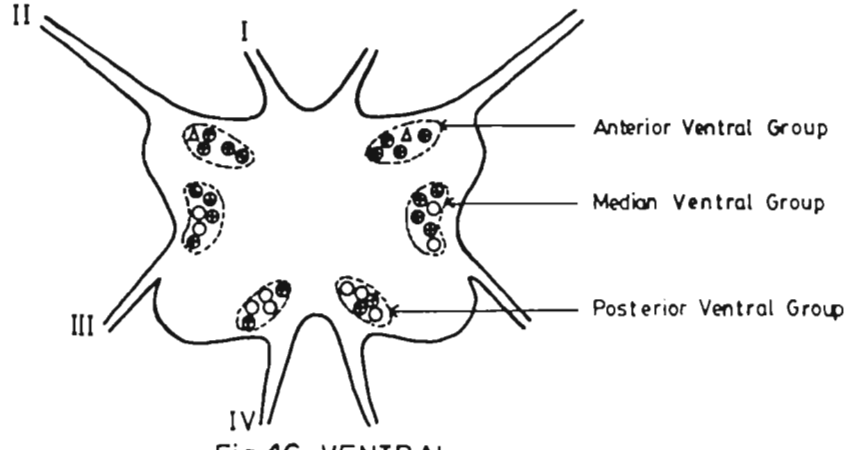


Fig.46- VENTRAL

KEY - I- Antennary nerve, II - Optic nerve, III - Maxillary nerve, IV - Circumesophageal connective, ● - GN Cell, o - A Cell, ● - B Cell, Δ - C Cell.

Ventrally the distribution of the NSC groups were similar to those seen on the dorsal side (fig.46). Anteriorly a pair of anterior ventral groups was present with B- and C-cells, the former being more predominant. A pair of median ventral groups was present in the middle. This was the largest NSC group on the ventral side and it comprised exclusively of A- and B-cells (fig.40). The posterior ventral group was diametrically opposite in position to the PDG and showed A- and B-cells, with relatively more number of A-cells. GN cells were absent in all the ventral NSC groups and C cells were found exclusively in the anterior ventral group.

In situ staining of the brain using victoria blue technique revealed darkly staining NSC soma and axons leaving it into the neuropile region (fig.42).

5.2. **Tritocerebral ganglion**

The tritocerebral ganglion was observed as a pair of extremely small ganglia lying midway between the tritocerebral connectives. It was characterized by the presence of two exceptionally large GN cells (figs. 50,43 and 44). Apart from the GN cells, the ubiquitous B-cells and one A-cell were also seen on its lateral side.

5.3. Subesophageal ganglion

The maximum number of NSC groups were observed in this ganglion. Histological preparations revealed the presence of 15 NSC groups on its dorsal, dorso-median and ventral sides.

The dorsal aspect of the subesophageal ganglion revealed 3 dorso-median cell groups (DMG), termed DMG I, II and III (fig.47). Occupying the anterior position, DMG I was the largest, and had only large GN and A-cells. Type B- and C-cells were absent. DMG II and III, lying immediately posterior to DMG I, were comparatively smaller and had only B and C cells. Unlike other ganglia, the subesophageal ganglion had NSC groups in its dorso-middle plane also. These cell groups were all found to be lateral in position and were termed the dorso-lateral groups (DLG). Totally 11 DLGs were observed each at the point where the nerve branches left the main ganglion (fig.48). B and C type cells were the NSC types present in all the DLGs (figs. 51 and 52).

Ventrally the subesophageal ganglion had only one cell group which was median in position (fig.49). This large oblong cell group, termed the ventro-median group, comprised

NSC MAPPING - Subesophageal Ganglion

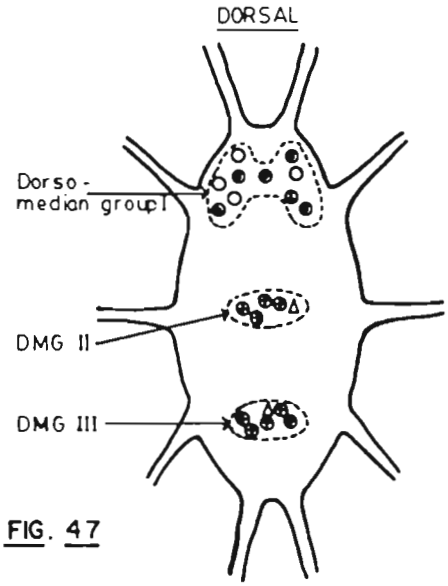


FIG. 47

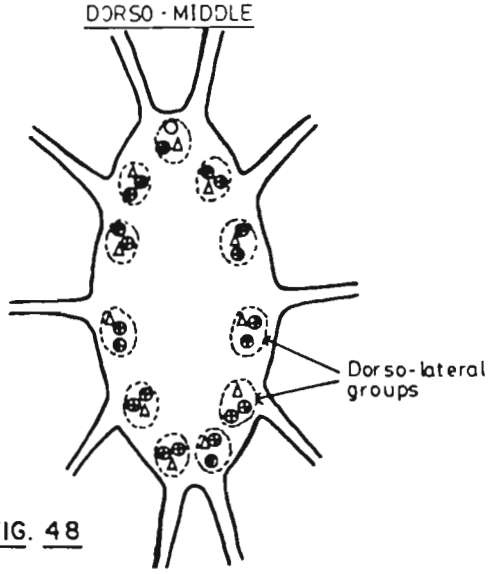


FIG. 48

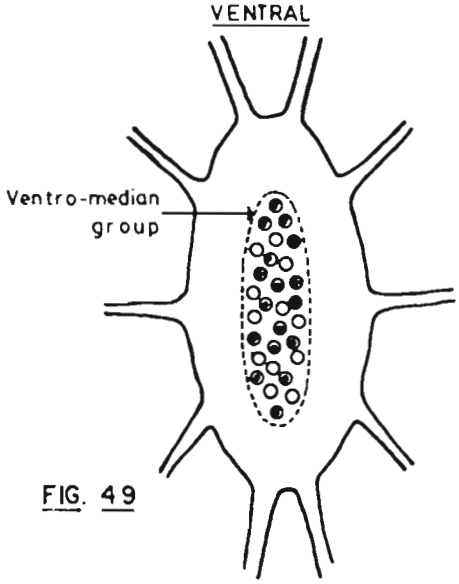


FIG. 49

Tritocerebral Ganglion

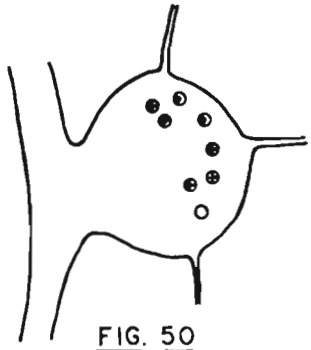


FIG. 50

KEY - ● - GN Cell, ○ - A Cell, ⊛ - B Cell and Δ - C Cell

Fig.51 Photomicrograph of the subesophageal ganglion showing the anterior most Dorso Median Group I with GN, A and B cells. Note an exceedingly well developed capillary plexus (CP) around a B cell almost forming a capillary loop. N - neuropile. MTP stain. x100.

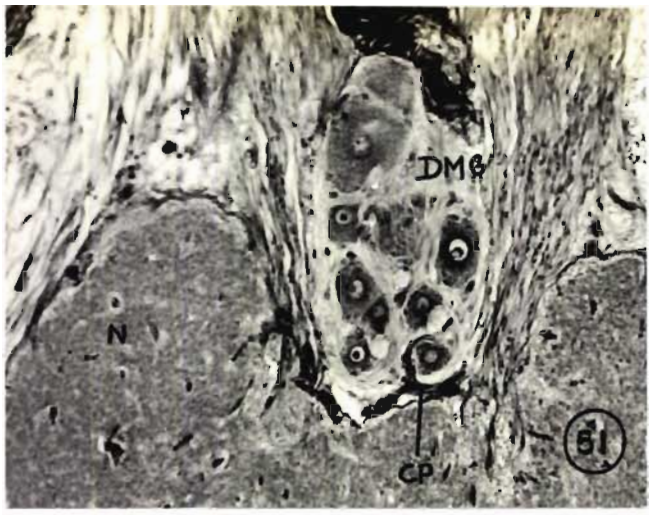
Fig.52 The dorsolateral groups of the subesophageal ganglion with B and C cells. CT - connective tissue sheath. MTP stain. x100.

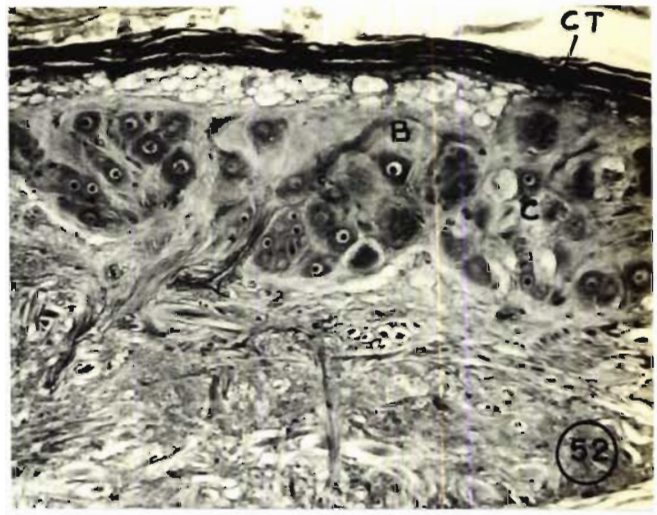
Fig.53 Photomicrograph of the Ventromedian Group in the subesophageal ganglion showing abundance of GN and MTP stain. x100.

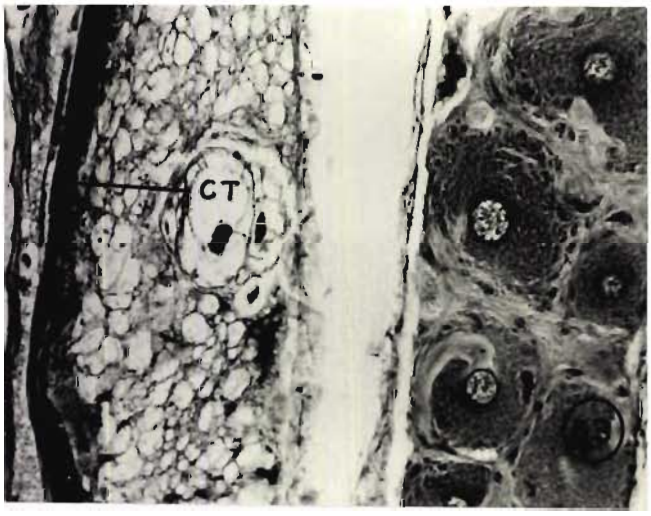
Fig.54 Photomicrograph of the Victoria Blue preparation of the subesophageal ganglion showing darkly stained neurosecretory axons (AX) and soma (NSC). x100.

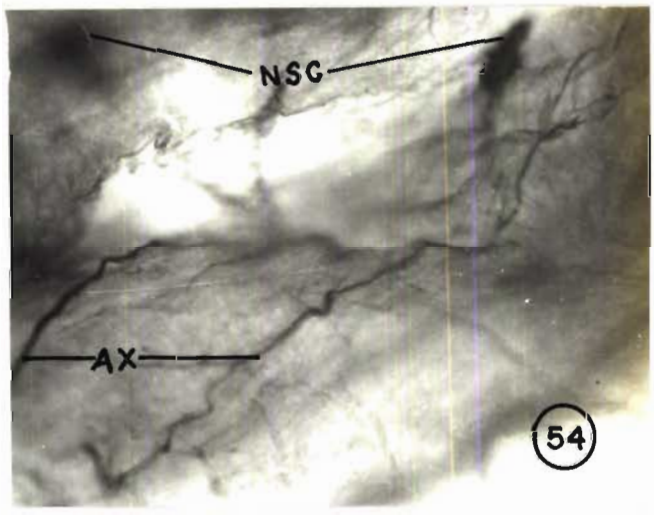
Fig.55 Photomicrograph of the Anterior Dorsal Group in the thoracic ganglion (IV) showing A, B and C cells. Also note a thin sheath covering the NSC group (NSC G). CT - connective tissue sheath. MTP stain. x100.

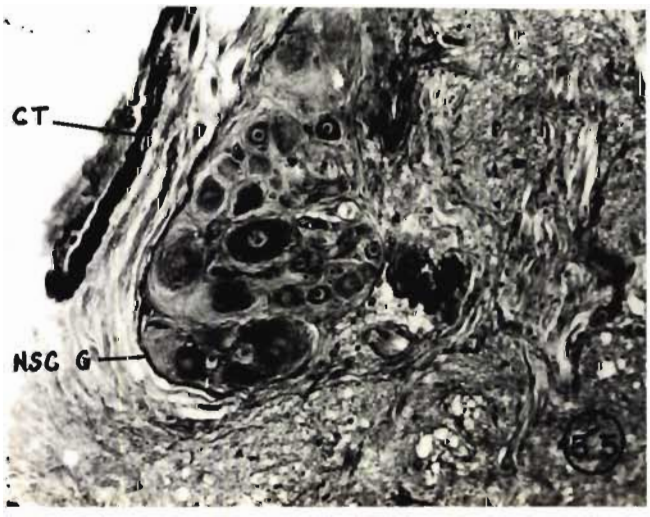
Fig.56 The anterior (ANT) and posterior (POST) lateral groups of NSC in the abdominal ganglion (I) showing A and B cells. MTP stain. x100.

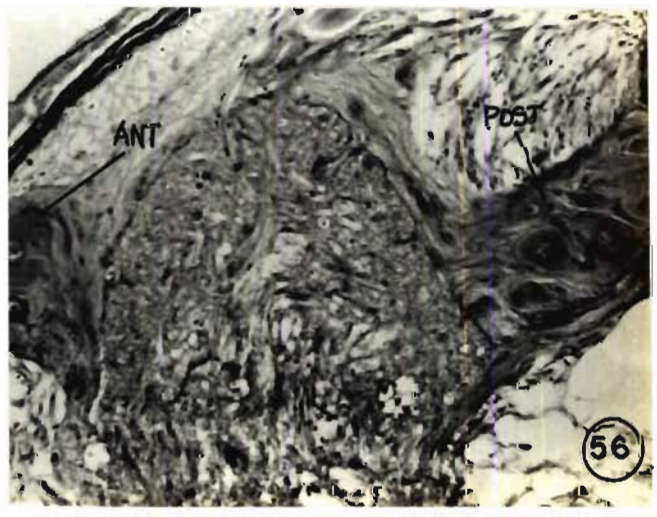

DMG
N
CP
51


CT
B
C
52


CT


NSG
AX
54


CT
NSC G


ANT
POST
56

NSC MAPPING - THORACIC GANGLION

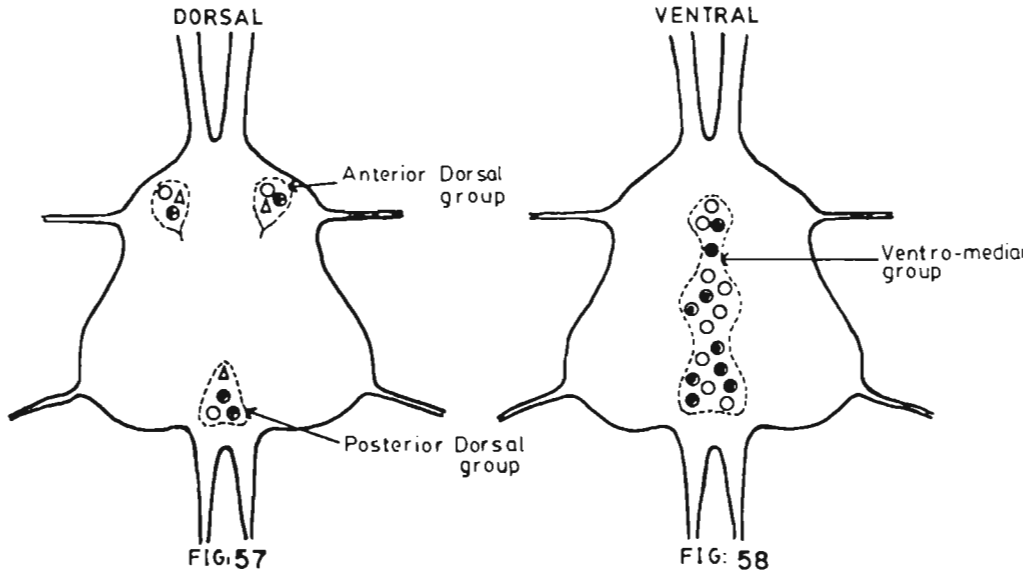


FIG: 57 FIG: 58

ABDOMINAL GANGLION

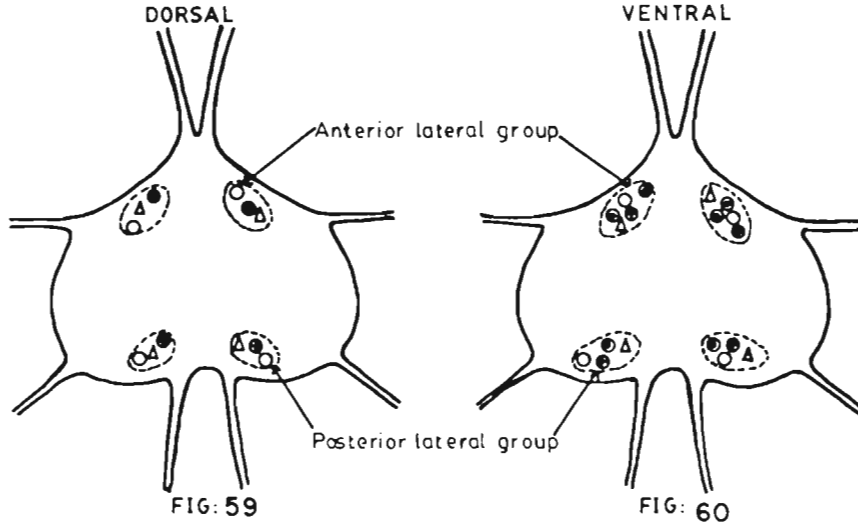


FIG: 59 FIG: 60

KEY: ◉-GN Cell, ○-A Cell, ●-B Cell and △-C Cell

exclusively of GN and A-cells (fig.53). In *in situ* preparations using the victoria blue stain, NSC soma and axons were clearly visible. Axon collaterals could also be clearly made out (fig.54).

5.4. **Thoracic ganglia**

All the thoracic ganglia were similar in the NSC group distribution; therefore the 4th thoracic ganglion, which is the largest, was taken as representative. Dorsally 3 NSC groups were observed (fig.57) with a pair at the anterior end termed as the anterior dorsal group (ADG). The ADG had few A-cells and comparatively more number of B- and C-cells (fig.55). Posteriorly there was only one NSC group named as the posterior dorsal group (PDG) which was located at the junction where the main nerve branch continued posteriorly to the next ganglion. Cell type distribution was similar to that observed in the ADG.

Ventrally, a single median NSC group was observed and it was termed as the ventro-median group (VMG). The VMG was oblong in shape with the flattenning on the anterior-posterior plane (fig. 58). Not unlike the subesophageal ganglion, the VMG in the thoracic ganglia also had only GN and A-cells with the B- and C- cells being absent.

5.5. Abdominal ganglia

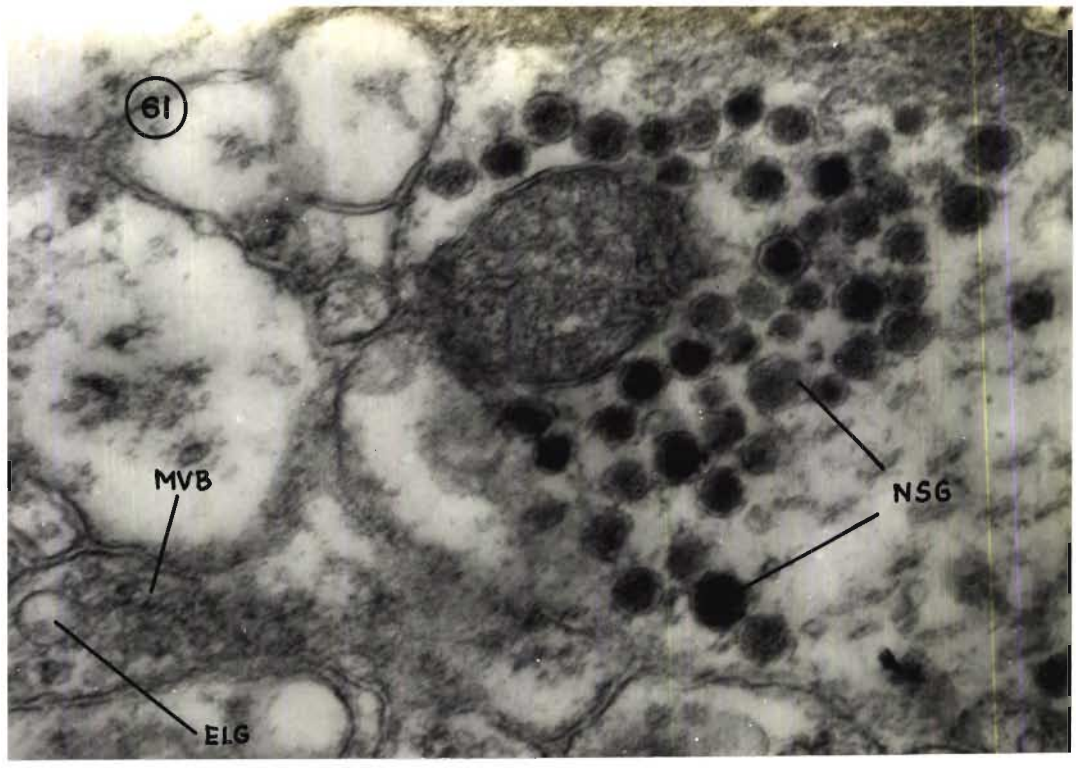
Of the 6 abdominal ganglia only the 1st abdominal ganglia was studied as a representative. The abdominal ganglion was found to be relatively poor in NSC groups. In histological preparations no basic differences in the NSC groups were observed in its dorsal and ventral aspects. Anteriorly and posteriorly a pair of lateral NSC groups termed as the anterior lateral group (ALG) and posterior lateral group (PLG) were observed (figs. 59 and 60). Both ALG and PLG had A-, B- and C-cells (fig.56). However, GN cells were not observed on both ventral and dorsal sides of these ganglia.

