THE DISTRIBUTION OF IODINE IN ASCIDIANS

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SUMMARY

Radioactive iodine was used to study the distribution of iodine in the tissues of several species of ascidians. Earlier work had shown that radioactive iodine was accumulated in certain zones of the endostyle and the cuticle of the tunic of *Ciona intestinalis* L. A higher concentration of radioiodine in the medium was used in this project.

Autoradiographs of histological sections of ^{131}I treated animals showed that protein bound iodine (P.B.I.) was present in the tunic,gonads,endostylar zones 7 and 8 and the blood and heart. It was also present in the endostylar appendix and pericardiac body of *Ciona*. The tunic contained ^{131}I in the matrix and cuticle. The ^{131}I was associated with the cellular and fibrous elements and it is postulated that these components of the tunic matrix carry the iodo-amino acids from the blood vessels to the cuticle where they are incorporated into scleroproteins. This implies that the blood is a primary iodine binding tissue,in contrast to Barrington's hypothesis that the tunic was the first tissue to contain organic iodine compounds. P.B.^{131}I. was also found to be abundant in the body wall of the larva of *Ciona*.

The nature of the iodine compounds in these tissues was investigated by chromatography of homogenised and hydrolysed samples. It was found that the dominant iodo-amino acid which was incorporated into proteins was monoiodotyrosine. Small amounts of diiodotyrosine,thyroxin and triiodothyronine were also found. The effects

of the anti-thyroid drugs methyl thiouracil and potassium thiocyanate were also studied. A considerable amount of iodide was accumulated in their presence and incorporated into amino acids and proteins. This is in contrast to their effect in higher vertebrates with a true thyroid gland. It is suggested that ascidians have a predominantly primitive system of iodine utilisation akin to that recently claimed to be present in mammals to a very small extent. It is possible that ascidians also have a more advanced system such as is dominant in the higher vertebrates and which is sensitive to the effects of goitrogens.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge and belief it contains no material previously published or written by another person, except where due reference is made in the text.

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Part 1. INTRODUCTION

In 1819 Fyfe found iodine in sponges and in 1825 Balard found it in corals. Since 1896, when Baumann noted its association with the vertebrate thyroid gland, studies on iodine in animals have been mostly confined to man and other mammals, and only a few workers have turned their attention to other sorts of animals. The work on animals other than mammals was reviewed by Gorbman (1955), Fleischmann (1951), and Vlijm (1958).

In vertebrates the greatest concentration of organically bound iodine is in the thyroid gland, though the iodine compounds formed in that gland are distributed and metabolised in other parts of the body. It seems that the compounds which occur in mammalian thyroid glands are also found in other vertebrates, although there may be differences, perhaps only in degree, in the function of these compounds as hormones.

Iodine compounds similar to those of the vertebrates are found in the invertebrates also, but in different parts of the body. The compounds occur most frequently in scleroproteins, mucus glands and gonads, but Limpel and Cassida (1957) found organically bound iodine in tissues other than these in <u>Periplaneta</u>.

It has not yet been demonstrated that the iodine compounds in invertebrates have any metabolic significamce such as they have in the higher vertebrates. The evolution of the hormonal function

of these iodine compounds (iodinated amino acids derived from tyrosine) may have been closely associated with the evolution of the thyroid gland but is quite likely to have developed before the gland assumed its follicular form. The non-vertebrate members of the phylum Chordata do not have a follicular thyroid gland,but they do have the morphological,and possibly physiological,precursor of it,the endostyle. It is now generally agreed that some part of an endostyle - like structure evolved into the typical thyroid gland of the vertebrates (Leach,1939; Gorbman and Creaser, 1942; Barrington and Franchi,1956). The Cephalochordata,such as _Amphioxus_,and the ammocoete larva of the Cyclostomata both have tracts of mucus glands and ciliated cells in the endostyle. A small group of cells in the endostyle of both of these types of animal accumulates radioactive iodine. This was demonstrated by Thomas(1956) and Barrington (1958) for Amphioxus,and by Gorbman and Creaser (1942) for the ammocoete larva. It has also been shown that the ammocoete larva produces the same iodine compounds as are found in the vertebrate thyroid gland - monoiodotyrosine, diiodotyrosine and thyroxin (Leloup and Berg,1954);similar work has not yet been done on _Amphioxus_.

