CHAPTER 2

INCORPORATION OF LABELLED IODINE INTO THE FOLLICLES

The discovery of the presence of iodine in the vertebrate thyroid gland stimulated a considerable amount of research concerned with the chemistry of iodine in relation to protein; and on the metabolism of iodine in a number of vertebrates, especially of mammals and a few invertebrates. It has been shown that many organisms metabolize iodine, forming at least mono-iodotyrosine and di-iodotyrosine. This is indeed true in the case of marine algae (Tong and Chaikoff, 1954; Roche and Yagi, 1952), though the mechanism of iodide accumulation and the subsequent oxidation of iodide to iodine might perhaps differ from what happens in the thyroid gland. Nevertheless, the first iodinated amino acid formed is mono-iodotyrosine. Mono- and di-iodotyrosine and iodinated histidiness have been reported to be constituents of the iodoprotein present in invertebrates (Roche, 1952; Gorbman et al., 1954; Limpel and Casida, 1957; Antheunisse and Lever, 1956).

The accumulation of iodine as di-iodotyrosine in sponges and gorgonians has been known since long (Vinogradov, 1935). Di-iodotyrosine was identified in the scleroprotein skeleton of corals (Drechsel, 1896). Utilization of

radioiodine in the mucoid layer of a nemertean Lineus ruber, was reported recently (Anoli and Major, 1966). The possibility of the occurrence of protein bound iodine in the setae of annelids has been suggested (Swan, 1950). In an extensive survey using radioiodine, similar storage of protein bound iodine has been observed in the pharyngeal teeth, cuticle and setae of polychaetes, Neanthes and Glycera, in the bysus threads of young Mytilus, in the growing exo-skeleton (carapace and mandible) of young Daphnia still in the brood pouch of the adult, and in the periostracum of gastropod and pelycypod molluscs (Gorbman, 1941; Gorbman et al., 1954). Iodine accumulation has also been found in considerable quantities in the dermis of the foot of the horse-clam, Schizothoerus nuttali, and in the opercula of the whelk, Polynices lewisii (Cameron, 1915). As early as 1898 Caubel reported very small amounts of iodine in the larvae of the house fly and Aronssohn (1911) in the ash of bees. The influence of iodine upon the development of a species of caterpillar, Deilephila euphorbiae, has been investigated by Gedroye (1923) who observed that iodine when fed to older larvae activated pupation but prolonged the larval period when fed to younger larvae. High concentration of radioiodine are known to get incorporated into insect cuticle (Wheeler, 1947; Slipka, 1952). Wheeler (1950) also succeeded in separating several iodinated intermediates from the larvae of Drosophila gibberosa by paper chromatography.

In a number of insects (Limpel and Casida, 1947) and in a bivalve Musculium partumeium, (Gorbman et al., 1954), however, as much of the total tracer iodine was found to occur in the form of thyroxine as in a mammal. In the American cockroach, which is the only insect examined organ by organ, concentrated thyroxine was found to be mainly in the nerve cord, fat body and muscle (Limpel and Casida,1947). There was indeed a possibility that some analogy might exist between the vertebrate thyroid and the ring gland of higher dipterus insects which is known to play an important endocrine function during metamorphosis (Hanstrom, 1939). Among the several species of crustaceans examined by Gorbman et al. (1954), for testing the regional capacity for the incorporation of radioiodine, only in Daphnia pulex, were they able to obtain satisfactory results. It should, however, be mentioned that on weight basis the eye-stalk of the lobster, Homarus americanus contained more than four times as much radioiodine as any other tissue.

Among the protochordates, the shallow integumentary gland of Balanoglossus, the septal tissue of the stolons of certain tunicates and the endostyle of Amphioxus were shown to be the storing centres of organically bound iodine (Gorbman et al., 1954; Barrington, 1958).

Inspite of the above evidence for the ability for fixation of iodine by invertebrates, however, there is no

evidence for the presence of any definite thyroidal structure as such. The available evidence rather points to the intake of iodine for the formation of iodinated scleroproteins such as spongins, gorgonins, constituents of the fibers of the byssus and of the periostracum (Roche, Fontaine and Lelop, 1963). Nevertheless, the presence in tunicates of thyroid hormones identical with those of vertebrates, namely L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyroxine (T_3) is firmly established. Its significance is great in terms of the biochemistry of evolution since, contrary to the very widely held opinion, there are no thyroidal structures in invertebrates of the group below protochordates (Öberg, Gorbman, and Kobayashi, 1959).