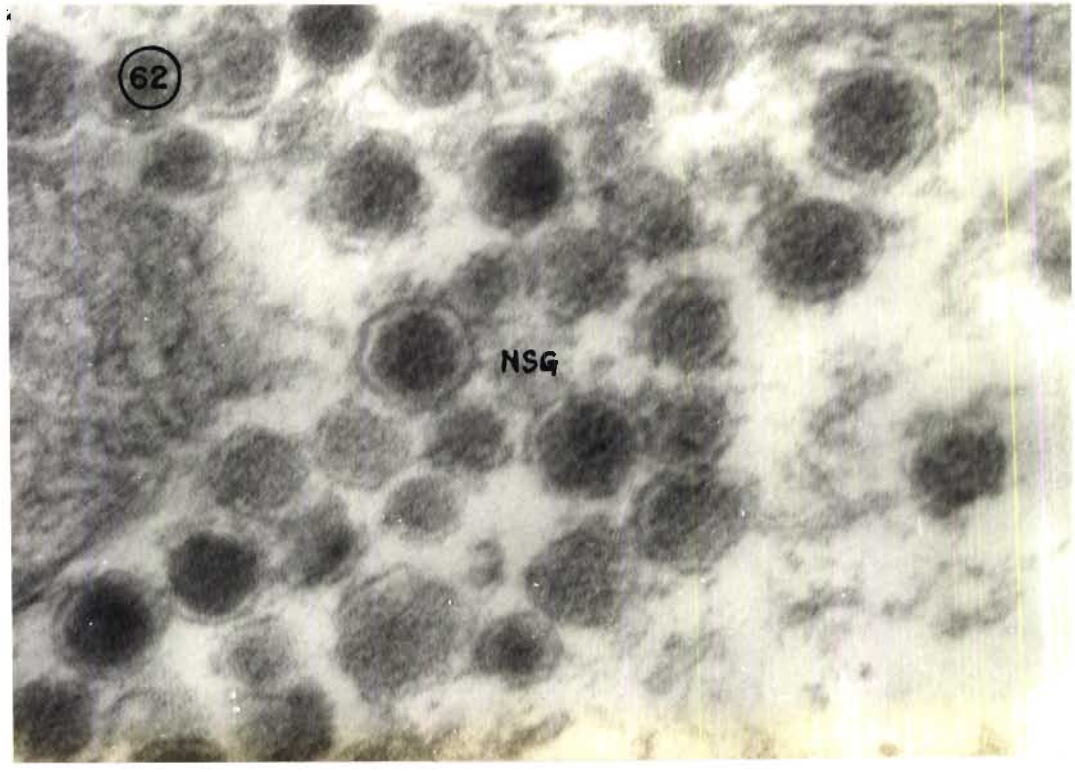
6. Ultrastructural investigations on NSC

The limited ultrastructural studies on the NSCs conducted in the present investigation revealed that the basic and most common element of the NSM was the elementary granulum, which was actually a vesicle with content. The elementary granulum (fig.61) which varied in size from 1400 to 1600 A^{o}, was enveloped in a single smooth membrane and contained an electron-dense material which was seperated from the membrane by a less electron-dense halo.

Fig.61 Election micrograph of a NSC perikarya in the optic ganglia indicating electron dense NS granules (NSG) Also note the multivesicular bodies (MVB) with electron lucent vesicles (ELV). x37000.

Fig.62 Electronmicrograph of the NSC perikarya showing the haloed dense core nature of the NS granula. Also note the differing electron density of the granula. x53000.


61
MVB
NSG
ELG


62
NSG

These haloed dense core neurosecretory granules were distributed in the neuron perikarya and synaptic junctions. Although the size range of the elementary granula was devoid of much variation, significant differences in their electron density could be noticed (fig. 62). Some of the granula were strongly electron dense, finely granulated and inhomogenous. The second granular type was moderately electron-dense and coarsely and loosly granulated. Multi-vesicular bodies with electron-lucent vesicles were also observed in the perikarya (fig.61).

7. Neuroendocrine control of reproduction

7.1. Relationship between NSC phases and maturity stages

A histological examination of the eyestalk and ventral ganglia (supraesophageal, subesophageal and thoracic ganglia) of the CNS in female P.indicus collected from the sea in different maturity stages revealed the significant role of the NSCs of these ganglia in controlling and influencing the process of gonadal maturation. The mean percentage occurrence of NSCs in different phases of their secretory cycle (ie., Quiescent, vacuolar and secretory phases) in relation to gonadal development in females is given in Table 4.

Table - 4 : Mean percentage occurrence of NSCs in different phases of the secretory cycle in relation to female maturity stages in different neuroendocrine centres of P. indicus.

Maturity Stages	Eyestalk (X-organs)			Supraesophageal			Subesophageal			Thoracic		
	Q.Ph	V.Ph	S.Ph	Q.Ph	V.Ph	S.Ph	Q.Ph	V.Ph	S.Ph	Q.Ph	V.Ph	S.Ph
I - Immature	23.4	35.3	41.3	64.3	28.9	7.1	59.1	30.6	10.3	77.7	12.9	9.5
II - Early Maturing	43.9	31.0	25.1	60.6	23.7	16.7	51.7	26.8	21.5	42.0	25.0	33.0
III - Late Maturing	42.2	33.8	24.0	34.3	33.5	32.2	40.0	37.7	22.3	34.7	26.8	38.5
IV - Mature	47.3	29.6	23.1	19.8	32.2	48.0	25.5	31.6	42.9	13.9	30.7	55.4
V - Spent	51.5	24.7	23.8	19.0	33.9	47.1	23.9	32.0	44.0	14.2	30.2	55.6

Q.Ph = Quiescent phase; V.Ph = Vacuolar phase; S.Ph = Secretory phase

Average of a minimum 3 observations.

As depicted in the table, in the eyestalk X-organ complex of immature females, more than 75% of the NSCs in MTGXO and MEGXO were in the physiologically active V and S phases (fig.63). The percentage of cells in the Q-phase almost doubled from 23.4% to 43.9%, with the initiation of folliculogenesis and vitellogenesis (fig.64). Concomitantly the number of active cells in the S-phase decreased from 41.3% to 25.1%. However, NSC in the V-phase showed slight decrease from 35.3 to 31.0%. Thereafter the variation in cell percentage was minimal and in animals with fully mature gonads, the ratio of inactive cells to active cells was almost 1:1. In the spent stage the cell percentages did not differ significantly from that of the mature stage.

In marked contrast to the pattern observed in the eyestalk, in the supraesophageal, subesophageal and thoracic ganglia, the trend was the reverse. In the supraesophageal ganglion of immature females a majority (64%) of the cells were in the Q-phase, with very few in the S-phase and approximately 29% in the V-phase (fig.65). In the early maturing stages although the number of S-phase NSCs doubled to 16.7%, there was little change in the percentage of Q-phase cells. In late maturing females, the number of Q-phase NSCs became halved (34.3%) and simultaneously the percentage of cells in the V-phase and S-phase increased significantly. In the

brain of fully mature females, physiologically active NSCs (V-phase - 32.2% and S-phase - 48%) together numbered more than 80% (Fig.66). There was however, little change in this status in spent females.

In the subesophageal and thoracic ganglia, the changes in NSC phases observed were similar to that observed in the supraesophageal ganglion. However, the quantum of NSC phase transition was considerably less in the subesophageal ganglion as compared to the supraesophageal and thoracic ganglia (Table 4). Consequently, in the subesophageal ganglia of mature females, the percentage of active cells was less than 75% in comparison to more than 85% in the thoracic ganglia of the same stage animals. Similarly in immature females, the subesophageal ganglia had considerably more number of cells in the active phases (40%) whereas in the thoracic ganlia the comparable value was only around 20%. As in the supraesophageal ganglion, in both subesophageal and thoracic ganglia too, there was no appreciable difference in the NSC percentage in spent stage animals as compared to the fully mature stage.

Hence, evidences derived herein indicated that the NSCs in the MTGXO and MEGXO of the eyestalk seemingly secrete a

Fig.63 Photomicrograph of the MTGXO-2 in the eyestalk of immature (Stage I) female showing preponderance of B and C cells in the active V and S phase. PAF stain. X100.

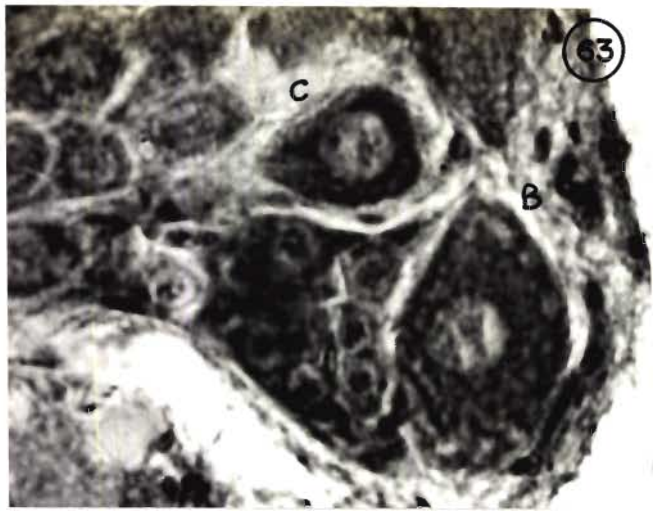
Fig.64 Light micrograph of the MEGXO in the eyestalk of a fully mature female (Stage IV). Almost all NSCs are in the inactive Q phase. MTP stain. x100.

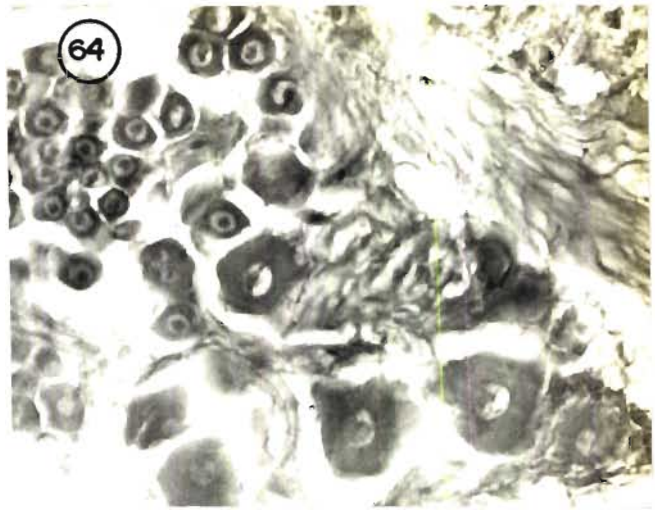
Fig.65 The Central dorsal group in the brain of an immature female (Stage I) showing GN, A and B cells in the inactive Q phase. MTP stain. x100.

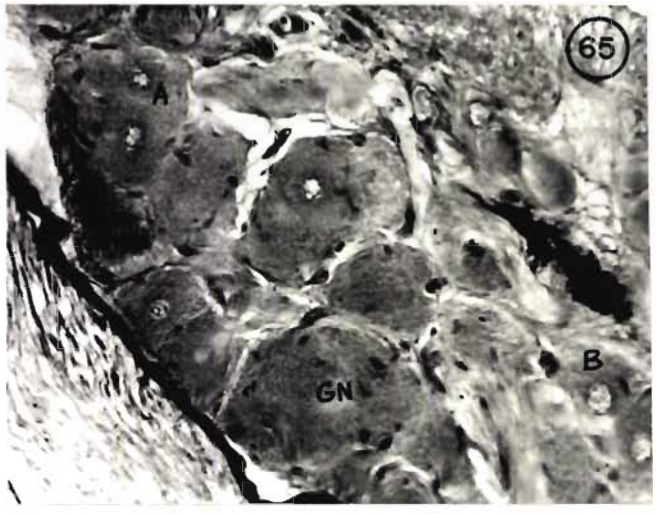
Fig.66 The same NSC group in the brain of a mature female (Stage IV). Note the change in the secretory activity of the NSCs. All cells are now in the active V and S phase with hypertrophied glial cells (GC). MTP stain. x100.

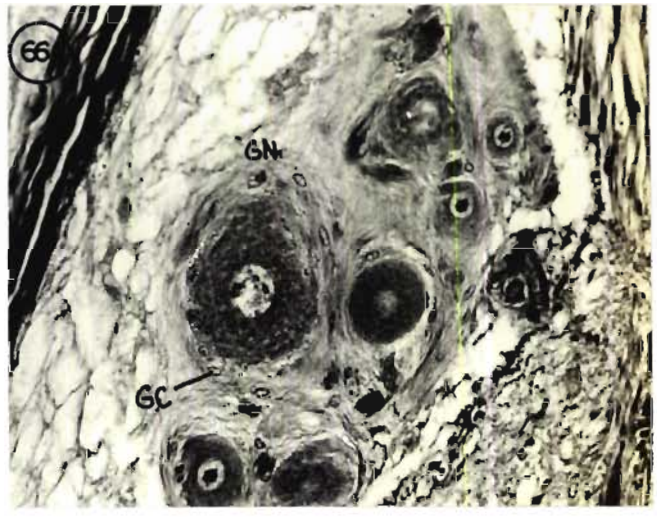
Fig.67 Photomicrograph of the MTGXO-1 in the remaining eyestalk of unilateral eyestalk ablated female. All the NSCs are in the suppressed Q phase of the secretory cycle. MT - medulla terminalis. MTP stain. x50.

Fig.68 Photomicrograph of the MEGXO in the same female. All cells have homogenous cytoplasm and are in the Q phase of the secretory cycle. ME - medulla externa. MTP stain. x50.


63
C
B


64


65
A
B
GN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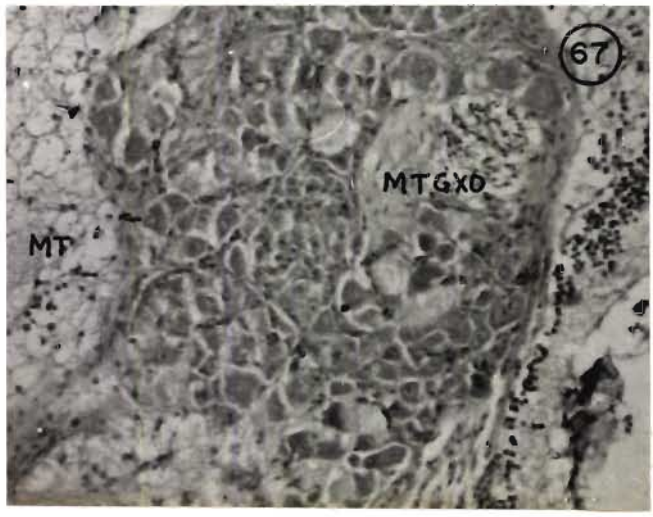
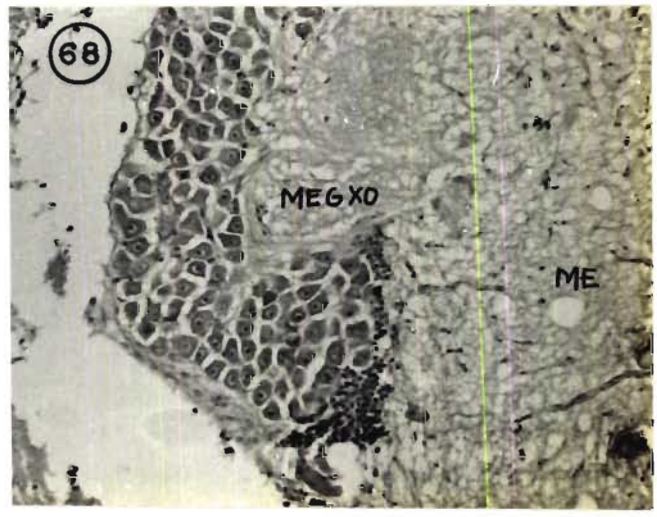
GN
GC


67
MTGXO
MT


68
MEGXO
ME

hormone or principle which inhibits gonadal development. On the other hand, the NSCs in the supraesophageal and thoracic ganglia and, to a considerably lesser extent, the subesophageal ganglion apparently secrete a hormone or principle which actively promotes gonadal growth in female P.indicus. Therefore, these two active principles, one from the NSCs in the eyestalk and the other from the NSCs in the ganglia of the CNS appear to act antagonistic to one another.

7.2. Histochemical responses of NSCs in relation to gonadal maturation

Remarkable variations in the histochemical response of NSCs were observed in relation to the progress in gonad development and reproductive maturity in females. The results of the histochemical tests in the eyestalk, supra-esophageal and thoracic ganlgia in relation to immature, mature and spent females are presented in tabular form in Tables 5,6 and 7.

7.2.1. Eyestalk

In the eyestalk, NSC perikarya and the sinus gland were moderate to strongly positive to all the tests employed (Table 5) except for the doubtful positivity to RNA. In the

Table - 5 : Histochemical responses of the perikarya of different NSCs and the sinus gland in the Eyestalk in relation to maturation in P. indicus.

Histochemical tests	A - cells			B - cells			C - cells			Sinus gland		
	IM	M	S	IM	M	S	IM	M	S	IM	M	S
1. Mercuric Bromophenol Blue test	++	+	+	+	+	+	+	+	+	++	+	+
2. Ninhydrin-Schiff Test	+	±	±	+	±	±	+	+	+	+	+	±
Control - Deamination	-	-	-	-	-	-	-	-	-	-	-	-
3. Ferric-Ferricyanide Test	++	+	++	+	±	±	+	±	+	+	±	±
Control - Mercaptide	±	-	-	-	-	-	-	-	-	-	-	-
4. PFAB Test	+	±	±	++	±	+	+	±	±	+++	+	++
Control - Alcian blue alone	-	-	-	-	-	±	-	-	-	±	-	±
5. PAS Test	+++	++	++	++	+	+	+	+	+	++	++	++
Control - Diastase	+	-	-	+	-	-	±	-	-	±	±	±
6. Sudan Black B test	++	+	++	++	+	+	++	+	+	++	+	+
Control - Delipidation	-	-	-	-	-	-	-	-	-	±	-	-
7. Methyl Green Pyronin	±(R)	±(R)	±(R)	±(R)	±(R)	±(R)	±(R)	±(R)	±(R)	-	-	±(R)

Key: IM = Immature; M = Mature; S = Spent recovery stage female P. indicus; R = Pale Red; - = Negative reaction; ± = Very mild reaction of doubtful nature; + = Positive reaction; ++ = Strong positive reaction; +++ = Intense positive reaction.

Table - 6 : Histochemical responses of the perikarya of different NSCs in the supraesophageal ganglion (Brain) in relation to maturation in *P. indicus.*

Histochemical tests	GN - cells			A - cells			B - cells			C - cells		
	IM	M	S	IM	M	S	IM	M	S	IM	M	S
1. Mercuric Bromophenol Blue test	+	++	+++	+	++	+++	±	+++	±	+	++	±
2. Ninhydrin-Schiff Test	+	+	+	+	+	+	+	+	+	+	+	+
Control - Deamination	-	-	-	-	-	-	-	-	-	-	-	-
3. Ferric-Ferricyanide Test	+	+	-	+	++	+	±	++	++	+	++	++
Control - Mercaptide	±	±	-	±	±	±	±	-	±	±	-	±
4. PFAB Test	+	++	+	+	++	++	±	++	+	±	+	+
Control - Alcian blue alone	-	-	±	±	-	±	-	-	-	-	-	-
5. PAS Test	+	++	+	++	++	+	+	+	±	+	+	±
Control - Diastase	±	±	±	+	±	±	±	±	±	±	±	±
6. Sudan Black B Test	+	±	+	+	+	+	+	+	+	±	±	±
Control - Delipidation	-	-	±	-	-	-	-	-	-	-	-	-
7. Methyl Green Pyronin	+(R)	++(R)	+(R)	++(R)	+++(R)	+(R)	+(R)	+++(R)	±(R)	+(R)	++(R)	±(R)

Key : IM = Immature; M = Mature; S = Spent recovery stage female *P. indicus*; R = Pale Red; - = Negative reaction
± = Very mild reaction of doubtful nature; + = Positive reaction; ++ = Strong positive reaction;
+++ = Intense positive reaction.

Table - 7 : Histochemical responses of the perikarya of different NSCs in the thoracic ganglia in relation to maturation in P. indicus.

Histochemical Tests	GN - cells			A - cells			B - cells			C - cells		
	IM	M	S	IM	M	S	IM	M	S	IM	M	S
1. Mercuric Bromophenol Blue Test	++	+++	+	+	+++	+	++	+++	+	+	++	+
2. Ninhydrin-Schiff Test	+	+	±	±	±	±	±	±	±	±	±	±
Control-Deamination	-	-	-	-	-	-	-	-	-	-	-	-
3. Ferric-Ferricyanide Test	±	++	+	+	+	±	+	++	+	±	++	+
Control-Mercaptide	-	-	-	-	-	-	±	-	-	-	-	-
4. PFAB Test	++	+++	+	+	++	++	+	+++	±	+	++	±
Control-Alcian blue alone	±	±	-	-	-	-	-	-	-	-	-	-
5. PAS Test	+	++	±	±	+	+	±	++	++	±	+	+
Control-Diastase	-	±	-	-	-	-	-	±	±	-	±	±
6. Sudan Black B Test	±	+	+	±	+	++	±	++	+	±	++	+
Control-Delipidation	-	-	-	-	-	-	-	-	-	-	-	-
7. Methyl Green Pyronin	±(R)	±(R)	±(R)	±(R)	±(R)	++(R)	±(R)	±(R)	++(R)	±(R)	±(R)	+(R)

Key: IM = Immature; M = Mature; S = Spent recovery stage female P. indicus R = Pale Red; - = Negative reaction; ± = Very mild reaction of doubtful nature; + = Positive reaction; ++ = Strong Positive reaction; +++ = Intense positive reaction.

eyestalks of immature females, A-cells were strongly positive to the mercuric bromophenol blue test indicating the presence of general proteins. Specific tests performed for the identification of the end group amino acids such as -NH2, -SH and -S-S groups indicated their presence in the cytoplasm in substantial quantity. A-cell perikarya in this stage were also intensely positive to lipids and PAS test. In fully mature females positivity to all the above groups diminished considerably. In spent and spent-recovery females the initial positivity was regained only for -SH groups and lipids.