The other class of the prochordates which has an endostyle in the pharynx is the Tunicata. In this class the endostyle has a basically similar structure to that of the _Amphioxus_ but it includes a zone of simple cuboidal cells very like those which line the

thyroid follicles of vertebrates. It was shown by Barrington (1957) that these cells accumulate radioiodine in <u>Ciona intestinalis</u> and he postulated that they may be homologous with the thyroid gland of vertebrates.

The purposes of this project were to find out if the accumulation of iodine in this group of cells is common amongst ascidians; to determine whether the iodine is accumulated by any other tissues; and to see if the iodine compounds are the same as those in the vertebrates.

The techniques of histology and chromatography were used, both of them in conjunction with autoradiography of radioactive iodine. The work was divided into a histological survey of the distribution of iodine in the species available locally and the larvae of <u>Ciona intestinalis</u>, and a chromatographic analysis of the organically bound radioiodine compounds. Estimations of the total iodine in the local sea water and ascidians were also done. A general discussion correlates the results of the various investigations.

Part 2. THE DETERMINATION OF THE CONDITIONS OF TREATMENT WITH RADIOACTIVE IODINE

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Figures 1 and 2

2.1

The usual way to apply ^{131}I to experimental animals, particularly terrestrial ones, is to inject it. The reasons for this are the nature of the isotope, which has a rather short half-life of 8.1 days (Francis, Mulligan and Wormall, 1954); also a high level of radioactivity would have to be maintained in the medium in many experiments and this must be avoided. A few experiments have been done, mostly with aquatic animals, in which the radioisotope has been dissolved in the medium and the animals allowed to absorb it 'naturally'.

A number of experiments with tadpoles have been reported but I was unable to discover the concentrations of ^{131}I which were used. The only work done with ascidians was that of Gorbman (1941) on Perophora annectans and that of Barrington (1956, 1957) on Ciona intestinalis. Barrington used a concentration of 0.2 μc/ml ^{131}I. Roche et al. (1961), working with cyclostomes, used 0.75 μc/ml. I decided to try concentrations higher than those used by Barrington because of the possibility that iodine could be accumulated to a lesser extent in tissues other than the endostyle and tunic and those accumulations might not be detectable at the lower concentrations in the medium.

It was observed that 1 liter of water was needed for about 20g (wet weight) of animals to ensure healthy survival over a period of treatment of up to four days. Since it was not practicable to use more than 1 mc of ^{131}I at a time, because of the

radiation hazard, the maximum concentration of radioisotope used was 1 mc/liter, or 1 μc/ml, in analytical experiments. Concentrations up to 2 μc/ml were possible for histological experiments using only a few animals and therefore less water.

2.2

To determine the optimum treatment time, the rate of uptake of the radioisotope was studied. This was done in two ways; 1) directly by determining the radioactivity in the tissues at various times, and 2) indirectly by estimating the loss of activity from the medium. The former method had several disadvantages which could lead to misinterpretations of the results.

The most important disadvantage was that the rate of uptake and total iodine content would vary in the individual animals. The use of a large sample would overcome this, but the amount of radioactive iodine required for such experiments was prohibitive. The samples used were of two or three animals only, and sometimes less at the longer time intervals of three or four days. This caused some uncertainty about the shape of the graph at the later times. The other main disadvantage was that a part of the medium was enclosed in the branchial cavities and unless this seawater was completely removed from the cavities before the radioactivity of the tissues was determined a false high count would have been recorded due to $Na^{131}I$ in the medium. It was not feasible to cut open the pharynx and atrium to remove enclosed seawater as considerable loss of blood would have occurred. Since <u>Ciona</u> has a filtering rate of 0.5 - 0.7 liters per hour (Jorgensen, 1949) it was considered advantageous to rinse them for a few minutes in non-radioactive seawater to help remove radioactive water from the branchial cavities. A large <u>Ciona</u> with a pharyngeal volume of

15 mls will replace the water in the pharynx in 1.3 minutes. The amount of radioactive iodine excreted during this time would be negligible.

