CHAPTER 1

A HISTOLOGICAL AND HISTOCHEMICAL STUDY OF THE THYROID-LIKE FOLLICLES IN THE VARIOUS TISSUES OF THE FRESHWATER CRAB, PARATELPHUSA JACQUEMONTII

Extensive studies have been carried out by several workers on the histology of the various tissues of numerous species of decapod crustaceans. Among the different tissues of the crabs, the hepatopancreas, gonads, and the central nervous system have received much attention. Jacobs (1928), and Hirsch and Jacobs (1929, 1930) have thoroughly investigated the process of secretion and restitution in the mid-gut glands or the hepatopancreas of Potamobius (Astacus) leptodactylus. The digestive glands in addition to their functions of digestion and absorption could also perform excretion, elimination and regulation (Cuenot, 1893). Reddy (1938, 1939) described the basic histologic structure of the hepatopancreas of the freshwater crab, Paratelphusa hydrodromous and suggested the possible roles of different types of cells in the cytology and physiology of digestion and absorption. The various cell types of the mid-gut gland (hepatopancreas) of Atya spinipes (New port) was best studied by Van Weel (1955) in relation to secretion, restitution and resorption whereas Andrew (1959) reviewed its histologic pattern in the blue crab, Callinectes sapidus.

Among the other tissues, Spaulding (1942) investigated the structure of the male reproductive system of Carcinus with special reference to the region of formation of the spermatophores and the intromittant organ. Cronin (1947) has described a number of histological details of the male reproductive system of the commercially important blue crab Callinectes sapidus. According to him the vas deferens is a complicated structure divisible structurally and functionally into three parts. Benjamin and Hobbs in 1958, presented a histological description of the testis and its annual cyclic pattern of activity in the male crayfish Cambarus monatanus-acuminatus (Faxon). Various light and electron microscopic studies have been carried out on the spermatogenesis in various decapod crustaceans (Nath, 1956; Yasuzumi, 1960; Moses, 1960). Ultrastructural studies on the sperm morphology and sperm-egg interaction in the decapod, Callinectes sapidus were made by George Brown (1966). Information on the histological structure of the female gonads in decapoda is very scanty. Huxley (1880) described the ovary of the crayfish, while, the various mechanisms regulating the female reproductive cycle in the crayfish Cambarus was studied by Stephens (1952). In the freshwater crab Paratelphusa hydrodromous ovarian maturation and the ovigerous state has been found to coincide with the secretory activity of the thoracic ganglion (Parameswaran, 1955).

A number of investigators have demonstrated histologically the presence of the neurosecretory cells in the eye-stalk ganglion, brain and thoracic ganglion of various species of crabs (Enami, 1951; Bliss et al., 1952;Matusumoto, 1954; Potter, 1954; Parameswaran, 1955;1956; Hodge and Chapman, 1958; Nirmal, 1964; Desmukh and Rangnekar, 1965). In the central nervous system of the freshwater crab Paratelphusa jacquemontii five different types of neurosecretory cells were demonstrated by Nirmal (1964).

However, in all these studies, using the various tissues as mentioned above investigators concentrated chiefly on the most important structures such as the tubules of the hepatopancreas and testis, and on the neurosecretory as well as the storing centres of the central nervous system. But another important structure, the connective tissue which serves as a connecting and filling tissue, lying beneath the epithelial layers of the tubular organs and between the masses of the secretory cells in the central nervous system, received very little attention. This fibrous tissue mainly consists of three components; cells, fibres, and matrix. In the present study certain follicles containing dense acidophilic colloid were observed in the connective tissue of the various tissues studied in the freshwater crab Paratelphusa jacquemontii. The colloid present in these follicles were comparable to the thyroidal colloid of higher animals in the various histological and histochemical staining reactions.

MATERIALS AND METHODS

The common freshwater crabs, Paratelphusa jacquemontii, collected from the local rivers were utilized in the present investigation. Animals of both the sexes were used and the various tissues such as the hepatopancreas, gonads, parts of the central nervous system and muscle were dissected out and fixed in different fixatives such as aqueous Bouin's fluid, 10% Formalin , Zenker's fluid and Formol saline for histological observations. Of all the fixatives used aqueous Bouin's fluid was found to give the best results. The tissues were embedded in paraffin after the usual procedures of dehydration and clearing. Serial sections of 6µ thickness were cut and stained with Ehrlich's haematoxylin -Eosin, Masson's trichrome, Mallory's triple and Heidenhain's Azan stains.