B-cells in the eyestalk of immature females displayed moderate positivity to general proteins, -NH2 groups and -SH group; and strong positivity to -S-S groups, glycogen and lipids. In mature females, the positivity of the B-cells to all the tests diminished substantially, except for the positivity to general proteins, which remained the same in all the stages. However, unlike the A-cells, in B-cells no reversion of positivity was observed in spent animals. C-cells displayed only moderate positivity to all tests with the exception of strong positivity to sudan black B test for lipids in immature females. There was however, no appreciable change in the affinity of the C-cell perikarya to these tests in mature and spent females.

The sinus gland of P.indicus, exhibited intense positivity to the PFAB test for -S-S group in the immature stage. Apart from this, strong positive reaction was also obtained for general proteins, glycogen and lipids. Only moderate positivity was observed for the amino acid end groups like -NH2 and -SH groups. In contrast, there was a drastic reduction in the positivity to -S-S groups, general proteins and lipids in the sinus glands of mature females. In spent animals the positivity was partially regained in the case of -S-S group. The sinus glands of immature and mature females were negative to RNA and surprisingly only spent animals displayed mild positive reaction to pyronin indicating presence of RNA. These results indicate that in the eyestalk, A-and B-cells are the most active cell types in relation to the affirmative function of inhibition of gonadal maturation by the x-organ-sinus gland complex.

7.2.2. **Supraesophageal ganglion**

In the cerebral ganglia of immature females, GN-cells exhibited only moderate positive reaction to all the tests employed including the methyl green pyronin test for nucleic acids (Table 6). In mature females, there was an appreciable increase in the positivity of the GN-cells to tests for

general proteins, -S-S group amino acids, glycogen and RNA. With the exception of general proteins all these compounds reverted back to their earlier levels in spent females. Curiously enough, the quantum of general proteins registered an increase from strong positivity to an intense positive reaction in spent females. In A-cells too a similar trend was observed with all the compounds except general proteins, attaining their peak levels in mature females. For general proteins, the maximum staining intensity was recorded in spent females similar to the condition met with in GN-cells.

The ubiquitous B-cells were characterized by their intense blue reaction to the mercuric bromophenol blue test indicative of general proteins in mature females. In immature and spent females the positivity was considerably less. There was however, no change in lipid levels in B-cells. Remarkable increase in positivity was noticed with tests for -SH and -S-S amino acid groups and RNA in mature females. Strangely enough, the diastase digestible PAS positivity which signifies the presence of glycogen, remained the same in immature and mature stages, but decreased in spent females. Almost similar conditions were observed in C-cells in the supraesophogeal ganglia, although the magnitude of reactions were considerably lower. The above results show that in the brain, GN-, A- and B-cells

are the most active in relation to gonadal maturity.

7.2.3. Thoracic ganglion

In the thoracic ganglia, GN- and B-cells were apparently the most active NSCs by virtue of the quantum of increase in positivity to histochemical tests observed with gonadal maturation (Table 7). The GN- and B-cells of immature females showed strong positive reaction to tests for general proteins and protein-bound disulphide (-S-S) groups. In this stage they were moderately positive to amino group and glycogen but their reaction to tests for sulphydryl groups, lipids and RNA were of doubtful nature. In mature females the magnitude of reactivity to tests for general proteins, aromatic amino acids, glycogen and sudanophilic lipids increased remarkably. In spent females these levels were reverted to the original status. However, spent animals displayed more number of pyroninophilic RNA granules in the perikarya than in immature and mature animals. This unique characteristic was exhibited by all the NSCs in the thoracic ganglia in varying degrees.

A pattern of change in the reactivity, similar to that described above was also observed in A- and C-cells. In C-

cells and to a certain extent A-cells, intense positive reaction was not observed for any of the components tested.

8. Eyestalk ablation and eyestalk extract injection experiments in relation to reproduction

These experiments were carried out to find the role of the x-organ sinus gland complex on gonadal maturity of the prawns. Results obtained are summarized in tables 8 and 9.

In the experimental group I, untreated females, which served as control, no ovarian development took place as evidenced by the low mean GSI of 0.488 (Table 8). The oocyte size ranged from 20 to 100 μ in diameter. During the 10 days duration of the experiment all the animals which were initially in intermoult (C) and/or early premoult (Do) stages advanced to premoult (D1',D1" and D1''') and late premoult (D2 - D3). However, none of them moulted during the course of the experiment and their feeding and swimming behaviour were normal. Histologically the ovary showed characteristics of a Stage I gonad (Fig. 73).

All the unilateral eyestalk ablated females (group II) exhibited significant increase ($p<0.05$) in their GSI and oocyte diameter in comparison to that observed in the un-

Table-8: Experimental details of eyestalk ablation experiments in relation to gondal maturity in P. indicus.

S NO:	TOTAL LENGTH (mm)	LATENCY PERIOD (Days)	MOULT STAGE (Initial)	MOULT STAGE (Final)	G.S.I	OVA DIAMETER RANGE(μ)
Group: I Control-Unablated						
1.	143.0	10	C	D1''	0.8320	25-60
2.	151.0	10	Do	D1''	0.4922	25-76
3.	141.5	10	C	Do	0.6095	30-90
4.	143.0	10	C	D1''	0.3581	25-50
5.	134.0	10	Do	D1''	0.2775	20-65
6.	141.0	10	C	D1'	0.2811	20-50
7.	145.3	10	C	D1'	0.4981	30-57
8.	147.0	10	Do	D1''	0.9514	43-100
9.	139.0	10	Do	D2-3	0.2088	25-63
10.	141.0	10	C	D1''	0.3720	25-55
Mean.		10	-	-	0.4880	20-100
Group: II - Unilateral Eyestalk Ablated						
1.	157.1	06	C	Do	5.3781	282-325
2.	141.5	09	Do	Do	5.0123	260-300
3.	147.0	05	C	Do	6.1078	295-330
4.	154.5	04	Do	Do	6.8965	293-335
5.	142.2	04	C	C	3.2147	164-239
6.	139.8	05	Do	Do	5.2246	285-315
7.	145.0	06	C	Do	6.3453	304-333
8.	152.4	05	C	Do	6.9531	289-326
9.	142.8	08	C	Do	5.3206	257-306
10.	145.0	07	Do	Do	6.5395	314-347
Mean.		5.9	-	-	5.6992	164-347

Table-9: Experimental details of eyestalk ablation experiments in relation to gonadal maturity in *P. indicus*

S NO:	TOTAL LENGTH (mm)	LATENCY PERIOD (Days)	MOULT STAGE (Initial)	MOULT STAGE (Final)	G.S.I	OVA DIAMETER RANGE (μ)
Group: III - Bilateral Eyestalk Ablation						
1.	151.0	03	C	Do	6.2809	318-346
2.	137.5	05	C	C*	4.9113	254-287@
3.	141.0	02	C	D2-3**	1.9215	108-139
4.	145.0	03	C	Do	5.2490	293-331
5.	142.3	05	C	B*	5.1032	278-324@
6.	145.0	04	C	D2-3	1.8795	87-126
7.	143.0	03	Do	Do	4.8189	157-235
8.	139.8	05	C	C*	3.4431	142-201
9.	140.0	03	C	D2-3**	2.1311	60-135
10.	138.0	04	Do	Do	4.6125	211-273@
Mean.		3.7	-	-	4.0351	60-346
Group: IV - Eyestalk Extract Injected						
1.	142.5	10	Do	D1''	1.0340	35-140
2.	144.0	10	C	D1'	1.6300	50-150
3.	139.5	10	C	D1'	0.9117	60-100
4.	141.5	10	Do	D1'	0.9898	50-110
5.	148.0	10	Do	D1''	0.4656	20-75
6.	155.0	10	C	Do	1.1960	75-115
7.	146.2	10	C	D1'	0.3218	30-66
8.	153.5	10	C	Do	1.1965	93-115
9.	140.5	10	C	Do	0.7200	25-60
10.	143.5	10	C	Do	0.5741	25-60
Mean.		10	-	-	0.9039	20-150

* - Moulted once during the period with developing ovary.
** - Died during the effort to moult or in moulting process.
@ - Fully mature ovary with light green colour (carotenoid deficiency).

treated females in group I (Table 8). In this experimental group, apart from an initial loss of equilibrium, no behavioural discrepancies were observed. However, the food intake was substantially increased with voracious feeding behaviour being displayed when clam meat with shell was placed in the pool. Unilaterally ablated prawns were able to completely devour the meat off the clam shell within a short span of time. The latency period for the maturation ranged from 4 to 9 days (average 5.9 days). Full maturity, with ovaries in the vitellogenic phase, was attained by 90% of the experimental animals (fig.74). The mean GSI was estimated to be 5.7 and the oocyte size ranged from 164 to 347 μ in diameter. There was no significant change in the moult stage during the period of the experiment. Intermoult or early premoult stage animals either remained in the same stage or as in some instances intermoult animals advanced to early premoult conditions.

The histology of the remaining eyestalk (one which was not ablated in the unilateral ablation group) revealed that all the NSCs in the MTGXO and MEGXO were in suppressed state. The cytoplasm of these cells were uniformly agranular and homogenous, as is characteristic of Q-phase NSCs (fig.67 and 68). The sinus gland of the animals were lacking in granular inclusions and stained lightly with neurosecretion

specific stains (Fig. 69). In contrast the majority of the NSCs in the cerebral and thoracic ganglia were in the secretory phase of activity as shown in Fig.70.

The bilaterally eyestalk ablated females (group III) showed significant behavioural abnormalities. Immediately after the extirpation of both the eyestalks, prawns were observed to swim at the surface in circles around the pool with abnormal speed. Almost within a few hours, these prawns became dark red in colour due to dispersion of the chromatophores. Feeding was observed to be voracious. Although unable to see, prawns were able to immediately perceive the introduction of clam meat into the water. However, unlike group I and II animals, they were not able to devour the food completely and hence invariably left-over meat was observed on the clam shells. In this experimental group 50% of the individuals attained full maturity (Table 9). The average latency period was 3.7 days, which is considerably shorter than what was observed for group II animals. The mean GSI was estimated to be 4.035 and the oocyte diameter ranged from 60 to 346 μ. This result was significantly different from that of the control. Interestingly 3 individuals out of 10 animals moulted with developing ovaries and 2 prawns died in the effort to moult or during

the process of moulting. Moulting itself was abnormal and was surprisingly observed during the daylight hours on some occasions. Ovaries of 3 fully mature (ovary in vitellogenic phase) prawns had light green colour unlike the fully mature ovaries of normal prawns which were dark green, indicating a possible defeciency in carotenoids.

The NSCs of cerebral and thoracic ganglia of group III animals were in a hyperactive state (Fig.71 and 72). Both V and S phase NSCs dominated in all the NSC groups in the ganglia.

In the experimental group IV where unilateral eyestalk ablated females were administered with an aqueous extract of the eyestalk the mean GSI was estimated to be 0.904 (Table.9). This value was marginally higher than that of the control (group I) but was significantly lesser ($P<0.05$) than that of group II and III animals. The oocyte size range varied from 20-150 μ, which was also significantly lower ($P<0.05$) than that of group II and III size ranges. The percentage inhibition of the gonad inhibiting hormone (GIH) present in the eyestalk extract used was estimated as 92.02% using the method of Bomirski et al. (1981).

Fig.69 Light micrograph of the lightly stained sinus gland of the same female. Note the lack of stainable granula and swollen axon endings around the internal blood sinus (IBS). Compare with Fig.34. PAF stain. x200.

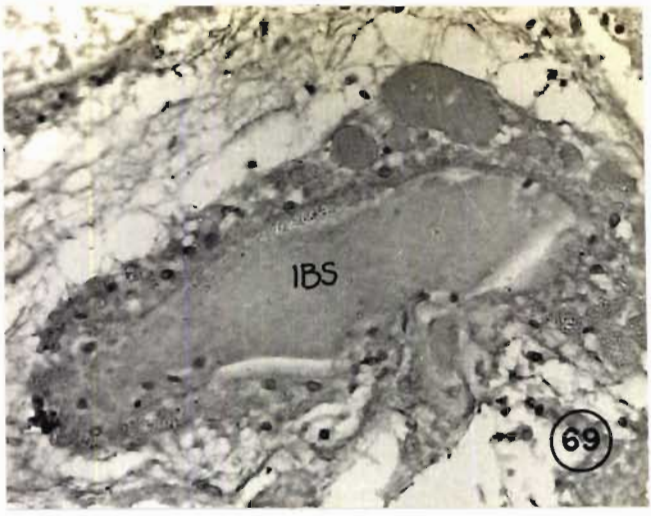
Fig.70 The very active B cells (S phase) in the thoracic ganglion of a unilateral eyeablated female in advenced stage of maturity. Note the abundance of capillary plexus (CP) around the cells. MTP stain. x200.

Fig.71 V and S phase B cells in the brain of a bilaterally eyeablated female. V - pericellular vacuole. PAF stain. x400.

Fig.72 Photomicrograph of the thoracic ganglion of a bilaterally eyeablated female. All NSCs are in the terminal stage (S phase) of the secretory cycle. PAF stain. x200.

Fig.73 Cross section of the ovary in Group I intact control females. All oocytes are in the previtellogenic phase. Haematoxylin and Eosin. x50.

Fig.74 Gross section of the ovary of a Group II unilateral eyeablated female revealing oocytes in ripe stage with peripheral cortical bodies. Haematoxylin and Eosin stain. x50.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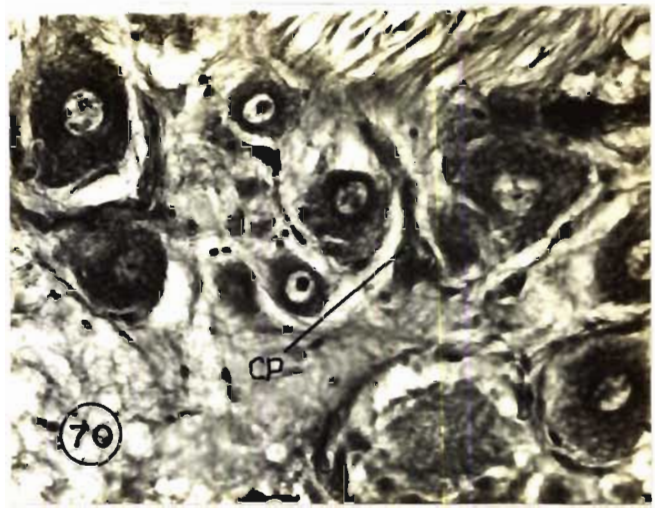
69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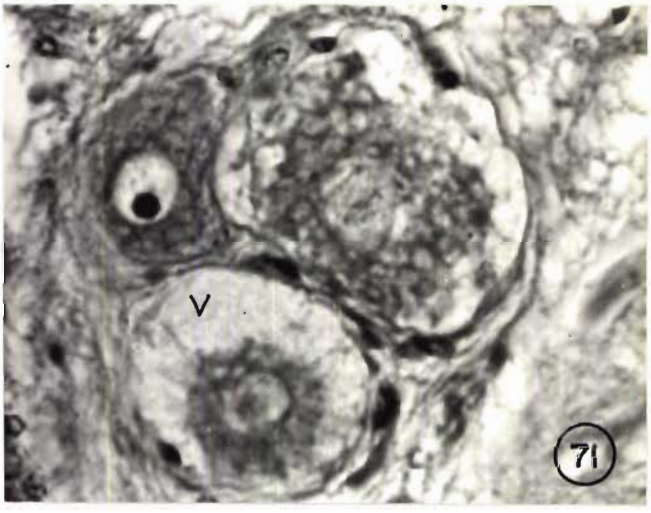
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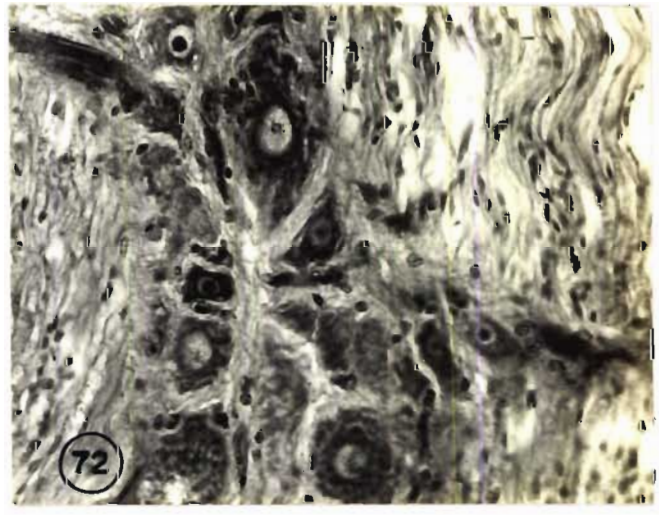
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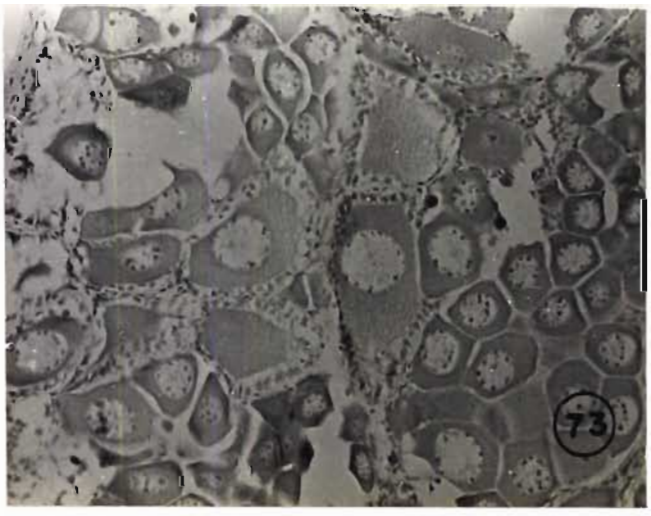
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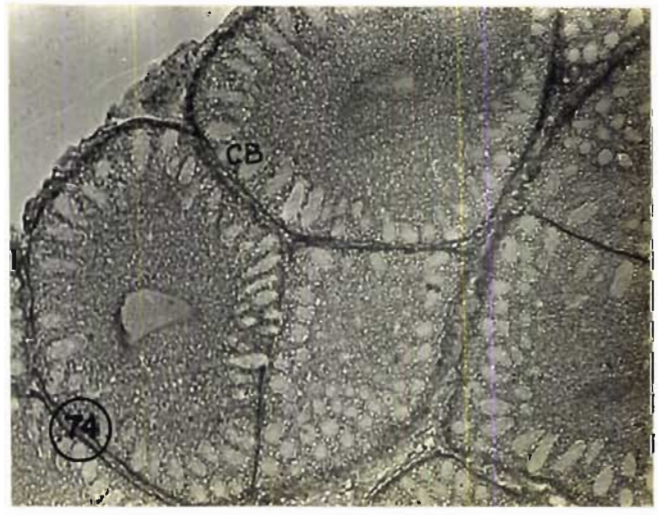
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9. Effect of CNS extract injection on maturity

In placebo (crustacean saline) administered group I (control) females, the mean GSI was 0.382 and the oocyte diameter ranged from 25 to 90 μ (Table. 10). The experimental group II (CNS extract injected) prawns showed small, but significant ($P<0.05$) enhancement in the GSI and oocyte diameter when compared to that of the control. Visual observations of the gonad through the dorsal cuticle showed that about 80% of the animals had advanced to stage II conditions by the seventh day itself, but further progress in gonadal development was not observed. A dose of I CNS equivalent was found to increase the average GSI to 1.518 and the oocyte diameter ranged from 25-160 μ, after a period of 10 days.

10. Relationship between gonadal maturation and moult cycle

The percentage occurrence of stage IV females observed in different moult stages is given in Table 11. In samples obtained from the wild population more than 80% of the ripe females were in early premoult (Do) stage. The remaining 18.03% were in intermoult (C) stage. Significantly, mature females were not observed in other moult stages. A similar pattern was also observed in unilateral eyeablated females,

Table-10: Experimental details of CNS extract administered female *P. indicus*.