Because of these sources of error in the direct estimation of iodine absorbed by the tissues, the results were checked by the indirect method of measuring the loss of iodine from the medium.

After rinsing and draining the animals were weighed, then cut into small pieces into about 10 mls 5N NaOH in a small beaker. The tissues were hydrolysed by gentle heating for five minutes. The hydrolysate was filtered through a loose plug of glass wool to remove any undigested tunicin remaining and the latter were washed with dilute NaOH and the washings added to the filtrate. After suitable dilution, to make the counting rate of the various solutions of the same order, (thus obviating corrections for mechanical deficiency of the scaler unit at high count rates) the activities of the hydrolysates were determined in a liquid counting tube (M6 or M6H).

The above method was followed with animals treated at various concentrations (0.1, 0.2, 0.7, 1.0 μc/ml) for periods up to four days. The results were corrected for decay and plotted as counts per hundred seconds per gram against time of treatment. The results are shown in Fig. 1. The graphs show that maximum tissue activity occurred after approximately two days treatment at 1.0 μc/ml but the activity did not appreciably lessen until after

three days. When this experiment was repeated with different sets of animals the graphs obtained were the same shape but the actual counts differed because several different combinations of Geiger counting tubes and scaling units were used.

The comparable loss of activity from the medium was also studied. An approximately equal weight of Ciona was kept in seawater containing 0.5 and 1.0 μc/ml ^{131}I. The initial and subsequent activities of the water were determined by taking ten aliquots of 0.01 mls by a micrometer syringe from the water, which was previously well stirred. The aliquots were drained onto eual sized pieces of Kleenex tissue on planchettes and the glass nozzles of the syringe washed out with equal quantities of dilute NaOH onto the Kleenex. The activity of each aliquot was counted and the mean taken. Each mean value was plotted against the time, as in Fig. 2. The graph shows that after the equilibrium between uptake and excretion was reached it was maintained for a short time before excretion exceeded uptake, as seen by the increase in activity of the medium. This indicated that there was insufficient iodide in the medium to replace the iodine excreted. From this it could be inferred that the excreted iodine was in a form not readily made available for reabsorbtion as iodide.

The main disadvantage of this method was that other organisms in the medium, particularly the algae, also accumulate iodine. A sudden increase in the amount of algae after three days always occurred and removed much activity from the medium. This growth occurred even after thorough initial cleansing of the ascidians;

it was impossible to remove some of thealgal thalli which were deeply embedded in the villous portion of the tunic without damagimg the ascidians and it was these remnants which gave rise to the later growths.

The very noticeable return of radioiodide to the medium, as indicated by the increasing count rate after equilibrium, was not detected by the tissue activity counts except at 1.0 μc/ml. The rather inaccurate sampling method,using very few animals,probably accounted for this. The data for Fig. 2 . were from experiments done within a few weeks of one another using the same counting euipment for all.

However,the purpose of studying the rate of uptake was to determine the time required for the animals to accumulate the maximum amount of radioiodine and this could be estimated from the graphs obtained,as is discussed in the next section.