Protein was demonstrated in Bouin's fixed sections by employing the Millon's reaction as modified by Baker (Pearse, 1960). The histochemical tests which were selected with special reference to the known properties of the thyroidal colloid included the Periodic Acid-Schiff (PAS) procedure and the Ferric ferricyanide test (Pearse, 1960). In the PAS procedure sections treated with saliva served as controls.

The effects of antithyroid compounds such as thiouracil and thiourea, which decrease the thyroxine levels in vertebrates were also studied. In certain cases the effect produced by these compounds is similar to those due to thyroidectomy.

The crabs were administered a total dose of 2 to 3 ml of a 2% solution of thiourea (0.2 ml/day/injection) for about a week. The tissues were excised, fixed in Bouin's and 10% Formalin for the histological studies. The control animals received the same quantity of glass-distilled water and were treated in an identical manner as the samples.

Eye-stalk extirpation was also carried out in order to see its effect on the follicles and the colloid. After the removal of the eye-stalk the animals were maintained for one to two weeks and then utilized.

Radioautographic experiments using labelled iodine (I^{131}) was also carried out to determine whether there was any incorporation of the labelled iodine into the colloid of these follicles (Chapter 2).

OBSERVATIONS

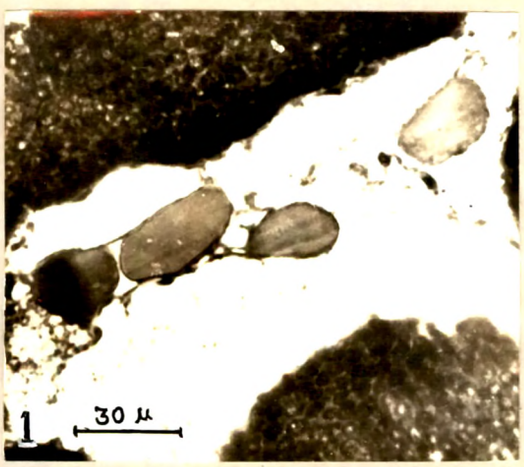
A number of follicles containing appreciable amounts of colloid were observed in the various tissues except muscle of both the sexes studied. These follicles, large and small, single or in groups, simple or sacculated, contained dense colloidal material which appeared somewhat similar to the colloid in the thyroid follicles of higher animals. They were widely distributed, being situated in the intertubular connective tissue of the hepatopancreas, in the interstitium present in

between the seminiferous tubules of the testis, in the lumen of the anterior portion of the vas deferens, in the ovarian stroma, and in the peripheral connective tissue of parts of the central nervous system viz: the eye-stalk ganglion, brain and thoracic ganglion (Figs. 1 to 6). The muscles on the other hand, were devoid of these follicles. In all the tissues of the male crabs these follicles were more in number when compared with those of the female, while in the immature ovary there were only very few follicles. An increase in the number and size of the follicles was noted in periods just prior to spermatogenesis and aestivation. The diameter and shape of these follicles was subject to wide variation even in the same animal, the large follicles occurring with increased frequency in mature ones. In the young animals the diameter ranged from 3 to 15μ whereas in the mature ones it ranged from 8.5 to 36μ. The shape was mostly sperical but in certain cases it was ovate. These follicles were covered by a thin limiting membrane with the acidophilic colloid inside. There were numerous thickenings on this membrane representing the spherical or flattened nuclei.

From the histological observations it was noticed that the stainable material (colloid) was coloured pink with Haematoxylin - Eosin, green with Masson's trichrome (Fig. 7) and bright red with Mallory's triple stain. With Azan stain it showed a bluish tinge in certain cases i.e. before the onset of spermatogenesis and aestivation period, whereas it was Orange G positive in most others. Only a few follicles showed an affinity towards

Aniline blue. With Baker's modified Millon's reaction the colloid stained deep pink in colour showing the presence of protein, probably containing tyrosine (Fig. 8). The colloid was also strongly PAS positive (Fig. 9). The saliva treated control sections and the untreated sample sections showed the same staining intensity and pattern. The colloid gave a clear blue colour with Ferric ferricyanide and followed the same staining pattern as revealed by the PAS procedure.