S NO:	TOTAL LENGTH (mm)	LATENCY PERIOD (Days)	G.S.I	OVA DIAMETER RANGE (μ)
Group: I - Placebo Injected (Control)				
1.	141.0	10	0.4057	30-80
2.	143.5	10	0.2820	25-75
3.	139.0	10	0.2822	25-75
4.	149.0	10	0.4156	25-90
5.	143.0	10	0.2374	25-75
6.	141.5	10	0.3118	25-75
7.	146.0	10	0.4167	30-65
8.	143.0	10	0.3479	30-90
9.	145.0	10	0.4400	25-85
10.	145.5	10	0.6919	30-90
Mean.		10	0.3821	25-90
Group: II - CNS Extract Injected (1 CNS Equivalent)				
1.	144.0	10	2.3595	55-160
2.	146.0	10	2.2820	35-145
3.	140.5	2*	1.2959	35-130
4.	160.0	10	2.9082	30-145
5.	157.0	10	1.4239	30-110
6.	144.5	10	1.2350	30-110
7.	142.0	10	1.3109	40-120
8.	142.0	10	0.5851	25-75
9.	147.0	10	1.4213	30-125
10.	142.5	10	0.3555	30-75
Mean.		9.2	1.5177	25-160

* - Died during the course of the experiment

Table 11: Maturation in relation to moult cycle for wild females and unilateral and bilateral eye ablated females in *P. indicus*

Moult-stages		% occurence of ripe (Stage IV) females		
		Wild (n=61)	Unilateral Eyeablation (n=10)	Bilateral Eyeablation (n=8)
Post-Moult	A	0	0	0
	B	0	0	12.5
Intermoult	C	18.03	10.0	25.0
Pre-moult	Do	81.9	90.0	50.0
	D1'	0	0	0
	D1''	0	0	0
	D1'''	0	0	0
	D2-3	0	0	12.5

with 90% of the treated prawns in mature condition in the early premoult stage and the remaining in intermoult stage. In the bilateral eye oblated group, notwithstanding the considerable variation in the above pattern, 50% of the mature females were in Do stage and 25% in C stage. However, 12.5% of the mature females were in late premoult (D2 - D3) which is the terminal stage prior to ecdysis. The remaining 12.5% of the animals were in late post-moult (B-stage). More importantly and perhaps uniquely, mature animals in B and C stages had moulted once with developing ovaries.

DISCUSSION

According to MacLaughlin (1983), the crustacean nervous system basically consists of a large supraesophageal ganglionic mass, often referred to as the brain, and a ventral nerve cord with a pair of ganglia corresponding to each embryonic somite. In P.indicus,a typical dendro-branchiate crustacean, a similar organisation of the nervous system was seen, inspite of the considerable variation in the number of segmental ganglia and structure of the ventral nerve cord in malacostracans.

Neurosecretory cells in P. indicus were found distinct from non-neurosecretory and by virtue of their characteristics, concur with Bargmann and Scharrer's (1951) concept of neurosecretion. The characteristics of the NSCs described here are basically in accord with those of neurosecretory elements described in other crustaceans by many workers (Enami, 1951; Matsumoto, 1958; Fingerman and Aoto, 1959; Lake, 1970; Nakamura, 1974; Diwan and Nagabhushanam, 1975 and Van Herp et al., 1977). Descriptions of the NSC types found in the optic and other ganglia in different species are varied, probably because of the differences in histological staining procedures, cyclic secretory activity, species differences and human subjectivity. Inspite of these, many attempts have been made previously to relate the variously described NSCs (Durand, 1956; Carlisle and Knowles, 1959; Passano, 1960; Adiyodi and Adiyodi, 1970 and Quackenbush, 1986).

All the four NSC types described in P.indicus seem relatively larger than those described in other dendrobranchiate species. Four types of NSC were described by Nanda and Ghosh (1985) in P.monodon. In contrast, 7 types were described by Nakamura (1974) in P.japonicus, 3 types in P.japonicus and P.kerathurus by Ramadan and Matta (1976), 6

types in Macrobrachium rosenhergii by Deitz (1982) and Palaemon paucidens by Hisano (1974) and 8 types in Parapenaeopsis stylifera by Nagabhushanam et al. (1986).

The GN cells described in the present study are comparable to cell type I and II of P.japonicus (Nakamura, 1974) and to the giant A cells described by Matsumoto (1954) in the thoracic ganglia of the Japanese freshwater crab Eriocheir japonicus. In its shape, size and histological features, A-cells of P.indicus are comparable with Enami's V cells (Enami, 1951), type II and III cells of P.paucidens (Hisano, 1974), type III cells of P.japonicus (Nakamura, 1974) and B-cells of P.serratus (Van Herp et al., 1977). The size and histomorphological features of A-cells detected in the present investigation showed close resemblance to the A-cells of P.monodon (Nanda and Ghosh,1985) and cell types I, II, III and IV of P.stylifera (Nagabhushanam et al., 1986). B-type cells of P.indicus were found to be similar to the type IV cells of Hisano (1974), B-cells of Nanda and Ghosh (1985) and cell types V, VI and VII of Nagabhushanam et al. (1986). The pyriform C-cells of P.indicus showed resemblance in size and cell histology to D-cells of Van Herp et al. (1977) and C-cells of Nanda and Ghosh (1985).

Almost all earlier investigators referred to the small

nerve cells (designated as non neurosecretory neurons in this study) as neurosecretory based presumably on their mere occurrence amidst the NSC groups. However, evidences derived from the present study are on the contrary, and no neurosecretory activity was detected in these cells. Matsumoto (1954) classified these cells as D-cells and emphatically stated the absence of secretory activity in them. These cells with a single conspicuous nucleoli had boundaries which were difficult to discern as noted by Matsumoto (1954). The non neurosecretory neurons described in the present study showed close resemblance to cell type V and VI Nakamura (1974), type VI of Hisano (1974) and type VIII described by Nagabhushanam et al., (1986). They were also similar to Enami's γ cells. Enami (1951) observed nuclear secretions and the formation of intra-nuclear droplets in them. No such phenomena were observed in these neurons P.indicus and hence neurosecretory status has not been bestowed upon them.

The neurosecretory material in crustaceans has been shown to be rich in proteins and carbohydrates by several workers (Lake, 1970, Diwan and Nagabhushanam, 1974; Nakamura, 1974; Rashan et al., 1976; Rashan and Gorgees, 1977 ; and Dabbagh and Baid, 1976; Rashan and Al-Mamory,

1983). The cytochemical tests applied to the NSCs of P.indicus revealed that the NSM was predominantly composed of a protein rich in sulfur containing amino acids like cystine (-S-S group) and cysteine (-SH group). The sinus gland being a storage site for the NSM in the eyestalk also showed similar characteristics. Similar results were obtained by Rehm (1959) in Carcinus maenas, Lake (1970) in Paragrapsus gaimardii, Rashan and Gorgees (1977) in the crab Potamon magnum and Nagabhushanam et al. (1986) in P.stylifera.

Nakamura (1974) referred to the NSCs in P.japonicus as 'PAS postives' because of their strong positivity to the PAS test. In P.indicus however, the PAS positivity of the NSM was only moderate indicating the presence of glycogen and neutral mucopolysaccharides or mucoproteins. In contrast, Rashan and Gorgees (1977) reported the absence of glycogen in the NSCs of P. magnum and the presence of acid mucopolysaccharides (AMP) in their perikarya. The glycogen content in the NSCs of P.indicus was confirmed when the perikarya was only PAS positive with the AB - PAS test. Nonetheless, the nucleus showed the presence of AMP.

A distinct cyclic secretory activity is indicative of the neurosecretory status of a nerve cell (Bargmann and

Scharrer 1951). In P.indicus two types of morphologically diverse secretory cycles were observed. Among crustaceans only few workers (Enami, 1951; Matsumoto, 1954 and 1958 and Williams et al., 1980) have discussed the cyclic activity of the NSCs in detail and have noted the formation of vacuoles as a significant part of the secretory cycle. In P.indicus vacuole formation is the first phase in the activation of the cell for synthesis of neurosecretory material. The functional significance of vacuoles in the NSCs is obscure and their probable role in providing an enlarged substratum for subcellular synthetic activity is yet to be investigated. In P.indicus, in general, the vacuolar size diminished with heightened secretory activity when granular inclusions also increased. Moreover peripheral vacuolization as observed in the secretory phase of GN-and A-cells is seen by most workers (Parameswaran, 1956; Matsumoto, 1954 and Chandy and Kolwalker, 1985) as the terminal phase in the secretory cycle of a NSC. Interestingly, coalesence of the peripheral vacuoles to form a pericellular vacuolar ring as observed in the B and C cells of P.indicus has not been reported previously and is therefore, seemingly unique. The role of neuroglia in the NSC secretory cycle of P.indicus also appears to be unique, although some indication of glial activity has been reported

by Lake (1970) in the brain of the crab P.gaimardii. Glial cells investiture around the NSCs begins in the vacuolar phase and is subsequently hypertrophied in the secretory phase. For the most part, the glio-neuronal relationship in crustaceans is vague, although the glial cells may serve as a source of nutritive material during the synthetic phase of the NSC (Dr.Fingerman personal communication). More significantly, they may also serve as a media between the NSC and the surrounding capillary network, thus forming a glio-vascular relationship similar to the one existing in the vertebrates (Glees and Meller, 1969).

Notwithstanding the open circulatory system that crustaceans possess, exceedingly well developed capillary networks were observed in all the ganglia of P. indicus. Capillaries were also observed to circumscribe individual NSCs forming extracellular plexus. These observations are in agreement with those of Matsumoto (1954) in the thoracic ganglia of the crab Eriocheir japonicus and of Smith and Naylor (1972) in the eyestalk of C. maenas. Neurosecretory cells are expected to have a high metabolic rate and therefore, may require rich blood supply. Again, it may serve as a medium for discharge of neurosecretory products by exocytosis from the cell as in vertebrates (Scharrer and Scharrer, 1954). More importantly, these pericellular

plexus may also serve to dispense feedback information to the NSCs.

Nuclear and nucelolar participation in cellular synthetic activity is well established in all active cells (DeRobertis et al.,1975). Although no nuclear and nucleolar changes were observed in the GN-and A-cells in the present study, considerable changes were seen in the nucleus of B-and C-cells during the secretory cycle. The nucleoli of secretory phase B-and C-cells underwent a peripheral migration to become closely applied to the nuclear membrane. This coupled with the mild positvity of these organelles to RNA, suggest their crucial role in supplying RNA, material, through probable nucleolar extrusions, for the cellular synthetic activity. Similar observations were made by Gorgees and Rashan (1976) in the B-cells in the thoracic ganglion of the crab P.magnum.

There is apparently a general agreement on the major structural components of the dendrobranchiate eyestalk, but controversy exists with regard to the NSC groups accociated with the ganglia. For instance, some studies have not recognized the MIGX - organ (Nakamura, 1974; Van Herp et al.,1977) while others do (Dall, 1965; Hisano, 1976 and

Deitz, 1982). In P. indicus too, no NSC groups were observed in the MI lobe. The discrepancies are perhaps a function of correctly identifying or associating a NSC group with a particular medullary lobe. The description of 3 distinct NSC groups, the MTGXO-1 and 2 and a single MEGXO in the present study is similar to that reported in P.serratus (Van Herp et al., 1977), P.japonicus (Nakamura, 1974), M.rosenbergii (Deitz, 1982) and P.monodon (Nanda and Ghosh, 1985). It could therefore be assumed safely that in general, the distribution of NSC groups in the optic ganglia of penaeids are analogous. The sensory and glandular organ of Bellonci (sensory pore X-organ) or the pars distalis X-organ was not observed in P.indicus. On the ventrolateral side, the sensory pore was poorly developed and the characteristic onion bodies and vacuoles were not seen. This observation is inconsistent with the reports in other dendrobranchiates by several workers (Van Herp et al., 1977; Nakamura, 1974 and Hisano, 1974). Smith and Naylor (1972) stated that the organ of Bellonci is distinct in dendrobranchiates but may be absent in other decapods. Evidence derived from the present study indicate that this statement is not necessarily true. A smiliar observation was also made by Dall (1965) in Metapenaeus sp. where he reported the absence of the characteristic onion bodies. Paradoxically, in Decapoda itself, the organ is diversely

located, i.e, at the level of ME in Lysmata secticaudata (Carlisle, 1953), enclosed within the ME in P.paucidens (Hisano, 1974), within the MT in Pandalus borealis (Carlisle, 1959) and superfical and partially surrounded by MT in P.serratus (Van Herp et al., 1977) and in M.rosenbergii (Deitz, 1982). The organs sensory and glandular functions are as yet not clear.

The sinus gland exhibted no peculiar morphology in the present study. Bulbous axonal endings, granula of various sizes and an internal blood sinus were the major components of the SG in P. indicus. This observation as well as its dorsolateral position in between the ME and MI lobes corroborates the findings of earlier workers (Dall, 1965; Nakamura, 1974 and Van Herp et al., 1977). The 'S' shaped X-organ - SG tract in P.indicus is identical to that of other decapod crustaceans (Adiyodi and Adiyodi, 1970). The pathway observed in the present investigation clearly indicated that the mode of discharge of NSM in the eyestalk is via axonal transport. Based on electrophysiological and cobalt ion iontophoretic studies, Andrew and Saleuddin (1978) have reported a similar pathway with branching axons in the crayfish, Orconectes virilis.

Mapping of the NSC in the CNS of decapod crustaceans has been more thoroughly investigated in pleocyematans (Adiyodi and Adiyodi, 1970). In dendrobranchiates such studies have been very few and limited to the optic and supraesophageal ganglia. In P. indicus, 3 NSC groups were identified in the optic ganglia. This contrasts numerically with the observations of Dall (1965) in Metapenaeus sp. (4 groups), Nakamura (1974) in P.japonucus (6 - 8 groups), Deitz (1982) in M.rosenbergii (4 groups) and Hisano (1974) in P.paucidens (4 groups) and agrees with the observations of Van Herp et al., (1977) in P.serratus (3 groups). Observations on the NSC type distribution among these groups are even more diverse and confusing, with differences present even at the genus level. However, the observation of 3 cell types in the MEGXO and MEGXO of P.indicus corroborates with that reported in P.serratus (Van Herp et al.,1977) and P.japonicus (Nakamura, 1974).

In comparison to optic ganglia, the NSC groups in other parts of the CNS has been only sparingly investigated in dendrobranchiates. Nakamura's (1974) studies on the neurosecretion in P.japonicus revealed 8 NSC groups in the cerebral ganglia. In contrast 11 NSC groups have been identified in P.indicus in the present study. The regions where NSCs occur in the supraesophageal ganglion of

P.indicus are basically similar to those observed in P.japonicus, P.stylifera and Caridina laevis by Nakamura (1974), Nagabhushanam et al., (1986) and Pillai (1961) respectively. However, the presence of a central dorsal group with large NSCs appears to be exceptional.

With regard to the remaining ventral ganglia, there are surprisingly no comparable studies among dendrobranchiates. In pleocyematans, these ganglia have been fairly well studied, probably by virtue of the fact that all of the ganglia in the thorax in this group have fused to from a single large thoracic ganglion. The situation in dendrobranchiates, especially penaeids is different with each somite being represented by a ganglion.

The tritocerebral ganglia are a pair of ganglionic swellings midway on the circumesophageal connectives. In crabs Sesarma dehaani (Enami, 1951) and Paragrapsus gaimardii (Lake, 1970) the tritocerebral ganglia were observed to have only the medium (B-cells) and small size NSC. However, in P. indicus apart from these, one or sometimes two exceptionally large GN-cells were observed. By virtue of its size, magnitude and number of NSC groups the subesophageal ganglion in P.indicus is comparable to the

fused thoracic ganglion of pleocymatans. Fifteen NSC groups were identified in the subesophageal ganglion, with GN and A-cells as the dominant cell type. Similarly in the thoracic ~~and~~ ganglia of crabs (Enami, 1951 and Matsumoto, 1954) large NSCs like the γ cells of Enami and A-cells of Matsumoto were dominant. The segmental thoracic and abdominal ganglia in penaeids are much smaller and have only a few NSC groups.

The most striking dissimilarity in the NSC distribution in P. indicus is the total absence of the GN-cells in the optic ganglia and their dominating presence in all the ventral ganglia. It is established that the optic ganglia and the ventral ganglia are responsible for the synthesis of the mutually antagonistic and putative gonad inhibting hormone (GIH) and the gonad stimulating hormone (GSH) respectively (Adiyodi and Adiyodi, 1970). Therefore, by virtue of the GN-cells being distributed only in the ventral ganglia, the possibility of these cells being the major source of GSH in P.indicus is more than likely .

The results of the limited fine structural studies conducted on the neurosecretory material in P.indicus corroborates the detailed investigations made by earlier workers in other crustaceans (Smith, 1975; Hisano, 1976; Nakamura,

1978 and 1980; Andrew and Shivers, 1976 and Bellon-Humbert et al.,1981). The 1400 to 1600 A^{O} size of the haloed dense core elementary granulam that was observed in the NSC of P.indicus is similar to that found in Orconectes virilis by Andrew and Saleuddin (1978), however, granules of wider diameters ranging from 1100 to 1900 A^{O} has been reported in P. japonicus by Nakamura (1980). The presence of granula with differing electron densities has also been reported in Carcinus maenas by Smith (1975). He classified the granula into 5 functional types based on their density alterations. The electron lucent multivesicular bodies seen in the present study are possibly clear synaptic vesicles of pre-synaptic neurites as observed by Andrew and Saleuddin (1978) in O. virilis.

In Crustacea, unlike the vertebrates and some invertebrate groups like insects, very few studies have been made to correlate changes in the neurosecretory system with physiological events particularly reproduction, although neurosecretory elements are known to control this phenomenon (Adiyodi and Adiyodi, 1970). In P. indicus NCSs in V and S phases dominated in the MTGXO and MEGXO in the eyestalk of immature females. Subsequently in the ripe stage, it was observed that the number of V and S phase NSC decreased and

Q phase NSCs dominated. Similar cytological differences in the activity of the X-organ NSCs was correlated with seasonal activity of the ovary in the shrimp Pandalus gracilis (Aoto and Nishida, 1956) and in the freshwater crab, Potamon dehaani (Hanaoka and Otsu, 1957). Recently, Kulkarni and Nagabhushanam (1980) have shown in Parapenaeopsis hardwickii that the activity of the ovary (gonad) inhibiting hormone was the highest in the eyestalks of females with inactive and spawned ovaries whereas it was negligible in those at full vitellogenesis. However, in P.indicus it was observed that spent stages had a NSC phase constitution similar to mature state. This is probably because of the fact that once P.indicus reaches reproductive maturity and spawns, it is able to undergo the process of rematuration within a short period of time (Muthu, 1983).

The NSCs of the supraesophageal and thoracic ganglia were maximally active in late maturing and mature P.indicus. In the thoracic ganglia of crabs, Matsumoto (1958) reported that the NSCs were maximally active in autumn when the ovary was generally in its early stages of maturation. Similarly in the crayfish Procambarus simulans, Perryman (1969) correlated the stages of ovarian development with varying amount of neurosecretory material in cell type III of the cerebral ganglion. Diwan and Nagabhushanam (1975) argued

for a seasonal activity of the thoracic ganglion in the freshwater crab Barytelphusa cunicularis. Such a high secretory activity during ovarian maturation has also been observed in specific NSCs in the thoracic ganglion of Macrobrachium lanchesteri (Rao et al.,1981), M. kistensis (Mirajkar et al., 1983), Squilla holoschista (Deecaraman and Subramoniam, 1983) and Potamon koolooense (Joshi,1989).

Histochemical evidence derived from the present study indicated that in P.indicus the B-cells and to a lesser extent A-cells in the X-organ were the most active during the sexually quiescent period. Likewise in the supraesophageal and thoracic ganglia, GN, A-and B-cells were the most active in relation to gonadal maturity. Protein bound disulphide and sulphydryl groups were the significant constituents of the neurosecretory material which showed variation with gonadal maturation. This observation is thus concordant with the fact that cystine and cysteine are the major constituents of neurosecretory material in vertebrates (Adams and Sloper, 1956), insects (Raabe, 1980) and crustaceans (Lake, 1970; Diwan and Nagabhushanam, 1974). The glycogen and RNA content of the NSC also increased substantially with increased secretory activity in P.indicus. RNA has been suggested to be involved in the

production of neurosecretory material (Scharrer,1966) as in other cells in the protein synthesis phase. From the histological and histochemical results obtained in the present study it is clearly discernible that the B- and A-cells in the X-organ complex of P.indicus are seemingly responsible for the elaboration of a factor which inhibits the maturation of the gonad. Added evidence to this statement comes from the histological evaluation of the remaining eyestalk in prawns induced to mature by unilateral eyestalk ablation. All the NSCs in the MTGXO and MEGXO, particularly B-cells, were in a suppressed state and the sinus gland was devoid of any secretory material. Perhaps the only study conducted in a similar manner has been that of Adiyodi (1967). She reported that in females of Paratelphusa hydrodromous, which respond by accelerated vitellogenesis to unilateral eyestalk excision, a bunch of C-cells in the remaining eyestalk were in a passive stage. Similarly, the GN, A- and B-cells in the supraesophageal and thoracic ganglia of P.indicus are apparently involved in the secretion of factors which stimulate gonadal maturity. The pyriform C-cells in all the ganglia are seemingly not involved in the control of reproductive processes.

Both unilateral and bilateral ablation of the eyestalk in P.indicus led to precocious maturation of the ovary

demonstrating that gonad inhibitory principles are present in the NSC of the eyestalk. Among penaeids, eyestalk abaltion has so far been synonymous with unilateral eyestalk ablation. Arnstein and Beard (1975) and Santiago (1977) observed that ablation of single eyestalk was sufficient to induce maturation in P.orientalis and P. monodon respectively. Similarly in P. indicus, Muthu and Laxminarayana (1977) also reported that unilateral eyestalk ablation was sufficient to induce precocious maturation as was observed during the present investigation. Bilateral ablation was observed to result in abnormal behaviour patterns and incomplete maturation of the ovary in the present study. Identical results were obtained by Caillouet (1972), Alikunhi et al., (1975) and Muthu and Laxminarayana (1977).

In P.indicus, on occassions, bilateral ablation also led to moulting with maturing ovaries, an event which Adiyodi and Adiyodi (1970) reported as rare in crabs. Aquacop (1975) noted that they had never seen a penaeid moulting with developed ovaries except for a bilaterally ablated P. aztecus. Accelerated moulting rate has been observed in P.merguiensis after bilateral ablation by Alikunhi et al., (1975) although they did not observe gonadal maturation side by side. In a recent study on the moulting physiology of

P.indicus, Vijayan (1988) reported that in immature females of total length varying from 80 mm to 120 mm, bilateral ablation significantly enhanced the moulting frequency and thereby growth. More importantly, unilateral eyestalk ablation did not evoke any significant response by way of reducing the moult cycle duration. These instances of apparently synchronous occurrence of moulting and reproductive activities due to bilateral eyestalk ablation suggest the possibility that the hormonal mechanisms involved in moulting and reproduction are the same. Such an eventuality was discussed by Adiyodi and Adiyodi (1970) when they argued the possibilty of both GIH as well as MIH being chemically related molecules produced from the same NSCs in the X-organs of the optic ganglia. Presumably at least in penaeids, apart from the physiological state of the animal, the circulating inhibitory hormonal level or titer is significant, with moulting occurring when all of the inhibitory hormone(s) are removed as in bilateral eyestalk ablation. In unilateral ablation only half of the inhibitory hormone present in the animal is removed and this probably aids gonadal maturation.