From these graphs it was decided that treatment for two to three days with the maximum concentration of ^{131}I which could be used would give the best general picture of radioiodine distribution in *Ciona*. For analytical experiments, in which a larger nember of animals were required, the concentration was usually 0.8 - 1.0 µc/ml for 60 - 70 hours. The duration of treatment was varied in some analytical experiments as is explained in the relevant section (4.4). It was discovered from such experiments that the amount of protein bound iodine (P.B.I.) was much higher in the tissues after three days treatment than it was after two days and subsequent histological experiments were all done with the longer time of treatment. In the earlier histological experiments, which were done before the analytical work was commenced, the time had mostly been two days, following Barrington's (1957) method. However, experiments done at ½, 1, 2, and 3 days treatment had shown that after ½ or 1 day little P.B.I. was present, especially in the tunic; after 3 days (in radioactive seawater) the density of radioiodinated proteins was often noticeably greater in most animals than after 1 - 2 days. This observation was substantiated by the subsequent analytical work (Part 4)

2.4

The injection of tracer amounts of $Na^{131}I$ into the body cavities or blood vessels was also tried, since it allowed the introduction of a large amount of radioactive iodine in a very short time. In *Ciona* a fine glass capillary needle could be used for piercing the body wall and heart through an incision in the tunic but in the other large, solitary ascidians (*Pyura, Styela, Ascidia*) a fine steel hypodermic needle was required. This method however had a serious drawback, in that the muscle tone of the heart walls and even the body walls was insufficient to retain the injected fluid and leakage occurred from the puncture, particularly in *Ciona* and also in the Pleurogonid species. For histological work this was not a serious defect and the method was used in some experiments. It was considered that a more accurate picture of the distribution of iodine could be obtained if the animals were allowed to accumulate the element by the natural means from the surrounding medium.

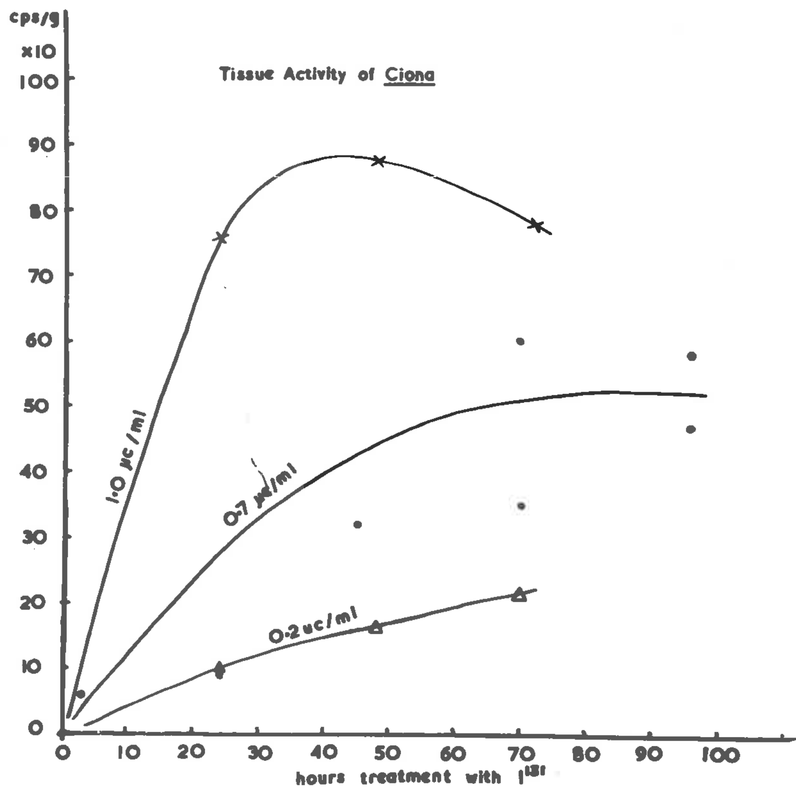

FIG. 1. Uptake of ^{131}I by Ciona at various initial concentrations.