In the present investigation, certain thyroid-like follicles present in the connective tissue of the various organs of the freshwater crab, Paratelphusa jacquemontii (Chapter 1) has been examined with the aid of labelled iodine (I^{131}) for testing the regional capacity for the incorporation of radioiodine.

MATERIALS AND METHODS

The crabs were collected from the local rivers and were maintained in the laboratory. A fairly equal distribution of male and female specimens was made for the study.

Animals from both the sexes were injected with a tracer dose of 25 to 35μc of carrier free iodine, I^{131} and were sacrificed at intervals of one to twenty four hours. Various tissues viz: the gonads, hepatopancreas, brain, thoracic ganglion and muscle were taken out and washed in 1% Sodium iodide solution in order to reduce the surface contamination. Since no follicles were observed in the muscle in histological preparations, muscle served as the control. Pieces of the tissues were fixed in aqueous Bouin's fluid and in Methanol for 24 hours. Then the tissues were washed and embedded in paraffin wax and 6μ thick sections were mounted on clean slides coated with 0.5% gelatine containing 0.05% chromealum. The sections were deparaffinized with xylene and brought down to distilled water through alcohol grades. The sections were then washed three times in 1% non-labelled Sodium iodide solution to remove excess of carrier iodine if any. All the sections were coated with Ilford's G.S. nuclear research emulsion. AR-10 Kodak stripping film was also used in certain cases. All the slides were kept in a light-proof container at 4°C. The sections were exposed to the emulsion for a period of 22 days, developed in Fieg's developer, stained with Erlich haematoxylin and eosin and finally mounted in Canada balsm.

Small portions of all the tissues were taken before fixing and weighed separately in tared weighing bottles.

The weighed tissues were then dissociated in 1 N NaOH and were used for the assay of radioactivity. Small quantities of the digested samples were spread separately on aluminium planchets and were dried under an ultraviolet lamp. The total radioactivity of each sample was then counted on a Geiger-Muller Counter. To each of the remaining digested solutions 3 ml of 10% trichloroacetic acid (TCA) were added in order to precipitate the protein, and was then centrifuged at 2500 rpm for 10 minutes. Supernatants were collected and the whole process was repeated three times with TCA and finally once with distilled water. Supernatants of all these repeated washes were collected and counted for radioactivity. The final precipitated protein was digested in 1 N NaOH and the radio-activity counted.

Protein was estimated quantitatively by the colorimetric micro method of Lowry et al. (1951).

OBSERVATIONS

The most constant and by far the clearest localization of organically bound iodine was obtained in the follicles. It was more precise in the male gonads. Radioautograph showing the accumulation of radioiodine in the follicles are shown (Figs. 1 & 2).

The results of the radioactive assay (Tables I & II), supported the histological observations. Almost all the tissues of the male crabs showed comparatively more radioactivity than those of females. In both the sexes the hepatopancreas, brain and thoracic ganglion, as well as the male gonads showed remarkably high activity, as the number of follicles in these tissues were also more, though comparatively less in females. As for the female gonads moderate radioactivity was obtained only in the fully mature stage, while the immature ovary did not show any activity. Parts of the central nervous system such as the brain and thoracic ganglion showed high radioactivity. Similarly the hepatopancreas of both the sexes and the male gonads also gave remarkably high counts. The protein precipitate of muscle which was taken as the control tissue gave only negligible counts showing the absence of organically bound radioiodine. It may be noted that no follicles were present in the muscle tissue. Nevertheless, the total radioactivity of the tissue which represents both inorganic and organic forms of activity was considerably high in all the tissues, even in muscle which served as the control. However, after precipitating the protein by adding 10% TCA and even after the repeated washes with TCA and distilled water the final protein residue showed very high radioactivity and that too only in the case of those tissues where the follicles were abundant. The

TABLE I

Incorporation of iodine^{131} into the various tissues of the crab, Paratelphusa jacquemontii