Precocious maturation of the ovary was prevented effectivetly in P.indicus by administration of an eyestalk extract into unilateral eyestalk ablated prawns. These

findings confirmed that the principle factors contained in the eyestalk are indeed gonad inhibitory. Earlier reports showed that the accelerated maturation of the ovary in eye ablated crustaceans is blocked either completely as in Palaemon serratus (Panouse, 1943 and 1947), B.cunicularis (Nagabhushanam and Diwan, 1974) and P.stylifera (Kulkarni et al., 1981) or incompletely as in the crab Scylla serrata (Rangenekar and Deshmukh,1968) after injection of the eyestalk extract. The 92 % degree of inhibition obtained in P.indicus by using an aqueous eyestalk extract is comparable to that observed by Bomirski et al. (1981) in the crab Cancer magister. Using various fractions of the eyestalk extract they obtained degrees of inhibition ranging from 3 to 106 % . Identical findings have also been reported by Quackenbush and Herrnkind (1983) while working on the lobster Panulirus argus using the same method.

In the present investigation, histological and histochemical observations of the supraesophageal and thoracic ganglia of P.indicus undoubtedly indicated that the secretions of the NSCs present in these ganglia are apparently responsible for the stimulatory effect on the ovary. Additional evidence for the elaboration of this stimulating factor comes from the observation of heightend neuro-

secretory activity in these ganglia after unilateral and bilateral ablation. Similarly in the crab Menippe rumphii bilateral eyestalk ablation resulted in the release of neurosecretory material from the NSCs of the brain and thoracic ganglion (Babu et al.,1980). Further they observed that the secretory activity of the NSCs of the thoracic ganglion as being more pronouced than the cerebral ganglion after bilateral ablation, suggesting the involvement of thoracic neurosecretory material in reproduction. In P.indicus however, both cerebral and thoracic neurosecretory cells appear to be equally involved.

Paradoxically in P.indicus complete confirmatory experimental evidence, by way of full gonadal maturation in response to administration of the CNS extract, was not forthcoming. Implantation of the thoracic ganglion into young females has been claimed to induce ovarian enlargement in Potamon dehaani (Otsu, 1963), P.hydrodromous (Gomez, 1965) B.cunicularis (Naghabushanam and Diwan, 1974) and Libnia emarginata (Hinsch and Bennett,1979). Gonadal development also occurred in juvenile females of P.hydrodrmous after implantation of brain (Gomez and Nayar, 1965), but other parts of the nervous system appeared to be ineffective. Again in the marine prawn P.hardiwickii injections of brain and thoracic ganglia extracts into both

eyestalkless and normal individuals led to acceleration of gonadal maturation (Nagabhushanam and Kulkarni, 1980). In the crab Potamon koolooense, Joshi (1989) reported that administration of the thoracic ganglion extract stimulated vitellogenesis but an extract of the brain failed to elicit a similar response. Although in P.indicus injections of the CNS extract containing brain, subesophageal and thoracic ganglia produced only partial gonadal development, it however served to confirm the gonad stimulatory role of the NSCs in these ganglia. Complete maturity was probably not obtained due to the inadequate dosage used and the limited experimental duration. Again most workers (Nagabhushanam and Diwan, 1974; Hinsch and Bennett, 1979; Eastman-Reks and Fingerman, 1984 and Joshi, 1989) have used repeated implantations or injections of the ganglionic extracts to stimulate ovarian development. In P.indicus, which is very sensitive to experimental manipulations, such an experimental technique resulted in high mortality and therefore only a single injection was administered.

In Crustacea, reproductive growth (gondal maturation) and somatic growth (moulting) has been at best described as antagonistic to each other, mainly because of the competition for organic reserves which are necessary for carrying

out the processes (Adiyodi and Adiyodi, 1970). In P.indicus sampled from the wild population it was observed that, maturation almost always occurred in the early premoult stage. Not surprisingly, this is the moult stage in which P.indicus stays the longest time (Vijayan, 1988). Identical results were obtained by Emmerson (1980) and Crocos and Kerr (1983) when they correlated events in the reproductive and moult cycles in P.indicus and P.merguiensis respectively. However, this pattern was observed, in the present study, to be grossly disrupted in the bilaterally ablated group, although unilateral ablation seemingly did not affect it.

A schematic representation of the control of reproduction by neurosecretory cells and the active principles elaborated from them is shown in fig. 75.

SUMMARY

1. The different neuroendocrine centres eyestalk, brain, subesophageal, thoracic and abdominal ganglia of P. indicus were studied in detail using histological, histochemical and electron microscopic techniques. Structural changes in the neurosecretory cells were correlated to the different phases of gonadal maturation and further the neuroendocrine control of gonadal maturation was studied under experimental conditions.

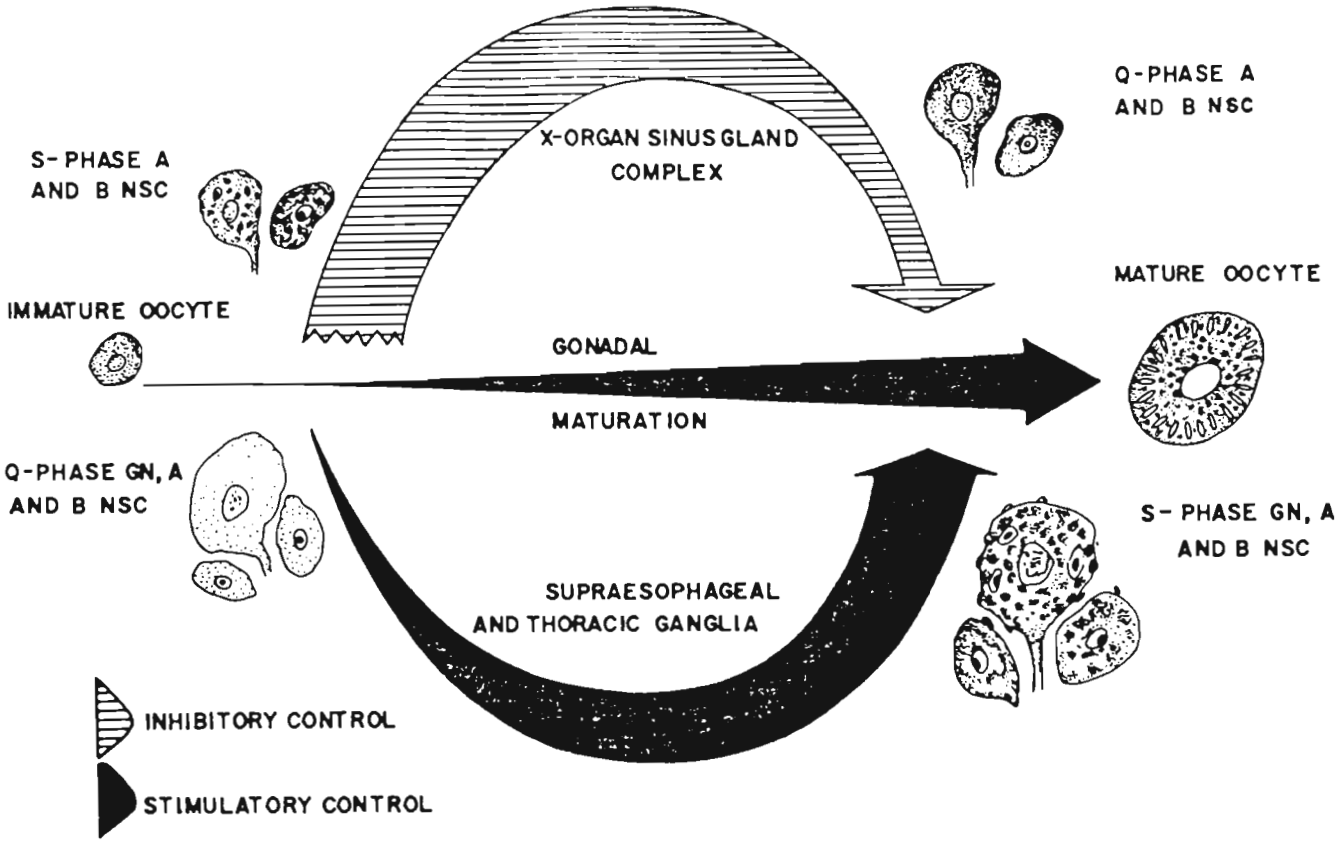

FIG. 75 SCHEMATIC REPRESENTATION OF NEUROENDOCRINE CONTROL OF OOGENESIS IN PENAEUS INDICUS

2. Using neurosecretion specific stains, neurosecretory cells were identified in the optic, supraesophageal, trito-cerebral, subesophageal, thoracic and abdominal ganglia. Based on cytomorphological differences 4 different types of NSCs were recognized viz., giant neuron (GN), A-cells, B-cells and C-cells.

3. Distinct cyclic changes were observed in the perikarya of the NSCs in relation to the synthesis of neurosecretory material. On the basis of the appearance of secretory granules in the cytoplasm, vacuolization and involvement of extracellular glial cells and capillary plexus, three phases were identified to denote neurosecretory activity viz., quiescent phase (Q), vacuolar phase (V) and secretory phase (S).

4. In the eyestalk, 3 NSC groups were recognized and they were termed as the medulla terminalis ganglionic X-organ (MTGXO) 1 and 2 and the medulla externa ganglionic X-organ (MEGXO) based on their location. Type B and C NSCs were the most common in the X-organs along with a few A-cells. GN-cells were absent in the eyestalk. The neurohaemal organ, sinus gland was found between the medulla externa and interna and axons from all the NSC groups were observed to

reach the sinus gland. The location of the neurosecretory cells in all the ventral ganglia were studied in detail. Totally 11 NSC groups were identified on both dorsal and ventral aspects of the supraesophageal ganglion. GN, A and B type NSCs were the most common in all the NSC groups. C-cells were present only in small numbers. The extremely small tritocerebral ganglion was found to be characterized by the presence of two exceptionally large GN cells and few B and A- cells. A maximum number of 15 NSC groups were recognized in the subesophageal ganglion on its dorsal, dorso-median and ventral aspects. GN and A-cells were predominant in the ventro-median group. Other cell groups had A, B and C type NSCs in them. Thoracic ganglia had 4 NSC groups on its dorsal and ventral aspects with GN, A,B and C-cells. The abdominal ganglia was relatively poor in NSC groups with A,B and C-cells. GN cells were absent in the abdominal ganglia.

5. Histochemical investigations revealed that the NSCs in P. indicus were in general, rich in proteins and carbohydrates and poor in lipids and nucleic acids. The principal component of the neurosecretory material was found to be a protein rich in cystine amino acid. The carbohydrate content in the cytoplasm was mainly glycogen. Glial cells were also intensely rich in proteins.

6. Electron microscopic studies on the neurosecretory material revealed that the primary NSM was composed of haloed dense core granules which measured 1400 to 1600 A in diameter.

7. The distribution of NSCs in the different phases of the secretory cycle was studied in relation to gonadal development. In the eyestalks of immature females more than 75% of the NSCs in the MTGXO and MEGXO were in the physiologically active V and S phases. In mature females majority of the NSCs were in a suppressed state (Q phase). Conversely, in the supraesophageal, subesophageal and thoracic ganglia of immature females almost 65% of the NSCs were in the Q phase, while in fully mature females physiologically active NSCs (V and S phase) together numbered more than 80%.

8. In relation to gonadal development, the variations in histochemical responses of NSCs were studied. The amino acid cystine was the major component which showed significant fluctuation in relation to vitellogenesis. Such investigations showed that in the eyestalks, A and B-cells were the most active cell types in relation to reproduction. Whereas in the brain and thoracic ganglia, GN, A and B-cells were the most active in relation to gonadal maturity. The

pyriform C-cells did not appear to be involved in the process of maturation.

9. Both unilateral and bilateral eyestalk ablation was found to lead to precocious maturation by significantly enhancing the GSI and ova diameter. Bilaterally ablated prawns exhibited behavioural discrepancies and were observed to undergo the process of moulting with developing ovaries. In unilateral eyestalk ablated females which were administered an aqueous extract of the eyestalk, the maturation process was found to be blocked due to the inhibitory nature of the neurosecretory principles contained in the eyestalk. Female P.indicus which were injected with an extract of the brain, subesophageal and thoracic ganglia, showed significant enhancement in the GSI and oocyte diameter although full gonadal maturity was not obtained.

10. The temporal relationship between gonadal maturation and the moult cycle was also studied. Gonadal maturation was invariably observed during the relatively long early premoult stage of the moult cycle. A similar pattern was observed in unilateral eyeablated females, however this pattern was found to be disrupted in bilateral eyeablated females.

CHAPTER IV

BIOCHEMICAL CHANGES IN RELATION TO OVARIAN MATURATION

INTRODUCTION

A survey of the literatutre on the biochemistry of crustaceans revealed that remarkable interspecific variations exist in the biochemical composition of tissues. Ovarian maturation places enormous demands on the energy reserves of females. In crustaceans, this problem is compounded by the energy requirements of the moult cycle, and therefore for the co-ordination of these two processes an endocrine control is essential (Adiyodi and Adiyodi, 1970). A gametogenic process in the female reproductive cycle of crustaceans includes the synthesis of nutritive yolk in the ooplasm to meet the basic requirements of embryonic development independent of the maternal organism (Adiyodi and Subramoniam, 1983). During this process considerable mobilisation occurs in the major organic reserves of the animals.

In crustaceans hepatopancreas or the midgut gland has been identified as the primary organ responsible for the storage of organic reserves, while the haemolymph plays the role of transporting these metabolites to the different tissues (Yamaoka and Scheer, 1970). It is well estabilished that glycogen and lipid are the major energy reserves

affected at the time of reproduction in crustaceans (Hohnke and Scheer, 1970 and Gilbert and O'Connor, 1970). However, information on quantitative and qualitative changes in these organic reserves is fragmentary, especially with reference to reproduction (Adiyodi and Adiyodi, 1970).

Studies on biochemical changes in relation to reproductive cycle in invertebrates have been pioneered by Giese and his co-workers (Giese and Pearse, 1974). Among crustaceans most of these studies are centered on the changes in metabolites like protein, lipid and carbohydrates in relation to different stages of maturity in pleocyematans like Portunus pelagicus (Rahaman, 1967), Charybdis variegata (Chandran, 1968), Paratelphusa hydrodromous (Adiyodi, 1968), Uca annulipes and P.pelagicus (Pillay and Nair, 1973), Barytelphusa cunicularis (Diwan and Nagabhushanam, 1974), Orconectes nais (Rice and Armitage, 1974), Menippe rumphii (Shyamsundari and Erribabu, 1979) and Clibanarius clibanarius (Varadarajan and Subramoniam, 1982). Similar investigations on dendobranchiate crustaceans are very few, although the annual reproductive cycle of penaeid prawns has been well described (Cummins, 1961; Rao, 1968; Thomas, 1974 Crocos and Kerr, 1983). The fluctuations in the biochemical constituents such as water, nitrogen, non-protein nitrogen, protein, lipid and glycogen in gonad, muscle and

hepatopancreas in relation to the different reproductive phases have been studied in the penaeid prawn Metapenaeus affinis by Pillay and Nair (1973). The percentage protein, carbohydrate and lipid in the ovary and hepatopancreas of Penaeus vannamei, Penaeus stylirostris and Penaeus setiferus were compared in captive and wild prawns by Lawrence et al. (1979). The levels of protein, glycogen and fat in the hepatopancreas and ovary were studied in relation to maturation in the marine prawn Parapenaeopsis hardwickii by Kulkarni and Naghubhushanam (1979). A brief study on the changes in body composition of water, protein, lipid, ash and energy during the moult cycle and ovarian development has been reported by Read and Caulton (1980) in P.indicus from the South African Coast. Proteins provide the basic structural material for tissue build up. The protein patterns in different tissues have been analysed electrophoretically by Kulkarni et al. (1980) in M.affinis, M.monoceros, P.hardwickii and P.stylifera in relation to sex. Studies on haemolymph protein content in relation to ovarian maturation in crustaceans are few. However changes in the serum protein during the reproductive cycle has been studied in the American lobster Homarus americanus by Barlow and Ridgeway (1969) and in Macrobrachium rosenbergii by Dietz (1982). Vitellogenin or the plasma precursor of yolk protein has been studied in crabs (Adiyodi, 1968; Kerr, 1968

and 1969 and Wolin et al., 1973) and the crayfish (Fyffe and O'Connor, 1974).

Recently, Yano (1988) studied the appearance of vitellogenin in the blood sera of female Penaeus japonicus. Lipovitellin which is the principal fraction of the crustacean vitellus has been investigated in relation to maturation of the ovary in a number of crustaceans (Adiyodi and Subramoniam, 1983). The accumulation and cyclic variation in the protein content of the gonad, hepatopancreas and muscle has been studied in M.affinis (Pillay and Nair, 1973), P. hardwickii (Kulkarni and Nagabhushanam,1979) and in P. setiferus (Lawrence et al., 1979).

Fluctuations in free sugars (glucose, galactose, and surcose) in the hepatopancreas of the crab P.hydrodromous has been studied in relation to the ovarian cycle by Adiyodi and Adiyodi, (1970, b). Similarly in P.hardwickii investigations have been made on the proportionality between haemolymph glucose level and ovary growth by Nagabhushanam and Kulkarni (1980). Recently Trujillo and Luna (1981) studied the variation in glucidic metabolism during the reproductive cycle of Penaeus notalis.

The chief storage site of lipid in crustaceans is found to be the hepatopancreas and the quantitative and qualitative composition of lipid detected at any given time in this organ has been reported to be the result of absorptive, synthetic, secretory and catabolic processes (Chang and O'Connor, 1983). Owing to the enormous quantity of lipids involved in crustacean vitellogenesis, many workers have studied the changes in the fat content of the gonad and hepatopancreas. Relatively very few investigations have been carried out on haemolymph lipids in crustaceans (Teshima and Kanazawa, 1978). The seasonal fluctuations in the fat content of the prawn P. indicus in relation to diet has been studied by Gopalakrishnan (1953). The metabolism of lipids, particularly phospholipids, in relation to reproduction and moulting has been investigated in the crab P.hydrodromous (Adiyodi and Adiyodi, 1970). The maturational changes in the ovarian lipid spectrum of the pink shrimp, P. duorarum has been studied by Gehring (1974). Guary et al.(1975) have investigated the seasonal variation of the fatty acid composition of P. japonicus. The metabolic profile of dietary lipids in relation to ovarian maturation in penaeid prawns was investigated by Middleditch et al.(1980). In the prawn P. japonicus the variation in lipid composition during ovarian maturation was studied by Teshima

and Kanazawa (1983) and the changes in tissue lipid composition during vitellogenesis in P. indicus was reported by Galois (1984). Crustaceans are not capable of de novo sterol synthesis but they do appear to modify dietary sterols to cholesterol (Morris and Culkin, 1977 and Morris et al., 1981). The variations in cholesterol content in the ovary and hepatopancreas in relation to reproduction has been studied by Adiyodi and Adiyodi (1971) in the crab P.hydrodromous and Middleditch et al., (1980) in penaeid shrimps. The changes in cholesterol content in the haemolymph of the female Macrobrachium kistensis during the reproductive cycle was investigated by Mirajkar and Nagabhushanam (1981).

The ovaries and eggs of fish and shellfish are almost invariably pigmented due to the presence of carotenoids and or carotenoproteins (Cheeseman et al., 1967; Gilchrist and Lee, 1972). However studies on carotenoid composition of crustacean ovaries are very limited and fragmentary (Miki et al., 1982). Among penaeids the biosynthesis of astaxanthin pigment in the prawn P.japonicus has been investigated by Tanaka et al., (1976) and the carotenoid content in the ovaries of P.orientalis has been described by Miki et al.,(1982). Nevertheless studies on changes in carotenoid content in relation to reproduction are few.

Investigations on the content and composition of nucleic acids in crustaceans are limited to the changes occurring in relation to the moult cycle (Skinner, 1968; Dall and Barclay, 1979). Surprisingly there are only few comparable studies on the variation in nucelic acid content in relation to reproduction in crustaceans (Nadarajalingam and Subramoniam, 1987), although RNA is known to aid in the synthesis of protein during the autosynthetic phase of many crustacean ovaries (Adiyodi and Subramoniam, 1983).

Crustaceans exhibit a general pattern in the fluctuation of metabolic components, but significant variations are known to occur at intrageneric and intraspecific levels. Therefore, a separate study on each species is essential for understanding the precise body metabolism in conjunction with the biosynthesis of yolk in the ovary. In the present investigation on female P.indicus, the quantitative variations in protein, carbohydrate, lipid, cholesterol, carotenoid, DNA, RNA and moisture in the various tissues like gonad, hepatopancreas, muscle and haemolymph were estimated during the different maturity stages.

MATERIALS AND METHODS

Collection of animals

Live females of P.indicus were collected from the sea off Cochin by trawling as described in chapter II. The prawns were transported to the laboratory and kept in plastic pools containing seawater (28-32 ppt) with continuous aeration. Prawns were then segregated according to the five maturity stages described earlier, viz. Stage I - immature, Stage II - maturing, Stage III - late maturing , Stage IV - mature and Stage V - spent and spent recovering and used for biochemical analysis.