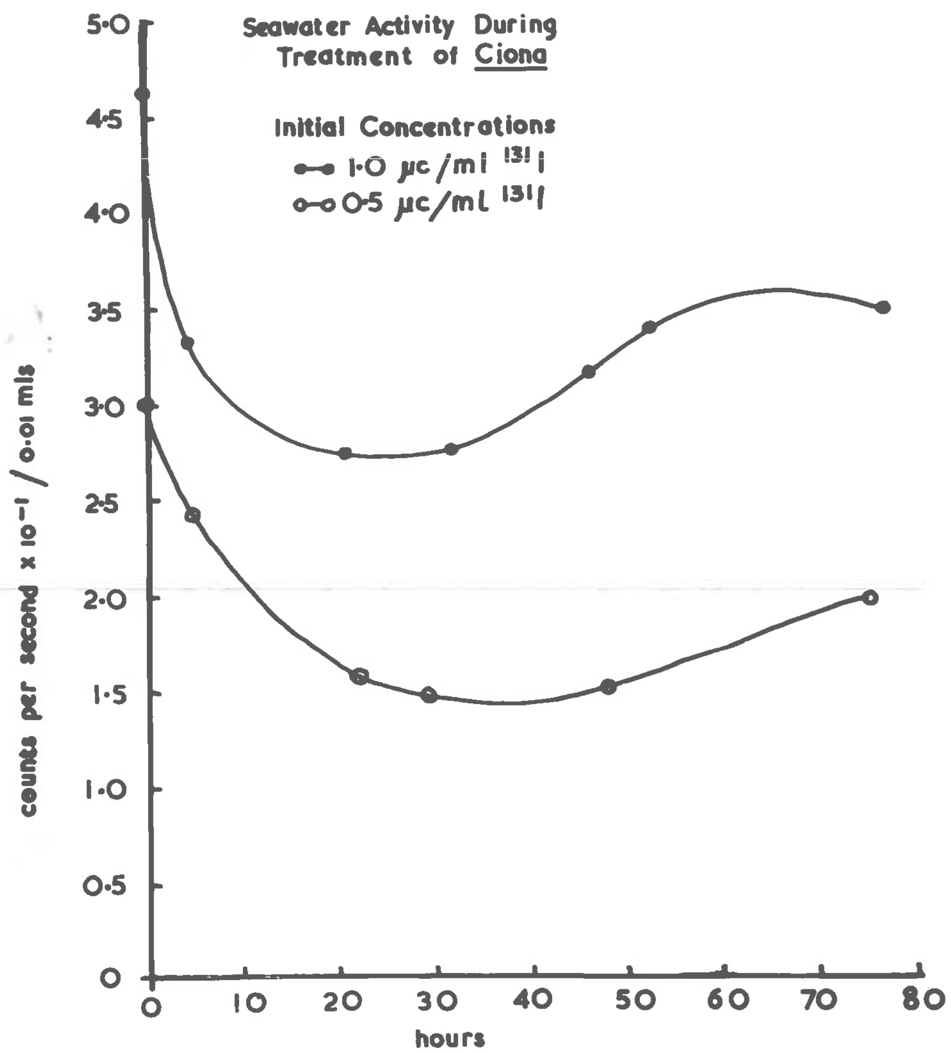

FIG.2. Radioactivity of the medium during treatment of

Ciona.

Part 3. HISTOLOGICAL SURVEY

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3.1 Material

The simple ascidian *Ciona intestinalis* L. occurs in the sheltered waters of the Inner Harbour of Port Adelaide. *Ciona* is usually found in estuaries and it flourishes in the tidal Port River during the winter,spring and early summer when some fresh water enters the harbour as rain and storm drainage. In cool temperate climates its life cycle occupies 12 -18 months; generations overlap and sexually mature animals occur throughout the year (Millar,1953). In Port Adelaide however it is not abundant throughout the year but dies off rapidly after mid-summer and from January to May is almost unobtainable,except by dredging. Exposure to the air by the greater fall in the low tide levels which occur from mid - summer to autumn is responsible for the death of the majority. Those which are not exposed also die off at this time and it is thought that this may be due to the high summer temperatures and the increase of salinity caused by these. The chlorinity varies from 15 ‰ to 21 ‰ during the cooler months but may rise as high as 24 ‰ during the summer. The temperature of the water at the level in which *Ciona* grows varies from 10°C to more than 27°C over a twelve month period.