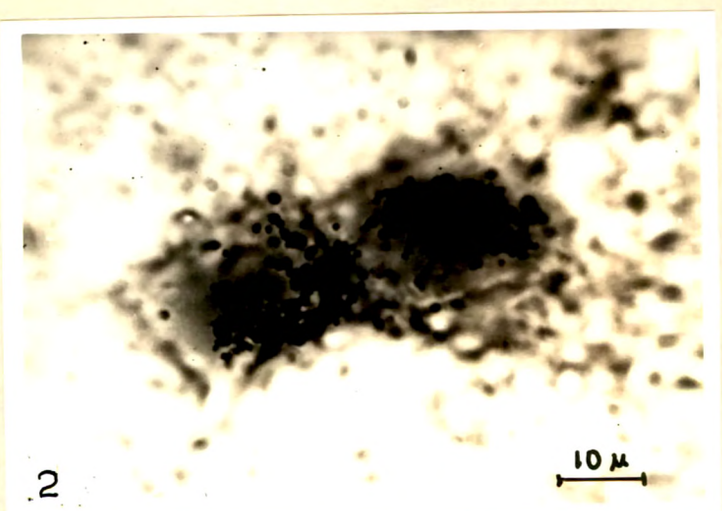
Sex	Injected dose	Treatment time	Tissue	Weight of tissue in grams	Counts per minute	Total cpm for the tissue	cpm/g wet weight	mg protein in the tissue	cpm/mg protein
Male	1.5 µc/g body wt.	3 hours	1. Hepato-pancreas	0.0559	863	4315	77191	0.6	1250
			2. Testis	0.0127	558	2790	219685	0.1	500
			3. Vas deferens	0.402	877	4385	109080	0.57	196
			4. Brain	0.0030	168	840	280000	0.039	641
			5. Thoracic ganglion	0.0117	275	1375	117521	0.087	920
			6. Muscle	0.1071	1109	5548	51989	1.18	24
Female	1.5 µc/g body wt.	3 hours	1. Hepato-pancreas	0.0583	1333	6666	131664	0.11	1500
			2. Ovary	0.0442	1192	5960	134842	0.6	50
			3. Brain	0.0033	178	890	269697	0.045	333
			4. Thoracic ganglion	0.0187	469	2345	171168	0.137	882
			5. Muscle	0.0726	1352	6750	93113	0.9	30

TABLE II

Incorporation of iodine^{131} into the various tissues of the crab, Paratelphusa jacquemontii

Sex	Injected dose	Treatment time	Tissue	Weight of tissue in grams	Counts per minute	Total cpm for the tissue	cpm/g wet weight	mg protein in the tissue	cpm/mg protein
Male	1.5 µc/g body wt.	5 hours	1. Hepato-pancreas	0.0947	4382	21910	231362	0.31	1871
			2. Testis	0.0186	1415	7075	380376	0.2	375
			3. Vas deferens	0.0550	2274	11370	206727	0.79	200
			4. Brain	0.0005	224	1120	2240000	0.032	470
			5. Thoracic ganglion	0.0123	800	4000	325203	0.123	1016
			6. Muscle	0.2241	4280	21400	95493	1.3	32
Female	1.5 µc/g body wt.	5 hours	1. Hepato-pancreas	0.1309	4904	24520	187318	0.49	1500
			2. Ovary (mature)	0.5697	2514	12570	22604	1.4	286
			3. Brain	0.0004	292	1460	3650000	0.028	536
			4. Thoracic ganglion	0.0126	1130	5650	448413	0.098	531
			5. Muscle	0.1223	5587	27935	228414	1.3	28


20 μ
1


10 μ
2

EXPLANATION TO THE FIGURES

Fig. 1. Photomicrograph of the follicles in the interstitium of the testis showing the incorporation of labelled iodine in the colloid.

Fig. 2. Part of the Fig. 1 magnified.

radioactivity of the supernatants of all the repeated washes were tested. Only the first supernatant showed certain activity, whereas the counts of all the other supernatants were negligible. However, the final protein residue of all the tissues except the muscle gave more counts than the supernatants of the repeated washes, thereby showing that the activity obtained from the protein residue actually represents the organically bound iodine and the activity of the supernatants that of inorganic iodine.

Regarding the percentage of activity, in the male hepatopancreas about 35 to 45% of the total activity in the whole tissue was protein bound, whereas in the female it was much less 15 to 22%. Testis showed 22 to 25% organically bound activity, but even in the fully mature ovary the protein bound activity was not more than 5%. The nervous tissues showed very high protein bound activity. 76% in the male brain and 38% in the brain of the female was found to be protein bound. In the thoracic ganglion, 72% and 27% of the total activity was bound to protein in the male and female respectively.