Animals treated with thiourea showed a considerable decrease in the number of follicles and in certain cases the follicles were totally wanting. The staining intensity of the colloid of the few follicles which were present in the treated animals was considerably less when compared with the normal follicles of the control animals. In general, after the thiourea treatment the whole tissue appeared to be shrunken and revealed atrophic and degneative changes. The entire ovarian tissue was seen to be a disorganized mass of homogenous yolk material after the thiourea injection. The anterior portion of the vas deferens where an appreciable number of follicles were present, showed remarkable changes after the treatment. Most of the follicles seemed to be empty showing the complete removal of the colloid. Some of them showed a gradual decrease in the amount of the colloid whereas in others only traces of the colloid was observed (Figs. 10 & 11). The control animals revealed neither atrophic and degenerative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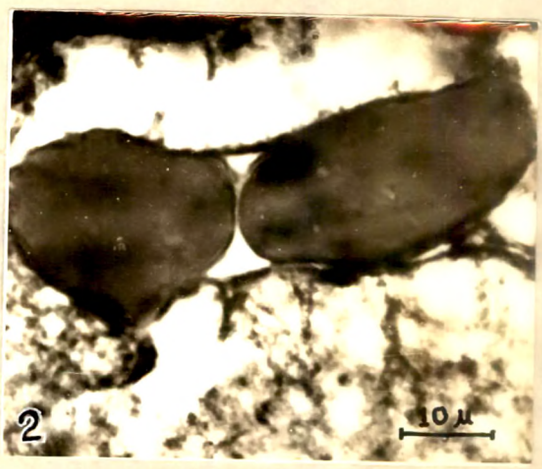
30 μ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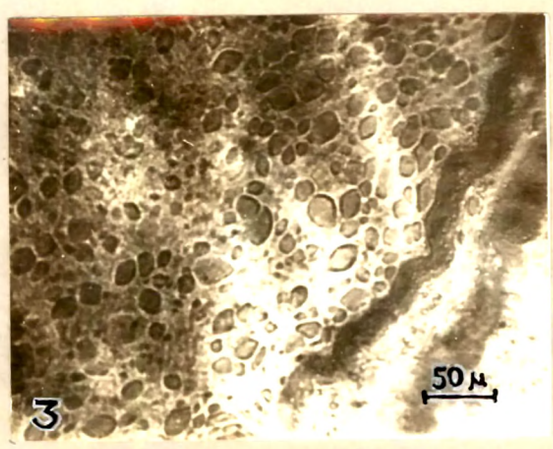
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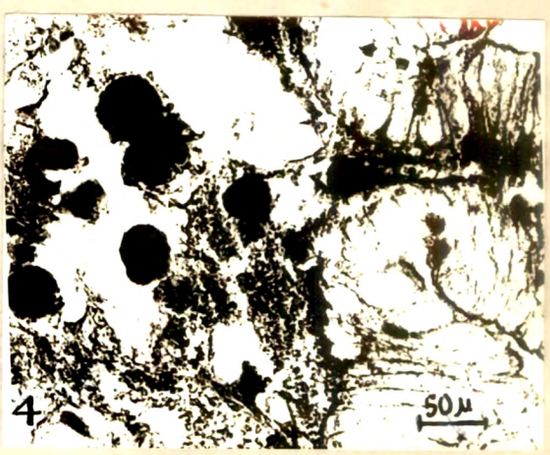
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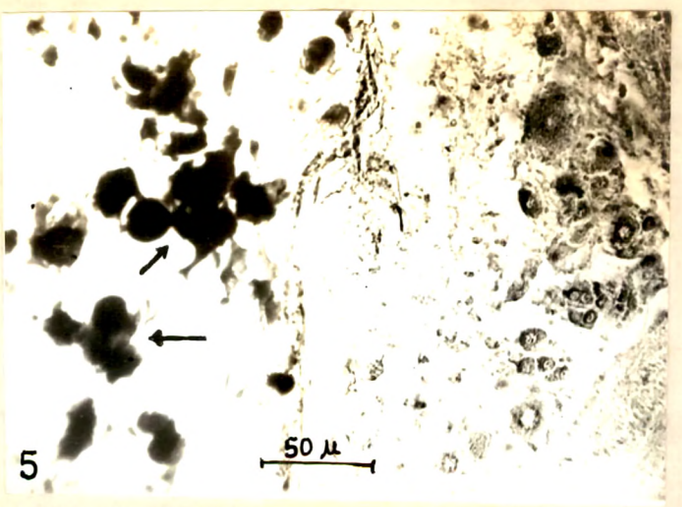
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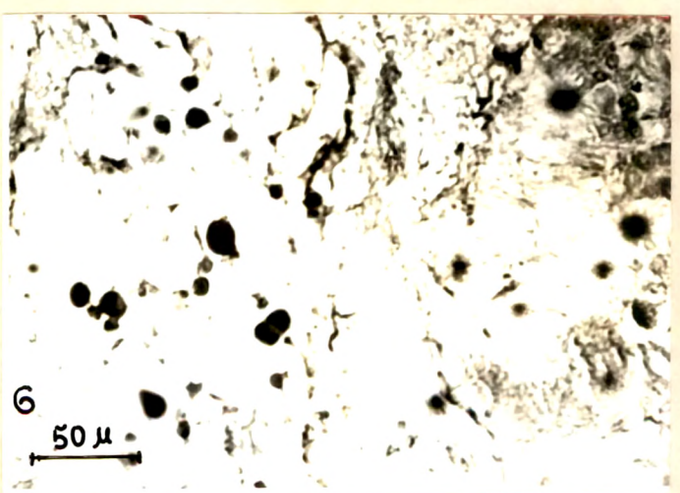
6
50 μ