Haemolymph collection and tissue sampling

Samples of haemolymph from prawns of all reproductive stages were analysed for total protein, lipid, carbohydrate, cholesterol and carotenoid contents. Gonad, hepatopancreas and muscle tissues from prawns belonging to different maturity stages were analysed for moisture, protein, lipid, carbohydrate, cholesterol, carotenoid, DNA and RNA contents. Carotenoids were not estimated in muscle tissues because of the very low levels present in them. Dried and powdered tissue samples were used, for the estimation of protein, lipid, carbohydrate and cholesterol, while for the

estimation of moisture, carotenoids, DNA and RNA content, fresh tissues were used.

Live animals were collected from the pool and blotted dry using a filter paper. Haemolymph samples from individual prawns were drawn by direct cardiac puncture using a hypodermic syringe fitted with a No.22 needle. The glass syringe and needle used for haemolymph collection were rinsed in an anti-coagulant (10% trisodium citrate) prior to each collection. The collected haemolymph samples were stored in sterilized glass vials at -20°C until analysis. After the extraction of haemolymph, the prawns were immediately dissected and the ovary, hepatopancreas and muscle tissues were quickly excised out. Tissues were dried to a constant weight at 60°C and then macerated using an agate mortar and pestle. They were then stored in a dessicator with silica gel until analysis. Six replicates were carried out for each estimation.

BIOCHEMICAL ANALYSIS

1. **Estimation of moisture content**

The moisture content of ovary, hepatopancreas and muscle were determined by keeping pre-weighed wet samples at 60 °C in a hot air oven till constant weights were obtained. The

loss in weight was taken as the water content and expressed as percentage.

2. Estimation of total proteins

Total proteins was estimated by the Biuret method of Gornall et al, (1949) using crystalline bovine serum albumin (Sigma) as standard. Pre-weighed dried tissue or a known aliquot of haemolymph was taken and deproteinized using 80% ethanol. The protein precipitate was dissolved in 2 ml 1 N NaOH and 8 ml of Biuret was added. The colour developed was read at 540 nm using a ECIL UV spectrophotometer against a reagent blank.

3. Estimation of total lipids

Total lipid content in the tissues and haemolymph was estimated gravimetrically using the method of Folch et al., (1957). Pre-weighed sample or a known aliquot of haemolymph was homogenized in a chloroform : methanol mixture (2:1 V/V) and placed in an amber coloured separating funnel. The phases were separated by the addition of 0.9% NaCl solution. The chloroform layer was collected and evaporated to dryness in a water bath. The dry weight of the lipid obtained was determined gravimetrically using a Mettler monopan balance.

4. Estimation of total carbohydrates

Total simple sugars, oligosaccharides and polysaccharides were estimated using the phenol - sulphuric acid method of Dubois et al., (1956). Haemolymph and tissue samples were deproteinized using 80% ethanol. To a known aliquot of the supernatent 0.05 ml of 80% phenol was added. Then 5 ml of concentrate sulphuric acid was added directly against the liquid surface to obtain good mixing. The stable orange-yellow colour developed was read at 490 nm in a ECIL UV spectrophotometer along with D-glucose standard and reagent blanks.

5. Estimation of total cholesterol

Total cholesterol was estimated following the ferric chloride glacial acetic acid method of Hestrin (1949). The cholesterol was either extracted directly from the tissue using glacial acetic acid or the total lipid was extracted using the method of Folch et al. (1957) and the lipid thus obtained redissolved in glacial acetic acid and a suitable aliquot taken for estimation. The colour developed was read at 560 nm in a ECIL UV spectrophotometer along with reagent blanks.

6. Estimation of total carotenoids

The total carotenoid content was estimated by following the extraction procedure of Olson (1979). Pre-weighed fresh tissue or a known aliquot of haemolymph was taken in a clean 10 ml screw cap glass vial and 2.5 g anhydrous sodium sulphate was added. The sample was gently mashed with a glass rod until it was well mixed with sodium sulphate. The caked residue was covered with 5ml of chloroform and placed at 0°C for 8-24 hours. An aliquot of the chloroform extract was diluted with ethanol and the absorption was read in a ECIL UV spectrophotometer at 450 nm.

7. Estimation of nucleic acids

The content of RNA and DNA in the ovary, hepatopancreas and muscle were estimated following the scheme proposed by Dagg and Littlepage (1972), which is based on the methods of Schmidt and Thaunhauser (1945) and Munro and Fleck (1966). Nucleic acids were extracted from pre-weighed fresh tissues using cold perchloric acid. RNA was measured directly by reading the absorbance at 260 nm in a ECIL UV spectrophotometer. The DNA content was determined by the indole method (Ceriotti, 1952). The colour developed by the indole reagent was read at 490 nm after removing the interfering colour by repeated extractions with amyl acetate.

RNA and DNA standard curves were prepared by using synthetic RNA and DNA obtained from SRL and Sigma biochemicals respectively.

Statistical analysis of data

The mean and standard deviations of the data were determined and values were plotted on graphs to obtain the trend of metabolites during the different maturity stages of the prawns. Analysis of variance (ANOVA) was performed to test the significance between treatments i.e., the effect of maturity stages on biochemical parameters (Snedecor and Cochran, 1968). The data was processed in a PC/XT computer with suitable programs.

RESULTS

Concentrations of the metabolic components like protein, carbohydrates, lipid, cholesterol, moisture, carotenoid, RNA and DNA showed significant variations in the various tissues of*P.indicus* during the progress of maturation. The analytical results of the parameters in tissues like heamolymph, hepatopancreas, ovary and muscle during the various stages

of maturation are given in tables 1 to 8 and graphically represented in figs.1 to 4.

1. Proteins

The variations in the concentration of total proteins observed in the haemolymph, ovary, muscle and hepatopancreas in the different maturity stages are given in Table-1.

The protein content in the haemolymph increased gradually from Stage I to Stage IV, where the maximum value of 105.14 mg/ml was observed. In stage V there was a sharp fall in the haemolymph protein content to 38.39 mg/ml. In stage I ovaries, proteins formed 16.15% of the total dry weight. The levels of protein in the ovary gradually increased and the maximum value of 39.27 mg/100 mg was observed in stage IV. Spent ovaries displayed a sudden decrease in protein content (fig.2). Muscle protein content was uniformly high in all maturity stages and the values showed an erratic behaviour without a definite pattern (fig.3). The protein content in the hepatopancreas was found to be generally poor. Maximum protein content was observed in stage I and V and the minimum value of 8.73% was obtained in stage III.

ANOVA showed that the differences in protein levels in haemolymph, ovary, muscle and hepatopancreas during the different maturity stages were statistically significant at 1% level.

2. Carbohydrates

The variations in total carbohydrates in the haemolymph, ovary, muscle and hepatopancreas during the different maturity stages are given in table 2.

In the haemolymph the total carbohydrate content was the lowest in stage I. A gradual increase in the carbohydrate content was observed and the maximum value of 1.98 mg/ml was noticed in stage IV. In stage V there was a drastic decrease in the carbohydrate level in the haemolymph (fig. 1). The carbohydrate content in the ovary showed a gradual increase from stage I to IV and a subsequent decline in stage V (Fig. 2). Peak carbohydrate level of 2.8% was observed in stage IV ripe ovaries. In the muscle, carbohydrate levels were generally poor, (0.9 to 1.2%) and it fluctuated without a definite pattern (fig. 3). Maximum carbohydrate content was observed in the hepatopancreas. Peak levels of 4.1% was observed in stage III and thereafter there was a drastic decline in the carbohydrate content in

stage IV(fig.4).

ANOVA revealed that the differences in total carbohydrate content in the haemolymph, ovary and hepatopancreas were statistically significant at 1% level. The carbohydrate levels in the muscle did not show any significant variation.

3. Lipids

The changes in lipid content in the haemolymph, ovary, muscle and hepatopancreas during the different maturity stages are given in table. 3.

High lipid levels in the haemolymph was observed in stage IV, while the lowest was seen in stage I. Haemolymph of spent animals showed a sudden decline in lipid levels (Fig. 1). In the ovary there was a drastic increase in lipid content from stage I to stage IV. Lipids constituted 29.14% of the ripe ovary. In stage V the lipid content decreased and was similar to the level found in stage I (Fig. 2). Muscle tissues were found to be uniformly poor in lipids with levels ranging from 5.5 to 7%. No definite trend could be observed in the muscle lipid content in relation to maturity stages (Fig. 3). Lipid content of the hepatopancreas during the different maturity stages ranged from

13.12 mg/100 mg in stage I to the maximum of 51.62 mg/100 mg in stage III. After this rapid increase in lipid content, there was a gradual decrease in the levels in stage IV and stage V. The hepatopancreas in spent animals also were remarkably rich in lipids with as much as 31.7% (Fig. 4).

ANOVA indicated that the variations in lipid levels in the haemolymph, ovary and hepatopancreas during the different maturity stages were statistically significant at 1% level. Changes in the muscle lipid content was not statistically significant.

4. Cholesterol

The differences in the concentration of cholesterol in the haemolymph, ovary, muscle and hepatopancreas during the different maturity stages are given in Table 4.

Cholesterol levels in the haemolymph rose steadily from stage I to peak at stage IV and thereafter the levels declined in stage V (Fig. 1). In the ovary also a similar trend was observed with the minimum content of 1.3 mg/100 mg observed in stage I and maximum of 4.14 mg/100 mg observed in stage IV (Fig. 2). The cholesterol in the muscle varied

from 1.28 to 1.48 mg/100 mg without any definite trend between the maturity stages (Fig. 3). Hepatopancreatic cholesterol content showed marked fluctuation with maturity stages. High cholesterol content of 5.51 mg/100 mg was noted in stage III and minimum values of 2.32 mg/100 mg was observed in stage I. The difference in hepatopancreatic cholesterol levels between stage IV and V were only marginal (Fig. 4).

The differences in the cholesterol content in the haemolymph, ovary and hepatopancreas during the different maturity stages were found to be statistically significant at 1% level using ANOVA. The changes in cholesterol levels in the muscle tissues were not statistically significant.

5. **Moisture**

The variations in the moisture or water content of the ovary, muscle and hepatopancreas during the different maturity stages are given in Table 5.

The moisture content in the ovary showed remarkable fluctuation with the maturity stages. Maximum water content was observed in stage V and stage I. From 74.16% in stage I, the water content declined to 65.41% in stage IV. Spent

ovaries were found to have the maximum water content (Fig. 2). The moisture content in the muscle was found to vary from 74% to 76% in the different maturity stages without a definite pattern (Fig. 3). In comparison to the other tissues, hepatopancreas was observed to have the least moisture content. In stage I the moisture content was 67.9% and thereafter the values showed a declining trend and the lowest value of 49.2% was recorded in stage III. From stage III to stage V the water content values showed a rising trend (Fig. 4).

ANOVA showed that the percentage variations of moisture in ovary and hepatopancreas during different maturity stages were statistically significant ($p<0.01$). However the changes in the muscle water content was not significant ($p>0.01$).

6. Carotenoids

The changes in the total carotenoid content of the haemolymph, ovary and hepatopancreas during the different maturity stages are depicted in Table 6.

In the haemolymph the carotenoid content showed a steady increase from the very low levels prevailing in stage I

(10.44 µg/ml) to the maximum of 28.51 µg/ml and 26.6 µg/ml in stage III and IV respectively. There was a fall in carotenoid content in the haemolymph in stage V (Fig. 1). In the ovary a slow build up of carotenoids was observed from stage I to stage IV. From the maximum of 5.87 µg/mg observed in stage IV there was a sudden drop in the level of carotenoids in stage V to 2.71 µg/mg. The dark green colour of the ripe ovary was observed to become dark red on extraction with chloroform. In the hepatopancreas, a rapid rise in the carotenoid content was observed from stage I to III. Subsequently the carotenoid levels stablized at 47.16 and 45.47 µg/mg in stage IV and V respectively (Fig. 4).

ANOVA showed that the differences in the carotenoid levels in the haemolymph, ovary and hepatopancreas during the various maturity stages were statistically significant at 1% level.

7. Ribonucleic acid (RNA)

The variations in the concentration of RNA in the ovary, muscle and hepatopancreas during the various maturity stages are given in Table 7.

In the ovary, the RNA content decreased steadily from an

inital peak of 50.7 µg/mg in stage I to 16.29 µg/mg in stage IV (Fig. 2). In stage V the RNA level was restored to the initial concentration (49.37 µg/mg). Muscle RNA content showed only very little variations, with stage I having the maximum and stage II the minimum (Fig. 3). The RNA content in hepatopancreas steadily increased from stage I (27.26 µg/mg) to stage IV (56.28 µg/mg) and thereafter a sharp fall in the value was observed (Fig. 4).

The variations in RNA levels in the ovary and hepatopancreas in different maturity stages were found to be statistically significant ($p<0.01$). Muscle RNA content showed significant variation at 5% level.

8. Deoxyribonucleic acid (DNA)

The concentration of DNA in the ovary, muscle and hepatopancreas during different maturity stages is indicated in Table 8.

In stage I ovary, the mean DNA content was 1.46 µg/mg, subsequently there was a drastic decline in the level and the lowest value of 0.14 µg/mg was observed in stage IV ovary. In stage V a very high DNA content of 2.39 µg/mg was

Table - 1 Variations in the concentration of total protein during the different maturity stages of *P. indicus*.

TISSUES		MATURITY STAGES				
		I	II	III	IV	V
Haemolymph (mg/ml)	N	6	6	6	6	6
	$\bar{X}$	31.82	48.76	72.04	105.14	38.39
	$\pm$ SD	4.72	7.83	14.83	11.43	7.39
Ovary (mg/100 mg) dry weight	N	6	5	6	6	6
	$\bar{X}$	16.15	25.77	31.97	39.27	23.08
	$\pm$ SD	4.96	3.46	1.19	2.66	3.97
Muscle (mg/100 mg) dry weight	N	6	6	6	6	6
	$\bar{X}$	51.95	51.45	54.50	45.79	51.52
	$\pm$ SD	2.92	1.48	3.41	3.41	4.54
Hepatopancreas (mg/100 mg) dry weight	N	6	6	6	5	6
	$\bar{X}$	13.02	10.81	8.73	11.81	13.24
	$\pm$ SD	1.90	1.75	1.64	1.49	1.94

ANALYSIS OF VARIANCE - PROTEIN

TISSUE	SOURCE	D.F	SUM OF SQR	MEAN SQRS	F - VALUE
Haemolymph	Treatment	4	21393.630	5348.408	54.68*
	Error	25	2445.352	97.814	
Ovary	Treatment	4	1850.047	462.512	39.05*
	Error	25	296.127	11.845	
Muscle	Treatment	4	244.625	61.156	5.60*
	Error	25	273.016	10.921	
Hepatopancreas	Treatment	4	81.691	20.423	6.88*
	Error	25	74.237	2.969	

* Significant at 1% level ($P < 0.01$)

Table - 2 : Variations in the concentration of total carbohydrate during the different maturity stages of *P. indicus*.

TISSUES		MATURITY STAGES I	II	III	IV	V
Haemolymph (mg/ml)	N	6	6	6	6	6
	$\bar{X}$	0.44	0.82	1.15	1.98	0.65
	± SD	0.10	0.09	0.31	0.34	0.16
Ovary (mg/100 mg) dry weight	N	6	6	6	6	6
	$\bar{X}$	2.27	2.20	2.45	2.80	1.89
	± SD	0.18	0.33	0.30	0.11	0.42
Muscle (mg/100 mg) dry weight	N	5	6	6	6	6
	$\bar{X}$	1.01	0.95	1.01	1.08	1.14
	± SD	0.07	0.12	0.12	0.18	0.31
Hepatopancreas (mg/100 mg) dry weight	N	6	6	6	5	6
	$\bar{X}$	2.49	3.29	4.10	1.53	2.16
	± SD	0.34	0.32	0.40	0.29	0.66

ANALYSIS OF VARIANCE - CARBOHYDRATE

TISSUE	SOURCE	D.F.	SUM OF SQR	MEAN SQRS	F - VALUE
Haemolymph	Treatment	4	8.693	2.173	40.49*
	Error	25	1.342	0.054	
Ovary	Treatment	4	2.646	0.662	7.74*
	Error	25	2.138	0.086	
Muscle	Treatment	4	0.122	0.031	0.94**
	Error	25	0.811	0.032	
Hepatopancreas	Treatment	4	23.998	5.999	33.95*
	Error	25	4.418	0.177	

* Significant at 1% level ($P < 0.01$) ; ** Not significant.

Table - 3 : Variations in the concentration of total lipid during the different maturity stages of *P. indicus*.

TISSUES		MATURITY STAGES				
		I	II	III	IV	V
Haemolymph (mg/ml)	N	6	6	6	6	6
	$\bar{X}$	7.10	10.50	14.69	20.40	14.29
	$\pm$ SD	1.37	2.32	2.17	1.93	2.24
Ovary (mg/100 mg) dry weight	N	6	6	6	6	6
	$\bar{X}$	11.80	16.13	22.04	29.14	13.93
	$\pm$ SD	4.17	2.34	4.00	2.21	3.20
Muscle (mg/100 mg) dry weight	N	4	6	6	6	6
	$\bar{X}$	5.58	6.17	6.69	7.02	6.76
	$\pm$ SD	0.73	1.93	2.11	2.02	1.84
Hepatopancreas (mg/100 mg) dry weight	N	6	6	6	6	6
	$\bar{X}$	13.12	23.62	51.62	45.69	31.77
	$\pm$ SD	3.28	5.74	10.25	6.39	5.15

ANALYSIS OF VARIANCE - LIPID

TISSUE	SOURCE	D.F.	SUM OF SQR	MEAN SQRS	F - VALUE
Haemolymph	Treatment	4	597.338	149.335	36.05*
	Error	25	103.573	4.143	
Ovary	Treatment	4	1182.484	295.621	27.30*
	Error	25	270.757	10.830	
Muscle	Treatment	4	7.785	1.946	0.61**
	Error	25	79.247	3.170	
Hepatopancreas	Treatment	4	5956.481	1489.120	31.38*
	Error	25	1186.406	47.456	

* Significant at 1% level ($P < 0.01$); ** Not significant.

Table - 4 : Variations in the concentration of Cholesterol during the different maturity stages of *P. indicus*.

TISSUES		MATURITY STAGES				
		I	II	III	IV	V
Haemolymph (ug/ml)	N	6	6	6	6	6
	$\bar{X}$	167.07	199.75	305.69	447.98	220.99
	$\pm$ SD	9.89	13.22	33.72	37.23	16.12
Ovary (mg/100 mg) dry weight	N	6	6	6	6	6
	$\bar{X}$	1.30	1.85	2.55	4.14	1.45
	$\pm$ SD	0.21	0.50	0.48	0.70	0.42
Muscle (mg/100 mg) dry weight	N	5	6	6	6	6
	$\bar{X}$	1.48	1.28	1.31	1.37	1.35
	$\pm$ SD	0.28	0.09	0.15	0.17	0.12
Hepatopancreas (mg/100 mg) dry weight	N	6	6	6	6	6
	$\bar{X}$	2.32	3.63	5.51	3.84	3.51
	$\pm$ SD	0.45	0.79	0.89	0.54	0.69

ANALYSIS OF VARIANCE - CHOLESTEROL

TISSUE	SOURCE	D.F.	SUM OF SQR	MEAN SQRS	F - VALUE
Haemolymph	Treatment	4	305257.300	76314.310	124.84*
	Error	25	15282.000	611.280	
Ovary	Treatment	4	32.223	8.056	34.54*
	Error	25	5.830	0.233	
Muscle	Treatment	4	0.147	0.037	1.34**
	Error	25	0.686	0.027	
Hepatopancreas	Treatment	4	31.422	7.855	16.39*
	Error	25	11.903	0.479	

* Significant at 1% level ($P < 0.01$) ; ** Not Significant.

Table - 5 : Variations in the level of moisture during different maturity stages of *P. indicus*

TISSUES		MATURITY STAGES				
		I	II	III	IV	V
Ovary (%)	N	6	6	6	6	6
	$\bar{X}$	74.16	70.69	68.51	65.41	79.99
	$\pm$ SD	2.19	2.46	1.86	5.98	0.96
Muscle (%)	N	6	6	6	6	6
	$\bar{X}$	74.62	76.18	74.60	75.43	74.60
	$\pm$ SD	1.45	2.87	2.38	2.25	2.91
Hepatopancreas (%)	N	6	6	6	6	6
	$\bar{X}$	67.93	56.70	49.20	59.49	70.62
	$\pm$ SD	6.17	8.35	5.41	5.63	11.92

ANALYSIS OF VARIANCE - MOISTURE

TISSUE	SOURCE	D.F.	SUM OF SQR	MEAN SQRS	F - VALUE
Ovary	Treatment	4	752.672	188.168	18.45*
	Error	25	254.969	10.199	
Muscle	Treatment	4	12.063	3.016	0.51**
	Error	25	147.703	5.908	
Hepatopancreas	Treatment	4	1801.242	450.311	7.25*
	Error	25	1553.828	62.153	

* Significant at 1% level ($P < 0.01$) ; ** Not Significant.

Table - 6 : Variations in the concentration of Carotenoid during the different maturity stages of *P. indicus*.