DISCUSSION

The results of the radioactive assay as well as the radioautographic studies show that the labelled iodine was incorporated into the follicles present in the connective

tissue of the various organs. This incorporation was seen to be bound to the protein molecules. The colloid of the follicles were strongly positive to Millon's reaction showing the presence of protein, probably containing tyrosine(Chapter 1). The strong radioautograph obtained in the follicles, indicating the incorporation of labelled iodine and considerably high radioactive counts obtained from the protein precipitate even after the repeated washes strengthens the view that the activity is bound to the protein fraction of the colloidal material. Under oxidative conditions and even in the absence of enzymatic intervention, iodine first adds to the tyrosine molecules in the protein and to a much lesser extent to histidine; the resulting mono-iodotyrosine and di-iodotyrosine, then if they are free to couple can form thyroxine compounds, including thyroxine (Roche et al., 1949). Mono-iodotyrosine, di-iodotyrosine and even thyroxine have been separated from various invertebrate tissues by several workers (Wheeler, 1950; Limpel and Casida, 1957; Roche and Michel, 1956). Moreover, since the fixed tissues and sections are passed through a series of aqueous and alcoholic solvents such as benzene, hot paraffin and alcohol-ether mixture before radioautography, it seems reasonable to conclude that only the protein bound iodine would remain in the sections to be detected (Gorbman et al., 1954). Judging from the relative insolubility of most of the I^{131} radioactivity and the recovery of only minute fractions of the radioactivity from Bouin's fixative, it is perhaps reasonable

to believe that most of the radioiodine we have been studying in the various tissues of the freshwater crab is in the protein bound form.

The ability to maintain a very high gradient of iodine concentration is well known to be one of the basic properties of the thyroid gland. However, the ability for iodine deposition in the organisms is found in some of the most unexpected sites which have nothing to do with thyroxinogenesis (Gorbman, 1955). In the vertebrates concentration of iodine compounds has been noted in the structures which are periodically shed, but definite organs identical with the thyroid gland of higher vertebrates have not so far been described. While thyroxine has become an integral part of a number of important metabolic and morphogenic mechanisms in vertebrates its functional significance in invertebrates is not clearly understood. Goldsmith (1949), has shown that thyroxine is without any clear effect in a large number of invertebrates. Moreover, the stockage of iodine compounds in structures which are periodically shed indicates that they are not available for any physiological purposes. However, it should be mentioned in this connection that in one oligochaete annelid, the setae are not lost (Stephenson, 1915), but accumulate in the coelom, where they are phagocytosed and dissolved. Furthermore, the scleroprotein bound iodine compounds in invertebrates would only require a local protease or catheptase to become available

for the organism. In vertebrates also iodine compounds are imprisoned in the thyroid follicles in combination with a very heavy globulin molecule from which they must be hydrolyzed by a protease to become available for the organism (Gorbman et al., 1954). In the present investigation, the thyroid like follicles, which could trap the labelled iodine, bound to protein, were seen in the connective tissue of the organs studied and were definite and permanent structures. Their possible role in relation to the various metabolic and neuroendocrine activities of the animal are discussed in Chapters 1, 7 and 8.

The results of the radioactive assay showing a relatively high incorporation of organically bound iodine in the parts of the central nervous system studied (brain and thoracic ganglion) is of special interest. It has been shown that in mammals thyroxine is both accumulated (Gross and Leblond, 1950), and metabolized (Tata et al., 1957; Ford and Gross, 1958), in the nerve cord. In cockroach also concentrated thyroxine was obtained mainly in the nerve cord (Limpel and Casida, 1957). Nevertheless, it is not clear, whether thyroxine is produced in the nervous tissues of these invertebrates or is produced elsewhere.

The various facts demonstrating the widespread occurrence of thyroid hormones and of hormonal precursors in the thyroidless invertebrates, must be taken into account in any

theory of evolution of thyroid function (Gorbman, 1955). The most basic properties of thyroid glands, iodine transport and protein binding of iodine, occur in many plant and animal tissues and it can be concluded that thyroxine and its precursors are rather common amino acids and that they have preceded the thyroid gland in phylogeny (Gorbman et al., 1954). However, it should be suggested that eventhough the suspected thyroid like tissue present in the freshwater crab is a rather unique structure in invertebrates, it adds one step further in the thyroidal evolution.