7
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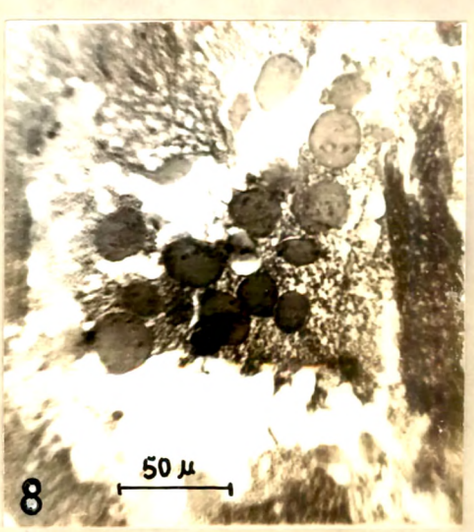
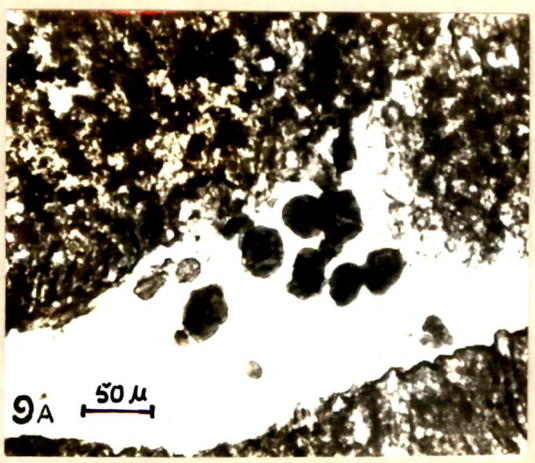
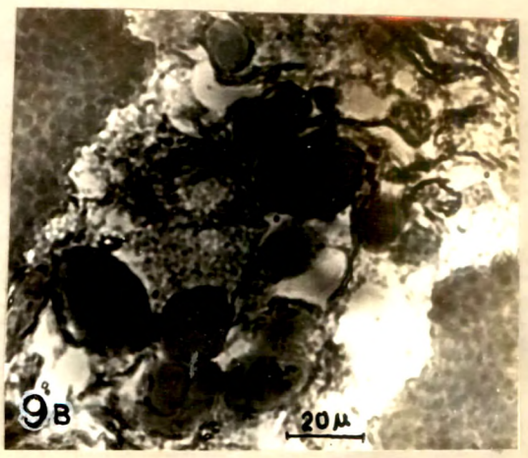