TISSUES		MATURITY STAGES				
		I	II	III	IV	V
Haemolymph (µg/ml)	N	6	6	6	6	6
	$\bar{X}$	10.44	15.99	28.54	26.65	19.70
	± SD	1.57	2.69	8.02	8.61	4.23
Ovary (µg/mg) wet weight	N	6	6	6	6	6
	$\bar{X}$	2.07	3.24	4.29	5.87	2.71
	± SD	0.19	0.46	0.45	0.48	0.41
Hepatopancreas (µg/mg) wet weight	N	6	6	6	6	6
	$\bar{X}$	14.88	27.68	63.86	47.16	45.47
	± SD	2.79	3.19	12.77	2.78	4.15

ANALYSIS OF VARIANCE - CAROTENOID

TISSUE	SOURCE	D.F.	SUM OF SQR	MEAN SQRS	F - VALUE
Haemolymph	Treatment	4	1345.332	336.333	10.36*
	Error	25	811.630	32.465	
Ovary	Treatment	4	53.340	13.335	78.48*
	Error	25	4.248	0.170	
Hepatopancreas	Treatment	4	8594.852	2148.713	45.03*
	Error	25	1193.051	47.722	

* Significant at 1% level ($P < 0.01$)

Table - 7 : Variations in the concentration of RNA during different maturity stages of *P. indicus*

TISSUES		MATURITY STAGES				
		I	II	III	IV	V
Ovary (µg/mg) wet weight	N	6	6	6	6	6
	$\bar{X}$	50.70	40.54	26.60	16.29	49.37
	± SD	5.82	4.98	4.50	3.71	4.48
Muscle (µg/mg) wet weight	N	6	6	6	6	6
	$\bar{X}$	5.27	3.22	3.84	4.01	4.16
	± SD	1.01	0.71	0.97	1.03	0.99
Hepatopancreas (µg/mg) wet weight	N	6	6	6	6	6
	$\bar{X}$	27.26	43.17	53.72	56.28	27.44
	± SD	3.06	5.04	7.40	7.11	3.88

ANALYSIS OF VARIANCE - RNA

TISSUE	SOURCE	D.F.	SUM OF SQRS	MEAN SQRS	F - VALUE
Ovary	Treatment	4	5340.949	1335.237	59.22*
	Error	25	563.695	22.548	
Muscle	Treatment	4	13.363	3.341	3.70**
	Error	25	22.594	0.904	
Hepatopancreas	Treatment	4	4626.785	1156.696	37.30*
	Error	25	775.305	31.012	

* Significant at 1% level ($P < 0.01$) ; ** Significant at 5% level ($P < 0.05$)

Table - 8 : Variations in the concentration of DNA during the different maturity stages of *P. indicus*.

TISSUES		MATURITY STAGES				
		I	II	III	IV	V
Ovary (µg/mg) wet weight	N	6	6	6	6	6
	$\bar{X}$	1.46	0.82	0.32	0.14	2.39
	± SD	0.28	0.13	0.05	0.05	0.45
Muscle (µg/mg) wet weight	N	6	6	6	6	6
	$\bar{X}$	0.42	0.29	0.25	0.24	0.29
	± SD	0.13	0.12	0.07	0.07	0.10
Hepatopancreas (mg/mg) Wet weight	N	6	6	6	6	6
	$\bar{X}$	0.08	0.15	0.14	0.20	0.35
	± SD	0.02	0.02	0.02	0.04	0.14

ANALYSIS OF VARIANCE - DNA

TISSUE	SOURCE	D.F.	SUM OF SQR	MEAN SQRS	F - VALUE
Ovary	Treatment	4	20.163	5.041	83.51*
	Error	25	1.509	0.060	
Muscle	Treatment	4	0.136	0.034	3.46*
	Error	25	0.245	0.010	
Hepatopancreas	Treatment	4	0.244	0.061	12.98*
	Error	25	0.117	0.005	

* Significant at 1% level ($P < 0.01$) ; ** Significant at 5% level ($P < 0.05$)

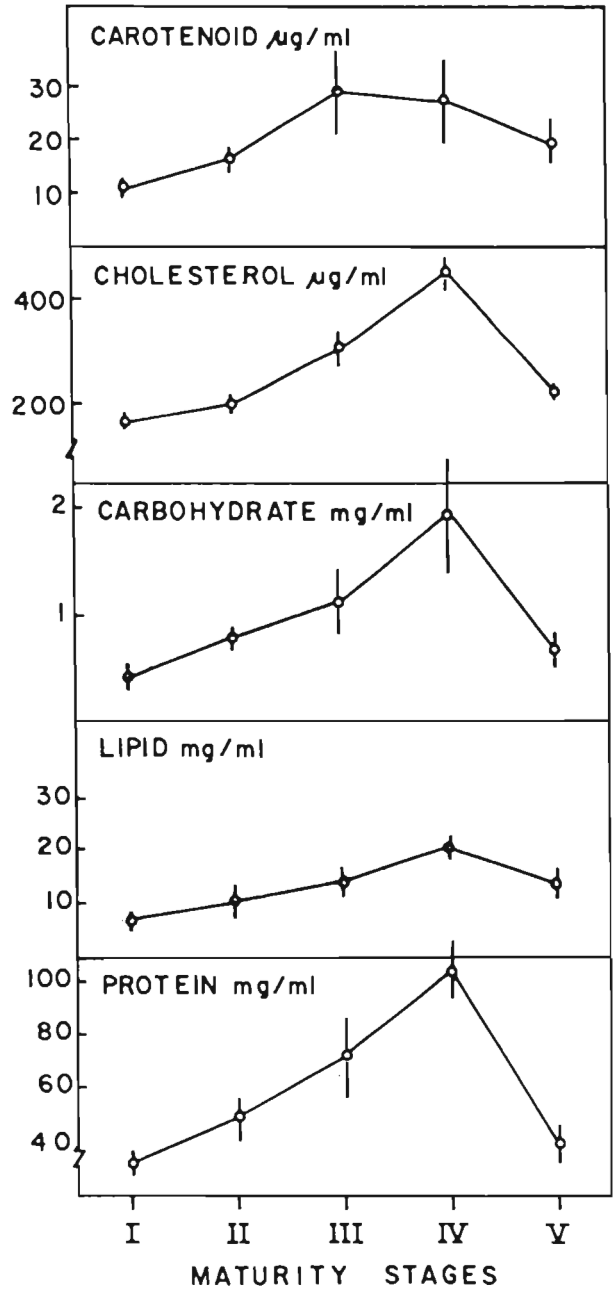

FIG. 1 Trends in the variation of biochemical parameters in the haemolymph during different maturity stages.

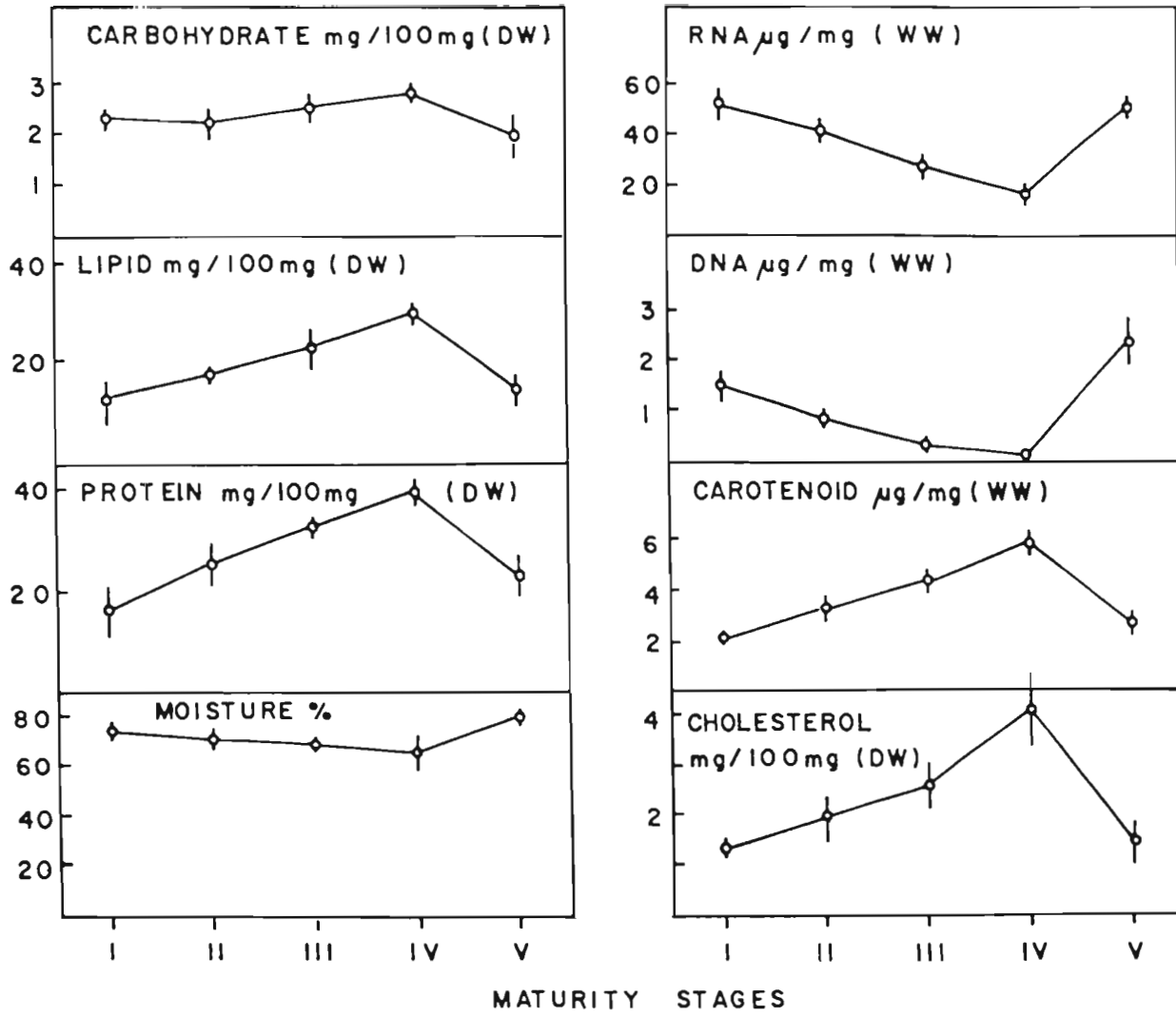

FIG. 2 Trends in the variation of biochemical parametres in the ovary during different maturity stages .

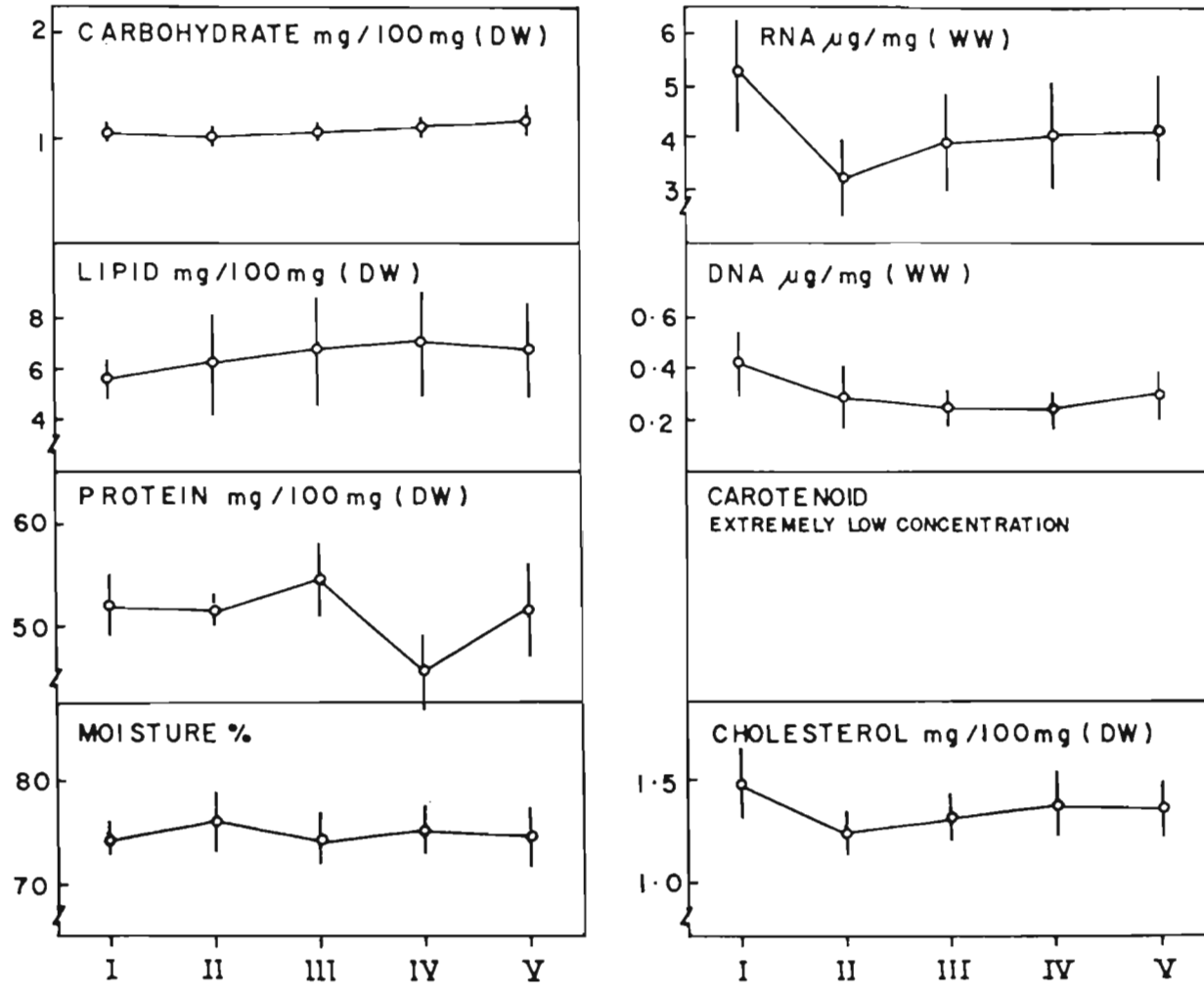

FIG. 3 : Trends in the variation of biochemical parametres in the muscle during different maturity stages.

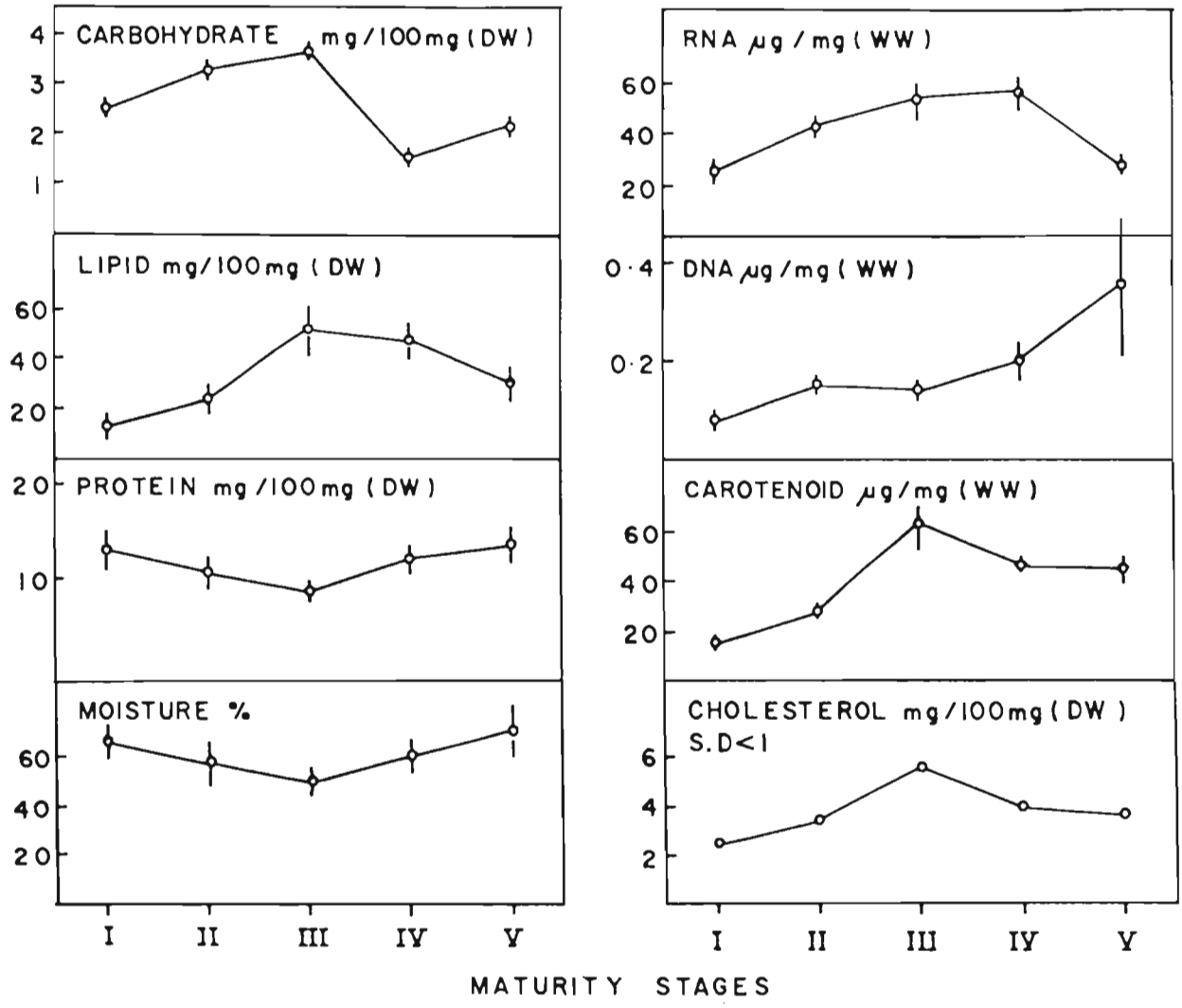

FIG. 4 : Trends in the variation of biochemical parametres in the hepatopancreas during different maturity stages.

observed (Fig. 2). In the muscle the DNA content fluctuated between 0.24 and 0.42 µg/mg without a definite pattern (Fig. 3). As shown in Fig. 4, in the hepatopancreas there was a gradual build up of DNA content from stage I, and the peak level of 0.35 µg/mg was observed in stage V.

ANOVA indicated that the variations in DNA content in the ovary and hepatopancreas with the different maturity stages were statistically significant at 1% level. The variations in the DNA content of the muscle was statistically significant at 5% level.

9. Composition of the mature yolk

Based on the analysis conducted, the biochemical composition of the mature yolk in P. indicus has been found and expressed in percentage as given below.

Metabolite	Percentage	
Moisture	65.41	wet weight
Protein	39.27	dry weight
Lipid	29.14	dry weight
Carbohydrate	2.80	dry weight
Cholesterol	4.14	dry weight

Carotenoid	0.587	wet weight
DNA	0.014	wet weight
RNA	1.629	wet weight

The major component of the yolk is found to be water and the next in order of dominance is protein and then lipid. The other metabolites are present only in small quantities.

DISCUSSION

The estimations of various biochemical parameters during the different maturity stages showed the cyclic variation and accumulation of organic reserves in the haemolymph, ovary and hepatopancreas of the prawn P. indicus. The biochemical composition of muscle tissues did not show any significant variation in relation to the maturation process. In general, these results are in agreement with that observed in other crustaceans by Rahman (1967), Adiyodi (1968), Pillay and Nair (1973), Diwan and Nagabhushanam (1974), Lawrence et al. (1979), Shyamsundari and Erribabu (1979) and Varadarajan and Subramoniam (1982).

In P. indicus during active phases of vitellogenesis there was a significant rise in the protein levels in the ovary as well as the haemolymph. Peak protein levels were observed in the fully mature stage. The hike in protein content of the haemolymph parallel to that observed in the ovary indicated probable plasmal transport of proteins from an external source to the ovary. In P. indicus the haemolymph protein levels varied from 31 mg/ml in the immature stage to 105 mg/ml in the mature stage. In other crustaceans, haemolymph protein concentration in general has been reported to range from as low as 28 mg/ml in Carcinus maenas (Gilles, 1977) to as high as 116 mg/ml in Panulirus longipes (Dall, 1974). In the American lobster H. americanus, Barlow and Ridgeway (1969) reported that the total serum protein was in peak levels during the mature stage due to the mobilization of proteins to the ovary. Electrophoretic studies on the haemolymph of some female crustaceans have revealed the presence of a lipoglycoprotein band, immunologically identical to the lipovitellin of the oocyte yolk (Adyodi, 1968; Kerr, 1969 and Wolin et al.,1973). Accumulation of proteins in the ovary of P. indicus is commensurate with the established role of proteins in tissue build-up during embryonic development. Similar accumulation of proteins in the ovary during the reproductive cycle has been noted in

cirripedes (Barnes et al., 1963), in P. pelagicus, M. affinis and Uca annulipes (Pillay and Nair, 1973), in B. cunicularis (Diwan and Nagabhushanam, 1974) and in the anomuran crab C. clibanarius (Varadarajan and Subramoniam, 1982).

The results obtained from the present investigation revealed that considerable mobilization of proteins takes place from the hepatopancreas. Although the content of protein in the hepatopancreas was observed to be very poor, there was a progressive loss in the protein levels in this organ during active vitellogenesis in P. indicus. But in the spent stage the protein content in the hepatopancreas was found to be restored to its original level. In contrast, in the hermit crab C. clibanarius, Varadarajan and Subramoniam (1982) observed only a slight fall in the hepatic protein content in the mature stage, which however was not statistically significant. Adiyodi (1969) reported that in P. hydrodromous the hepatopancreas was not the source of major vitellogenic proteins, but could well be the source for vitellogenic precursors. However in P. indicus, the present study indicated some degree of transport of hepatic proteins to the ovary through the haemolymph, and additional evidence to this statement comes from the observation of low initial concentration of protein found in the ovary during stage I

(16%). Conversely in C. clibanarius, Varadarajan and Subramoniam (1982) found high initial concentration of protein (22%) in the ovary obtained through autosynthetic means which therefore percludes the need for proteins from an external source. In P. indicus apparently autosynthetic proteins alone are not sufficient for the formation of yolk and therefore heterosynthetic proteins derived from the hepatopancreas are also probably incorporated into the yolk. Added support to this view is obtained from the results of the histochemical and ultrastructural studies conducted on the oocytes of P. indicus reported in chapter II. From the histochemical studies it was evident that some of the amino acids like cystine and cysteine were derived from sources external to the oocyte. Moreover the presence of pinocytotic vesicles along the oolemmal wall as observed in the present study indicated active transport of organic materials from outside into the oocytes.