Occasional specimens of *Ascidia aspersa* (Muller) and *Styela stolonifera* (Herdman) also occur at the Inner Harbour. *Styela* is more tolerant of the summer conditions than *Ciona* and replaces it as the dominant species during the late summer,but in much smaller numbers.

At the Outer Harbour of Port Adelaide,which is on the open

coast, the following ascidians are found at various seasons:-
Pyura stolonifera Heller, *P. irregularis* (Herdman), *Podoclavella australis* (Herdman), *Botrylloides leachii* Savigny, *B. nigrum* Herdman, *B. magnicoecus* Hartmeyer, *Diplosoma macdonaldi* (Herdman). Small colonies of a Didemnum species are occasionally found; also sporadic specimens of *Ciona* and *Ascidia aspersa*.

Most of these species could only be obtained at low water spring tides during those months of the year when the water level fell to within 2.5 feet of datum. When the specimens were collected the temperature of the water was noted, and the chlorinity of a sample estimated by titration with silver nitrate.

A special rake was used to facilitate collection of specimens from several feet under water. It consisted of a handle four feet long which could be extended to eight feet, the two parts being joined by brass ferrules. The lower end was fixed to a raking device of cadmium plated steel. The rake was rectangular in section, 9 x 12 inches, being fixed to the handle at the centre of one of the longer sides. The opposite side was serrated along the upper edge. A strong net of ½ inch mesh, closed at the bottom, was attached to the lower edge of the rake frame.

At the commencement of this work some difficulty was experienced in obtaining *Ciona* and other species from the Inner Harbour because of the removal of the old hulls and barges on which the ascidians were growing. Therefore a wooden raft, thoroughly coated with 'Estapol', and fitted with vertically suspended, sandblasted,

glass plates was constructed. This was lowered into the water under the wharf piles by a strong copper wire and attached to a convenient horizontal member of the wharf. It could thus be raised to the surface of the water whenever required.

Ciona in particular grew abundantly on this raft, the greatest concentration being on the under surface of the wooden parts. Very few *Ciona* settled on the glass plates, but overcrowding on the raft was severe. The compound ascidians which were sometimes found in the Port River on barges and bouys brought in from the coast, were the only ascidians which chose to settle on the vertical surfaces in preference to the horizontal. Chittleborough (1952) found a less definite selection of sites in his work on the marine fouling organisms of the same harbour. In the absence of horizontal beams Ciona grows abundantly on the vertical wooden piles, but the crevices on the surface of the piles, and adherent weeds and molluscs probably provide ample 'overhead' shelter for the tadpole larvae to settle on.

When the old wooden piles were replaced with metal sheet piling in 1958 - 59 the usual sources of *Ciona* were no longer available. Another area, further up-river, was discovered in which *Ciona* was undisturbed and could be easily obtained with the rake. It was noticed that there was a sharp decline in the population of compound ascidians during the years 1958 - 61, and the species studied by Chittleborough could hardly be considered as causing serious fouling now.

3.2 Method

The conditions of treatment with radioiodine were decided upon as stated in Part 2. Specimens for histological examination were frequently treated in the same radioiodinated seawater as were those for chemical analysis, the quantity of seawater used being adjusted to the number of animals used. A minimum of 0.5 liters to five Ciona less than 5 cm long was allowed. The radioiodine concentration in the seawater was 0.8 - 1.0 μc/ml, added as carrier free $Na^{131}I$.

The ascidians were left in this water for three days, being aerated all the time. At the end of this time the faeces had all been voided, but there was still some mucus in the alimentary canal with some food particles in it. Dried liver powder (Wisely, 1961) was sparingly added as a plankton substitute.

Species such as Ciona, which usually produced larvae in the labor atory aquarium, still did so after treatment. Some difficulty was experienced in keeping botryllid colonies alive for three days and treatment time was usually shortened to two days for them.

Introduction of the tracer iodide into the blood stream by injection was also tried, the animals being fixed a few hours later.

Two anti-thyroid drugs were used to test the effect of goitrogens on the binding of iodine. Methyl thiouracil (0.005g/1) was put into the medium 24 hours before the $Na^{131}I$ was added; KCNS (0.02g/1) was added similarly.