8
50 μ


9A
50 μ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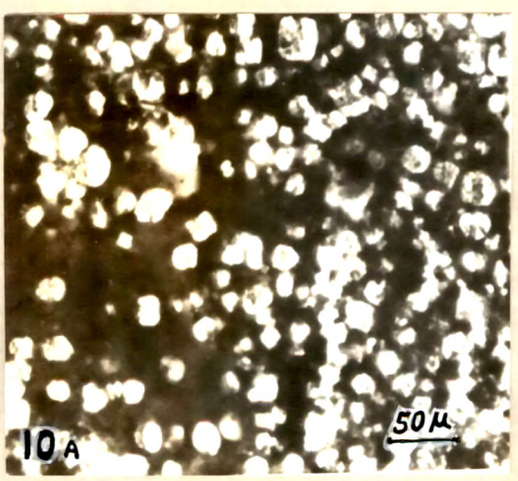
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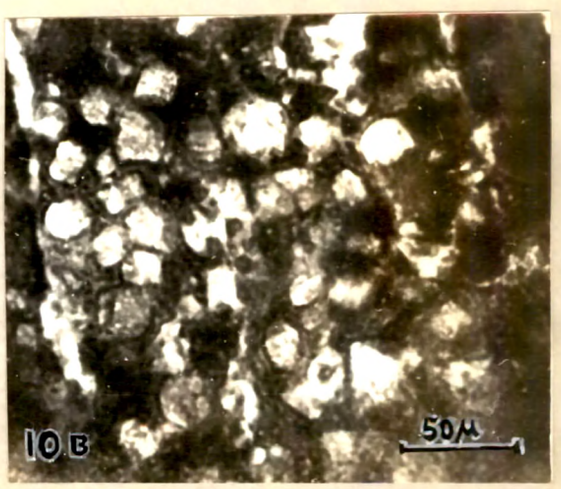
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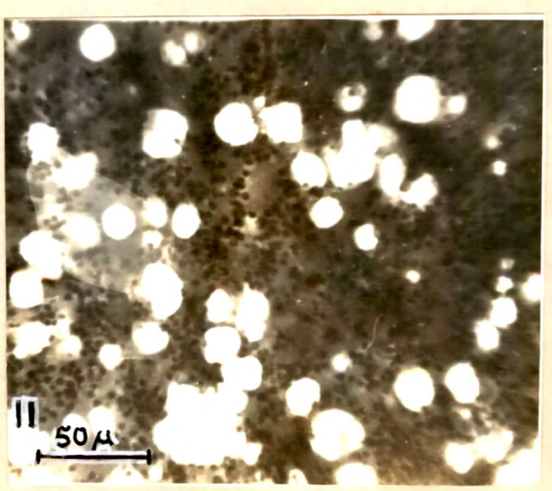
11
50 μ

EXPLANATION TO THE FIGURES

Fig. 1. Photomicrograph of the thyroid-like follicles in the interstitial connective tissue of the testis. Haematoxyline-eosine stain.

Fig. 2. Higher magnification of the follicles.

Fig. 3. Follicles in the lumen of the vas deferens. Haematoxyline-eosine stain.

Fig. 4. Follicles in the inter tubular connective tissue of the hepatopancreas. Mallory's tripple stain.

Fig. 5. Follicles in the peripheral connective tissue of the brain. Note the neurosecretory cells on the right side of the photograph and follicles on the opposite side. Aldehyde-fuschin stain.

Fig. 6. Follicles in the peripheral connective tissue of the thoracic ganglion. Aldehyde-fuschin stain.

Fig. 7. Follicles in the interstitium of the testis stained with light green of the Masson's trichrome stain.

Fig. 8. Follicles in the interstitium of the testis showing the presence of tyrosine. Millon's reaction.

Fig. 9A. Follicles in the intertubular connective tissue of the hepatopancreas treated with Periodic Acid-Schiff's reagent.

Fig. 9B. Follicles in the interstitium of the testis treated with PAS reagent.

Fig.10A. Effect of thiourea on the follicles in the lumen of vas deferens. Haematoxyline-eosine stain.

Fig.10B. Fig. 10A magnified. Note the irregular shape of the follicles and the considerable decrease of the colloid content.

Fig.11. Effect of thiourea on the follicles. Note the complete removal of the colloid from the follicles after continuous treatment with the drug.

changes of the tissues nor any histological alterations of the follicles. It is known that the various physiological activities are controlled by the neurosecretory centres of the eye-stalk ganglion, but in the present investigation after the removal of the eye-stalk ganglion no far reaching histological changes in the nature of the follicles or the colloid were seen.

Radioautographic experiments showed incorporation of labelled iodine (I^{131}) in the follicles. Quantitative measurements of the radioactivity supported the results of the radioautographs. According to the radioactive assay, remarkably high radioactivity was obtained in those tissues wherein the follicles were abundant and an almost negative or negligible activity in the muscle which was taken as the control. A detailed account of these observations are presented in chapter 2.