Muscle protein content in the present investigation did show significant variation but without any definite trend in relation to the stages of maturation and therefore this could not be correlated to the process of vitellogenesis. It has been reported that muscle proteins are mainly involved in the process of tissue growth and metabolism in crustaceans (Claybrook, 1983). Therefore the irregular trend

observed in the muscle protein content might be due to its involement in growth and metabolism rather than in reproduction.

A doubling of the haemolymph glucose level was observed by Dean and Vernberg (1965) in Callinectes sapidus and by Telford (1968) in Cancer borealis as the crabs became ovigerous. In the haemolymph of P. indicus, the carbohydrate content increased fourfold during stage IV but decreased drastically in spawned prawns. Similarly in P. hardwickii, Nagabhushanam and Kulkarni (1980) reported an incessant hike in haemolymph glucose level as ovarian development proceeded from Stage I to IV and then a sudden decrease in the levels was observed in Stage V. Adiyodi and Adiyodi (1970 b) reported that in P. hydrodromous, the sugars present in the hepatopancreas and haemolymph were also found in some abundance in the ovary during early stages of vitellogenesis, but disappear progressively as the proteins in the ovary became conjugated in the course of yolk formation. It was suggested by Nagabhushanam et al. (1980) that the sugars present in the haemolymph and hepatopancreas may be utilized mainly for the construction of yolk proteins and perhaps also as a fuel during the active vitellogenic process. The increasing trend of haemolymph carbohydrate content observed in P. indicus with the advancement of maturity stages also

seemed to indicate transport of carbohydrate substances to the ovary for identical reasons.

In the ovary of P. indicus the carbohydrate content remained more or less static, with a small peak in the ripe stage. In C. clibanarius, Varadarajan and Subramoniam (1982) observed that the carbohydrate levels in the ovary diminished to very low levels during the ripe stage. They suggested the possibility of carbohydrate levels in the ovary being constant but the relative amount of other macromolecules reducing its percentage in later stages. In the hepatopancreas of ripe females a drastic decline in the carbohydrate content was observed in the present study, indicating the possible transport of carbohydrate substances from this storage organ to the ovary. Identical conclusions were drawn by Adiyodi and Adiyodi (1970 b) when they found that free sugars like glucose, galactose and sucrose in the hepatopancreas of P. hydrodromous underwent quantitative and qualitative cyclic fluctuation related to the ovarian cycle. In a recent study on the glucidic metabolism of Penaeus notalis, Trujillo and Luna (1981) also suggested that during ovarian development glycogen is mobilized from the hepatopancreas to the gonads.

In several crustacean species, the haemolymph lipid

exists as a complex lipoprotein moeity (Chang and O'Connor, 1983). In P. indicus, the changes in the levels of different biochemical parameters in the ovary and hepatopancreas were reflected in the haemolymph. Thus the haemolymph lipid levels rose to a peak in stage IV and subsequently decreased slightly in spawned prawns indicating transport of lipidic material to the ovary and its utilization in the process of maturation. Similar variations in haemolymph lipid content in relation to the reproductive cycle was observed by Teshima and Kanazawa (1978) in P. japonicus. In spent recovering P. indicus the lipid content remained at comparatively high levels due to the possible resorption of relict oocytes, which involves the retransport of nutrient material from the ovary back to the storage site. Although no comparable studies are available on the resorption of lipid material via the haemolymph, Adiyodi (1985) attributed the protein levels of the haemolymph of crustaceans during spent stages to be due to the proteins from resorbing oocytes. Again the high haemolymph lipid levels in spent P. indicus may be due to the rapid rematuration capabilities of this species reported by Mohamed et al. (1981). Additional support for this view comes from the comparatively high lipid levels present in the hepatopancreas of spent prawns.

In P. indicus an inverse relationship was observed between ovarian and hepatic lipid content. As the content of ovarian lipids increased, there was a concurrent decrease in the lipid levels in the hepatopancreas. The hepatopancreas has been indentified as the principal storage site for lipids in crustaceans (Chang and O'Connor, 1983). Apparently large amounts of this stored lipid is mobilized to the ovary in P. indicus during active vitellogenesis through the haemolymph. In the pink shrimp P. duorarum, Gehring (1974) followed the changes in total lipids, neutral lipids, phospholipids, triglycerides and sterols in the ovary with maturation and found peak levels in the late maturing stage. In P. japonicus, Guary et al. (1975) reported that the increasing trend of various fatty acids was related to ovarian maturation. Similar variations in the lipid composition during ovarian maturation was reported by Middleditch et al. (1980) in P. setiferus and Teshima and Kanazawa (1983) in P. japonicus. In a recent study on P. indicus, Galois (1984) observed large accumulation of phospholipids and triacylglycerols in the ovary during vitellogenesis, but discounted the role of hepatopancreas in supplying these to the ovary. He suggested that direct input of lipids from feeding to be the preponderant source. However, the trend in variation of hepatopancreatic lipid in conjunction with maturation observed in the present study seems to suggest

that the hepatopancreas is the major source of ovarian lipids. Similar conclusions were drawn by Gilbert and O'Connor (1970) in their review on lipid metabolism in arthropods and Varadarajan and Subramoniam (1982) in the anomuran C. clibanarius.

Cholesterol has been demonstrated to be the dominant sterol in the ovary of penaeids (Middleditch et al.,1980). The haemolymph and ovary of P. indicus showed considerable accumulation of cholesterol during vitellogenesis. In contrast there was a concomitant decrease in cholesterol levels in the hepatopancreas indicating the transport of cholesterol from the hepatopancreas to the ovary via the haemolymph. Crustaceans lack the ability to synthesize cholesterol although these sterols are necessary to suppress the permeability of phospholipid membranes to water, glucose and proteins (Morris et. al., 1981). Therefore the cholesterol in the present study is apparently derived solely from the diet. Thus, Middleditch et al. (1980), could induce ovarian maturation and spawning in penaeids by supplementing the diet with lipids cointaining cholesterol. However Adiyodi and Adiyodi (1971) were unable to detect cholesterol in the ovary of P. hydrodromous using chromatographic techniques.

The deepening colouration of the ovary of P. indicus with vitellogenesis was found to be due to the increasing presence of carotenoid pigments. The fluctuation in the carotenoid content in the haemolymph observed during vitellogenesis in the present study is apparently owing to the transfer of pigments from the hepatopancreas to the ovary. The hepatopancreas plays a major role in the absorption of carotenoids from food and has been observed to show fluctuation during vitellogenesis (Castillo et al., 1982). In C. maenas, Ceccaldi and Martin (1969) showed that carotenoid pigments are concentrated from the hepatopancreas into the haemolymph during vitellogenesis. In the ovary of P. indicus the steady build-up of carotenoids is apparently used for the formation of the glycolipocaroteno-protein yolk. Simultaneous increase of weight and carotenoid content of the ovary was observed during vitellogenesis in the shrimp Plesiopenaeus edwardsianus (Establier, 1966). The dark green colouration of the ripe ovary in P. indicus is apparently due to the high content of astaxanthin pigment, since the green or blue complexes are often found to contain astaxanthin as the prosthetic group (Tanaka et al.,1976; Castillo et al.,1982).The precise biological function of carotenoids in crustaceans is still vague (Goodwin, 1984). Paradoxically, carotenoids appeared unutilized during the

development of the eggs of the sand crab Emerita analoga (Gilchrist and Lee, 1972). Among the various functions attributed to carotenoids are, as a cross-link between lipid and protein to stabilize the lipoprotein molecule, to protect the protein from catalytic action of the enzymes and to protect the eggs from high illumination and solar radiation (Castillo et al., 1982).

Water was observed to be the principal component in the ripe ovary of P. indicus. Both in the ovary and hepatopancreas the water content showed a declining trend during vitellogenesis. In an earlier study on P. indicus from South African Coast, Read and Caulton (1980) observed a decrease in fresh mass, despite the increase in ovarian mass due to a loss of water during ovarian maturation. George and Patel (1956) observed in the case of the Bombay lobster an inverse relationship between water and fat content of the gonad, and this increase in fat content was evidently associated with gonadal development. In M. affinis and P. pelagicus, Pillay and Nair (1973) also observed an inverse relationship between water content and gonad development. In general the water content in the ovary tended to decline along with an increase in total mass of organic substances in the ovary. Considerable variation in water content takes place during the moult cycle in crustaceans and control of water in

tissues has been indicated as a possible mechanism by means of which the animal achieves tissue growth (Yamaoka and Scheer, 1970). Similarly it is probable that water is lost in ovarian and hepatic tissues due to the continued deposition of organic materials resulting in an increase in dry weight of the body.

Despite their importance to crustacean biology, nucleic acids have been investigated to only a limited extent (Claybrook, 1983) and in relation to reproduction they are yet to be studied in detail. In P. indicus, the RNA content in the ovary showed a declining trend during vitellogenesis and in spent-recovering stage the RNA level was restored to its original level. During pre- and early vitellogenic phases of the oocytes the RNA level was consistently high due to the autosynthetic manner of protein synthesis taking place within them. The content of RNA in crustacean tissues are known to be consistently high corresponding to the period of maximum protein synthesis during the early growth phase of the animals (Skinner, 1968; Buckley, 1984). The RNA level was found to be drastically reduced in the present study during late vitellogenic phases when heterosynthesis is predominant. Unfortunately, there are no reports of such studies among other crustaceans. In a study on the

influence of light on crab (Ocypoda sp.) reproduction, Nadarajalingam and Subramoniam (1987) reported that RNA reduction in the ovary coincided with oocyte resorption and elevated RNA and DNA levels coupled with increased protein content coincided with oogonial proliferation. However in the purple sea urchin Strongylocentrotus purpuratus, Giese et al. (1959) reported a steady increase in the RNA content of the gonad due to the constant protein synthetic activity in the oocytes during vitellogenesis. In the hepatopancreas of P. indicus, the RNA levels steadily increased during maturation and then declined in the spent stage. Since hepatopancreas is the major synthetic and storage organ in crustaceans, the high RNA content observed is probably due to the high metabolic rates prevailing in this organ.

The DNA concentration in the ovary of P. indicus showed a decreasing trend during vitellogenesis but spawned prawns showed a sudden spurt in DNA content. Normally the DNA content of an undividing cell is not expected to change and remains static (Bulow, 1970) and therefore it is difficult to explain the decline in DNA values. Since oocytes increase in size during vitellogenesis, each sample of ovary taken at different stages of maturity for analysis of DNA, would contain less and less number of cells. This probably explains the progressive decrease in DNA content. Identical

studies were made by Giese et al. (1959) in the purple sea urchin S. purpuratus, where they observed a steady decrease in the DNA content with advance in maturity. In spent prawns, the ovaries undergo quick recovery with mitotic multiplication of oogonial cells and this explains the remarkable increase in DNA content during this stage. Stevenson (1985) attributed the high DNA levels in late premoult and post moult of crustaceans to the cell division occurring in connection with the rebuilding of the cuticle. In the hepatopancreas the DNA levels were observed to steadily increase from stage I to V in the present study due to the constant cellular synthetic activity taking place in them.

Fluctuations in muscle RNA and DNA content observed in the present study were statistically significant, but the trends could not be related to the process of vitellogenesis. This observation is consistent with that reported by Lawrence et al. (1979) in P. setiferus where components of the muscle are not directly mobilized for penaeid reproduction.

In crustaceans, yolk is the nutritive material accumulated in substantial quantities in the ooplasm to meet the basic requirements of embryonic development and moreover

the composition of yolk varies from species to species (Adiyodi and Subramoniam, 1983). The biochemical studies on the mature ovary of P. indicus revealed that water formed the most significant portion of the yolk and proteins and lipids constituted the major organic reserves. It is generally accepted that yolk proteins provide the basic structural material needed for tissue build up during embryonic development, while lipids serve as the major fuel (Adiyodi and Subramoniam, 1983).

Evidences derived from the present study indicated that a significant portion of the proteins and carbohydrates and almost all of the lipids, cholesterol and carotenoids found in the yolk of P. indicus are probably derived from the hepatopancreas via the haemolymph through heterosynthesis. However, during the initial stages in the maturation of ovary some amount of autosynthesis apparently takes place as indicated by the presence of large amounts of RNA which is known to be derived from the nucleolii. The biosynthesis of yolk is seemingly carried out through dual processes i.e, autosynthesis and heterosynthesis and the latter appeared to be the predominant mode. Although such a study has been conducted for the first time among penaeids, a similar bias in the synthesis of yolk has been reported in the crab P. hydrodromous (Adiyodi, 1969), Libnia emarginata (Hinsch and

Cone, 1969), Uca pugilator and Procambarus clarkii (Wolin et al., 1973) and in the amphipod Orchestia gammarella (Zerbib, 1976).

S U M M A R Y

1. The metabolic components like protein, carbohydrate, lipid, cholesterol, moisture, carotenoid, RNA and DNA in the haemolymph, ovary, muscle and hepatopancreas of female P.indicus during the five maturity stages were estimated.

2. Significant variations in the biochemical parameters in relation to the maturity stages were observed in the haemolymph, ovary and hepatopancreas. The biochemical composition of muscle tissues did not show any significant variation in relation to the maturation process.

3. In P. indicus, the changes in levels of different biochemical parameters in the ovary and hepatopancreas were always reflected in the haemolymph where the content of proteins, carbohydrates, lipids, cholesterol and carotenoids increased steadily from very low levels prevailing in Stage I to peak levels in Stage IV. In spent recovering prawns (Stage V) there was a significant drop in the levels of all

these parameters.

4. The content of protein, carbohydrate, lipid, cholesterol and carotenoids in the ovary gradually increased from Stage I to maximum levels in fully ripe prawns (Stage IV). A significant decrease in the levels of all these biochemical components was observed in the ovaries of spent recovering prawns. In contrast, the levels of moisture, RNA and DNA were observed to decrease from an initial high concentration in Stage I to very low concentration in stage IV. Subsequently, the levels of all these parameters registered an increase in the Stage V. Maximum moisture and DNA content was observed in the ovaries of spent recovering prawns.

5. The carbohydrate, lipid, cholesterol and carotenoid contents in the hepatopancreas, gradually increased from low levels in Stage I to maximum levels in Stage III and thereafter there was a gradual decrease. Maximum content of hepatic proteins was observed in Stage I and V and minimum in Stage III. The moisture content in the hepatopancreas also showed a similar trend. Peak RNA values were observed in Stage IV after which there was a sharp fall in the value. The content of DNA increased steadily from Stage I to peak in Stage V.

6. The content of proteins, carbohydrates, lipids, cholesterol, moisture, RNA and DNA in the muscle tissues were found to vary during the different maturity stages without a definite pattern or trend.

7. Moisture was found to be the principal component of the fully formed yolk in P. indicus. Proteins and lipids found the next major organic compounds, while the other metabolites were found only in small quantities .

CHAPTER V

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The reproductive endocrinology of the penaeid prawn P. indicus has been investigated by adopting a comprehensive approach to the problem. The major aspects of the study included the indepth investigations on the process of oogenesis, spermatogenesis, spermatophore formation, neuro-endocrine control of reproduction and the variation in bio-chemical components in relation to ovarian maturation.

The prawns used in the present investigation were collected from the sea off Cochin or from the perennial prawn farms in Vypeen, Cochin. The studies were carried out using modern histological, histochemical, electron micro-scopic and biochemical methods. The salient findings of the present study are listed below.

The morphological features of both male and female reproductive system of P. indicus has been described. The maturation of the ovary was found to be accompanied by distinct colour changes as well as an increase in GSI and diameter of the oocytes and nuclei. Based on these characters five different maturity stages (I to V) were identified. Again based on the manner in which oocytes accumulated yolk and the concurrent changes in the ooplasm

and cytoplasmic organelles, five vitellogenic phases are described.

Histochemical studies revealed the yolk to be a glyco-lipoprotein complex which was deposited sequentially in the ooplasm. Proteins were the first to be associated with the yolk complex followed by carbohydrates and finally lipids. Among proteins, tryptophanyl, aromatic and amino end groups were the first to be formed. Thiol-disulphide end groups were incorporated into the yolk platelets from external sources. The cortical bodies which finally forms the jelly layer of the spawned eggs were found to be rich in sulphur containing amino acids (cystine and cysteine) and sulphated and carboxylated AMP. Their probable function in controlling the permeability of water, protection against bacterial attack and aiding in cytokinesis in the eggs of penaeids is discussed.

Electron microscopic studies revealed the presence of nuclear pores through which RNA rich nucleolar material is extruded into the cytoplasm, and mitochondria and rough endoplasmic reticulum during pre and early vitellogenic phases. The presence of these organelles indicated the auto-synthetic capability of the ovary during the initial phases. During the late vitellogenic stage the oocytes were

decidedly heterosynthetic as evidenced by the presence of micropinocytotic vesicles along the oolemmal wall and the absence of cellular synthetic machinery. Folliculogenesis was observed to be accomplished during the early stages of vitellogenesis. Ultrastructural studies showed that follicle cells had considerable protein synthetic ability and histochemical evidences were present for the follicle cells being a major source for the carbohydrate moeity of the yolk.

No specific maturity stages were ascribed to the process of spermatogenesis, which was found to include spermatogonia, spermatocyte, spermatid and spermatozoa stages. The sperm in P.indicus was found to be of the altered vesicular type with a spherical main body that was partially encompassed by a morphologically diverse cap region containing the acrosomal complex, from which extended a single spike. Histochemical studies revealed that the spermatozoa had a remarkable abundance of glycogen and basic proteins like arginine and lysine which are persumably used for the transmoult maintenance of the spermatozoa in the thelycum of the female.

The spermatophores were observed to be formed in the vas deferens with the aid of the glandular epithelial cells lining the duct. The electro-ejaculated spermatophore was

found to be oval in shape with a parachute like wing at the apical end of the body. Sperms were observed to be packed inside the spermatophores in lobules and were encompassed by two structurally diverse layers composed mainly of acid mucopolysaccharides.

The different neuroendocrine centres were identified on the basis of the presence of neurosecretory cells in the optic, supraesophageal, tritocerebral, subesophageal, thoracic and abdominal ganglia. Based on cytomorphological differences 4 different types of NSCs were recognized viz, giant neuron (GN), A-cells, B-cells and C-cells. All the four types of NSCs were observed to undergo cyclic changes in their perikarya in relation to the synthesis of neurosecretory material. On the basis of the appearance of secretory granules, vacuolization and involvement of extracellular glial cells and capillary plexus, three phases were identified viz., quiescent (Q) vacuolar (V) and secretory (S).

In the eyestalk, 3 NSC groups, viz., the MTGXO 1 and 2 and MEGXO were identified. The neurohaemal organ, sinus gland, was found between the medulla externa and interna lobes. Totally 11 NSC groups were recognized on the dorsal and ventral sides of the supraesophageal ganglion, 1 NSC group in the tritocerebral ganglion, 15 NSC groups in the

subesophageal ganglion and 4 NSC groups each in the thoracic and abdominal ganglia. The distribution of the different NSC types in these groups was worked out.

The neurosecretory material was found to be constituted mainly by a protein rich in cystine and the carbohydrate content observed was mainly glycogen. Electron microscopic studies revealed that the neurosecretory material was composed of haloed dense core granules which measured 1400 to 1600 A in diameter.

In the eyestalks of immature females more than 75% of the NSCs in the MTGXO and MEGXO were in the physiologically active V and S phases. In mature females the majority of the NSCs were in a suppressed state (Q phase). In contrast in the supraesophageal, subesophageal and thoracic ganglia of immature females almost 65% the NSCs were in the Q phase while in fully mature females, active NSCs dominated. Histochemical studies showed that significant fluctuation in the cystine content took place in the eyestalks, indicating that A and B-cells were the most active cells types in relation to reproduction, whereas in the brain and thoracic ganglia, GN, A and B-cells were the most active in relation to gonadal maturation. The pyriform C-cells did not appear to be involved in the process of maturation.

Both unilateral and bilateral eyestalk ablation significantly enhanced the GSI and ova diameter leading to precocious maturation of the ovary and this process was blocked in ablated prawns injected with an extract of the eyestalk, thus confirming the inhibitory nature of the neurosecretory principles contained in the eyestalk. Extracts of the central nervous system stimulated significant enhancement in the GSI and oocyte diameter although full gonadal maturation was not obtained.

A temporal relationship was found to exist between gonadal maturation and moult cycle. Gonadal maturation almost always occurred during early premoult period of the moult cycle. However, this pattern was altered in bilaterally ablated females.

The various biochemical components like protein, carbohydrate, lipid, cholesterol, moisture, carotenoids, RNA and DNA were found to show significant variation and accumulation in the haemolymph, ovary and hepatopancreas during the different maturity stages. The biochemical composition of muscle tissues did not show any significant variation in relation to the maturation process. In general the ovary showed accumulation of protein, carbohydrate, lipid, cholesterol and carotenoids and loss of moisture, RNA

and DNA during vitellogenesis. High RNA content during the early stages of vitellogenesis indicated an autosynthetic mode of yolk synthesis. However, in the later stages significant amount of protein, lipid, carbohydrate, cholesterol and carotenoids were found to be mobilized from the hepatopancreas to the ovary through the haemolymph. The yolk in P. indicus was found to be composed predominantly of water. Protein, lipid, carbohydrates and cholesterol were the major organic reserves. Carotenoids , DNA and RNA were found only in small quantities.

In general, the information generated from the present study clearly establishes the neurosecretory control of reproduction in the penaeid prawn P. indicus. Moreover, the precise cellular factors controlling reproduction has been identified and their roles established, and the neurosecretory mechanism involved in the technique of eyestalk ablation for induced maturation has been found out. The observation that the ovary in P.indicus had only limited autosynthetic capabilities indicated that brood-stock nutrition has to play an even more important role in the effort to achieve earlier induced maturation in penaeid prawns. The basic knowledge on the structure of the male gamete generated from this study should help in the future

research on artificial insemination of these prawns. The findings obtained from the present investigation should go a long way in streamlining the hatchery production of prawn seed using endocrine manipulations. It should also pave the way for future research on the endocrine hormones in penaeids using modern separation techniques.

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