After treatment with radioiodine the specimens were allowed to expand in clean seawater to wash out radioactive seawater from the peribranchial cavities. Chlorbutol or menthol was added to this water as a narcotic. The animals were then fixed, either whole or dissected into separate organs, in Bouin's in seawater or Susa. The ascidians with a thick tunic were removed from it before fixation.

After fixation the specimens were embedded in paraffin wax and sectioned at 7 - 10μ. Some difficulty was experienced with the *Pyura* species as they went brittle even after shortened dehydration times and it was difficult to cut good sections of them.

Sometimes the sections were stained with haematoxylin or haemalum before autoradiography. Otherwise adjacent sections were stained with Mallory's Triple stain or Erhlich's haematoxylin -eosin for comparison with autoradiographed sections. McManus' P.A.S. technique (1948) and Lison's (1953) Alcian blue - Chlorantine red stain for mucins were also used.

Auto radiography was done with Kodak Autoradiographic Stripping Film (A.R.10). The exposure time was two weeks, during which time the slides were kept at less than 5°C. The films were subsequently developed with D19b.

3.3 Results

The interpretation of autoradiographs of histological sections is based on the assumption that the only radioactive iodine present in the tissues after the processes of fixation and embedding is protein bound iodine. According to Leblond and Gross (1948) all inorganic iodine and free amino acids are removed from the tissues during fixation and subsequent dehydration by alcohols,whilst only protein bound iodine is precipitated in the tissues by the fixative.

Apart from the accumulation of radioactive iodine in the endostyle and tunic as ascertained by Barrington (1956,1957,1959) and confirmed for the species here investigated,it has also been found in the gonads and in the blood and associated tissues.

A few specimens of various species (Styela,Ascidia,Pyura) have shown a definite uptake of ^{131}I around the epithelium lining the atrial cavity,particularly in association with the muscle blocks. The ^{131}I appeared to be concentrated between the muscle blocks and rarely extended into them. In some cases (specimens of Styela) this uptake of iodine could have been associated with the gonadial epithelium but in others this was not so. It is interesting to note that Barrington (1956) also mentioned that he thought he had detected iodine in the body epithelium,but he later discounted his evidence. In my work it has only been detected in a few specimens,all of which showed an unusually heavy uptake in general. It is not yet known whether it has any significance in connection

with the binding of ^{131}I by the epidermis of the larvae.

3.4 <u>Endostyle</u>

The results published by Barrington (1957,1959) on the uptake of iodine by the endostyle of <u>Ciona</u> have been generally confirmed;an accumulation of radioiodine was associated with zones 7 and 8 of Barrington (Fig.3,a+b.) and some was often seen on the free ends of the long cilia of zone 1. This has been observed in a variety if genera - <u>Ciona</u>,<u>Pyura</u>,<u>Styela</u>,<u>Ascidia</u>, <u>Podoclavella</u>. The picture was not so clear in the small zooids of the colonial genera but the pharyngeal lips of the endostyle gave a radiographic image (Fig.4a). The difficulty of producing a good section of a botryllid endostyle was mentioned by Barrington (1959). It was even more difficult to find a good endostyle section which also gave a radiographic image. It was observed in all colonies investigated that only a small proportion of the zooids present gave an autoradiographic image from the endostyle. This applied also to members of the Didemnidae. There is no mention in the general literature on ascidians,or in Herdman's monograph on 'Botryllus' (1926) that the zooids do not all feed together,but that would be a reasonable explanation of the sporadic uptake of ^{131}I by the endostyles of a colony.

Some variations on the distribution of iodine over zones 7 and 8 were noted. A few specimens showed an almost complete absence of iodine from zone 7,whilst it was accumulated over zone 8. Variations in the density of the image over the width of zone 7 were frequent. A 'centre' of iodination was seen usually at

one part or another of the row of cells but it rarely extended over the whole row. This suggests that some regulation may be imposed on the uptake of iodine by the cells. It is possible that they exhibit a periodicity in their ability to accumulate iodine and that this periodicity affects a group of cells and not just a single cell;or it could be that the rate of iodination is actually very low and the localised higher density of the radiographic image is due to ineffiecient clearing of the iodinated compounds from the surface of the cells.