DISCUSSION

The occurrence of follicles such as described here in the connective tissue of the various organs of the freshwater crab, Paratelphusa jacquemontii, seems to be quite an unusual feature for the invertebrates. The ciliated groove called the endostyle of the Protochordate, Amphioxus shares some functional properties with the thyroid (Barrington, 1958). In adults of the primitive vertebrates, like the Cyclostomes the thyroid follicles are embedded in the dense fibrous tissue

along almost the full length of the pharyngeal floor (Marine, 1913;Fontaine, et al., 1952). But the basic histological unit of this thyroid, the follicles, resemble quite closely the equivalent structures in higher vertebrates (Eggert, 1938). The epithelial cells of the endostyle of the Ammocoetes larvae are also characterized as possessing throid like properties based on the localization of bound iodine by radioautography and cytological staining behaviour (Barrington and Franchi, 1956). Barrington and Franchi (1956) have shown the binding of iodine to be correlated with the production of a PAS-positive "thyroidal secretion", which showed a positive Ferric ferricyanide response together with some affinity for Orange G of the Azan stain. These properties gave considerable support to the view that the secretory process involved here is homologous to that which produces thyroidal colloid in higher forms. However, in the present investigation also similar results were obtained. The colloid was strongly positive to PAS reaction in both control and sample sections. Leblond, et al. (1957) consider that once glycogen has been removed, only one class of material could give a positive PAS reaction. These are referred to as carbohydrate - protein complexes, and they include the neutral mucopolysaccharides (in part) and the muco- and glycoproteins. It is also well known that the amount of colour developed by this reaction is dependent on the amount of the reactive glycol structure present in the tissues. In addition to this, the colloid was also positive to Ferric

ferricyanide giving a clear blue colour. A wide range of substances may be expected to respond positively to the Ferric ferricyanide test, but thyroidal colloid is one amongst them and it has in fact been recommended for the identification of the latter (Fisher, 1953; Lille and Burtner, 1953). The colloid appeared orange with the Orange G of the Azan stain in most cases, but just before the onset of spermatogenesis and aestivation some of the follicles showed a bluish tinge. This observation is in accordance with the results of other authors. Barrington and Franchi (1956) showed both Aniline blue positive and Orange G positive colloids in the endostyle of the Ammocoetes larva. George and Naik (1964) also noticed both acidophilic and basophilic colloids in the thyroid follicles of the migratory starling Sturnus roseus, in sections stained with Azan stain. In the present investigation too, both acidophilic and basophilic colloids were seen in the follicles. It has been suggested that the thyroidal colloid stains with Orange G or Azocarmine in the resting stage and with Aniline blue in the activated glands (Pickford, 1953; Dempsey, 1954). Thus it may be assumed that the follicles were in an active stage just before the spermatogenesis and aestivation periods, after which an increase in the stored colloid (Orange G-positive) was seen. In females these types of colloids were seen only towards the aestivation period. In the immature ovary only very few follicles were present and they did not show any affinity to the Aniline blue i.e. activated follicles were not

seen towards the production of the ova. This is supported by the observations of Maqsood (1964) in relation to the role of thyroid in maturity and sterility in males. The mechanism whereby the thyroid gland influences the various male reproductive functions in different species of animals probably involve a complex series of interactions (Maqsood,1964). In the present study, follicles showing affinity to the Aniline blue dye, representing the active stage of the follicles were seen towards the starting of spermatogenesis. Following this an increase in the Orange G positive follicles representing the storing stage was seen. However, in the light of these findings it can be suggested that these follicles are directly or indirectly involved in the process of spermatogenesis, probably in the maturation and growth of the sperms.

An increased number of follicles were noted in all the tissues during this period. Active follicles followed by an increase in the storage of colloid were again noted in all the tissues towards summer, prior to aestivation. At this time the ovary attains maturity and the stored colloid (Orange G-positive) gets accumulated as it is seen in other tissues. In April/May which are the dry months of the year the crabs aestivate due to the drying up of the rivers. A release of the neurosecretory material from the neurosecretory cell centres of the central nervous system was also found to take place prior to this (Chapter 8). This is accompanied by an increase in the number of follicles noted towards summer. It is well

known that in migratory birds, such as Sturnus roseus first the release of the neurosecretory material takes place which circulates through the blood and stimulates the thyroid to release its hormone just prior to migration (George and Naik, 1965). The results of the present study are in agreement with the above observation, since before the aestivation period a release of the neurosecretory material followed by an increase in the follicles with Orange G positive colloid was noted. But in this case the colloid is not released as that of the birds.

In the hepatopancreas, which is the storage organ of the various metabolites viz: protein, glycogen and fat a large number of follicles with stored colloid were observed in the intertubular connective tissue towards the aestivation period. Since this is the non-feeding period for the animals it is most likely that they utilize the stored up fat at that time, which accounts for its progressive reduction during this period (Chapter 3). Myant (1964) had reported that the thyroid influences the distribution of the neutral fat and fatty acids in tissues probably by modifying the rate of release of free fatty acid from the glycerides of the adipose tissue. This action of thyroid hormone may be due indirectly to its effect on the oxidative metabolism of the tissues which may in turn provide the stimulus for increased mobilization of fat. So the increased number of follicles seen among the fat storing

tubules of the hepatopancreas towards the aestivation might help in the utilization of the stored fat by increasing the oxidative metabolism during the non-feeding time. The same might be the case with ovary also. Among the fully mature yolk laden ova a large number of follicles were seen towards aestivation. As they are present only in the mature ovary, their role in the formation of ova is doubtful.

The colloid stained green with the light green of the Masson's trichrome stain in all the cases. The thyroid tissue of the hag fish, Myxine glutinosa, found in the fatty connective tissue along the course of the ventral aorta beneath the pharynx, also showed affinity to light green stain. (Waterman and Gorbman, 1963).

In addition to all these histological staining reactions the colloid was also strongly positive to Millon's reaction showing the presence of protein containing tyrosine. This reaction is due to the presence in the protein molecule of a hydroxy phenyl group, and is given by any phenolic compound which is unsubstituted in the position meta to the hydroxyl group. But such compounds are not found free in the tissues and the only known aminoacid containing the hydroxy phenyl group is tyrosine. This together with the strong radioautograph supported by the radioactive assay, showing the incorporation of labelled iodine (I^{131}) in the colloid strengthens the opinion that the activity is actually bound to the protein molecule

containing tyrosine. However, iodine binding capacity was noted in follicles, comparable to that of the vertebrate thyroid gland.

Another important fact that leads to the comparison of these follicles with the thyroid follicles of higher animals was the similarity in the effects of antithyroid compounds. After injection of thiourea, atrophic and degenerative changes in the tissue as well as a decrease in the follicular number and colloid content was noted in the present study. The mechanism responsible for concentrating inorganic iodide was inhibited by 0.001M thiouracil in marine algae Nereocystis leutkeana as shown by Tong and Chaikoff (1954). Merkel(1958a) also showed that the migratory activity of the birds could be inhibited by a dose of 0.2 cm^3 of 5% solution of methyl thiouracil, whose action on thyroid is to decrease the thyroxine levels. According to him Methyl thiouracil interferes with the formation of the thyroid and acts like the removal of the gland. Again Maqsood (1964) showed that the feeding of thiouracil (2-thio-6-Oxypyrimidine) as 0.1% of the ration to the young male mouse and rabbit at different age groups for short periods resulted in a significant decrease in the weights of the male sex organs with arrested spermatogenic activity, and some atrophic and degenerative changes. Prolonged thiouracil treatment or thyroidectomy in young male rabbit and sheep

arrested the onset of sexual maturity. Moreover, any interference with the normal production of the thyroid hormone by the thyroid gland such as the administration of thiouracil or thyroidectomy adversely affected the various male reproductive processes in young animals. In the light of the above findings the present observations agree with the widely held opinion that thiouracil or thiourea decreases the thyroxine levels and in certain cases interferes with the formation of the tissue influencing changes similar to those produced by thyroidectomy. Atrophic and degenerative changes of the tissues were also observed in the present investigation.

In the eye-stalk removed animals there was no noticeable changes in the follicles or the colloid. This is indicative of the fact that eventhough the various metabolic and neuro-endocrinological activities of the animal are controlled by the X-organ sinus gland system of the eye-stalk ganglion, these follicles are not directly under its control.

These follicles showed the presence of the enzyme alkaline phosphatase. McAlpine (1955) showed a marked increase in the phosphatase activity during the differentiative phase of thyroid development. Moog (1962) working on the chick embryo has also demonstrated an increase in the phosphatase activity just before hatching, which is considered to be due to the thyroid and adrenal activity.

In summing up the data with respect to the evolution of thyroidal function, the most obvious conclusion that could be drawn is that these tyrosine containing, PAS positive, follicles showing incorporation of labelled iodine (I^{131}) have preceded the thyroid gland in phylogeny.