Barrington (1957) asserts that the radiographic image of zone 7 extends over the cells,whereas that of zone 8 is limited to the distal surface of the cells. His argument is that the radio-iodine is intracellular in zone 7 but entirely extracellular at zone 8. My own observations do not,at first sight,agree with his. Allowing for the effect of scattering of the β-particles through the tissue,it was often observed that the radiographic image was wholly extracellular at zone 7 also. This could be explained, however,by allowing the suggested periodicity in accumulation and subsequent secretion of radioiodine. Barrington's observations require that the cells be continually accumulating iodine,but this does not account for the uneven distribution along the width of the zone. It seems necessary to postulate some periodicity, although the controlling factor is unknown. If it could be shown that ascidians are repsonive to thyroid hormones then some kind of self-regulating mechanism might be inferred. However the metabolism

of iodine by zone 7 cells may be subsidiary to some other process which is definitely regulated.

The Periodic Acid - Schiff reagent (P.A.S.) and Alcian Blue-Chlorantine Red stains for mucins were used in order to detect any such secretions from the endostyle. Since the endostyle is a part of the pharynx one would expect to find that its secretions are of a type usually found in alimentary epithelia; that is they would contain the carbohydrate polymers known as acid mucopolysaccharides or amino polysaccharides. The zone 1 cells show a strong positive reaction apically with Alcian Blue, which indicated the presence of amino polysaccharides. None of the other secretory zones gave a definite positive reaction. Occasionally some small droplets at the surface of zone 7 cells have been seen to give a weak reaction with the stain, as they do with P.A.S.

The experiments done by injecting tracer iodine into the blood stream gave an interesting result in the uptake by the endostyle. Several animals were injected and fixed at different time intervals. Those which were fixed 6 - 10 hours after injection showed very little iodine in the endostyle except at the basal or posterior end. An interval of at least 24 hours was needed before ^{131}I was found in the anterior endostyle. This suggests either that the iodine may be carried to the endostyle by the blood stream and passed into the zone 7 cells as iodide or iodinated aminoacids for ultimate incorporation into P.B.I. - or that the endostyle was taking up iodine excreted from the coelom and blood stream into the pharynx. The experiments on the rate of uptake however

suggested that iodine is excreted in a form not quickly made available for reabsorption and the analysis of excreted iodine compounds shows the excretory products to contain amino acids, possibly with some iodide (Fig.24). Unless these amino acids were trapped by the endostylar mucus and converted there to P.B.I. it seems unlikely that the ^{131}I in the endostyle of injected specimens came from the medium. Some detailed work on the organic iodine compoumds excreted and the _in vitro_ and _in vivo_ iodination of the endostylar mucus is needed to elucidate the situation. It has already been shown by Thomas (1962) that the mucus of the house of _Oikopleura_ does not take up ^{131}I directly from seawater.

The possibility that the endostylar mucus could have been the 'original' iodine binding substance in the lower chordates must not be neglected in spite of the negative evidence so far obtained about the _in vitro_ iodination of such mucins. Barrington, in particular, is of the opinion that fragments of iodinated tunic scleroprotein, ingested by the early tunicates, set up a metabolic dependence on iodine in that class. Thomas, however, (personal communication) has advanced the idea that surface mucins might also be easily iodinated, as apparently happens in the Hemichordata, and as has been reported for some invertebrates, especially annelids (Gorbman, 1954). Such an origin for iodinated amino acids implies that the mucins concerned must contain a relatively non-specific oxidising system capable of dealing with the sea water iodide. A system of this kind has yet to be demonstrated. Barrington's hypothesis necessitates that it

exists in the tunic also. It is just as likely to exist in the endostyle in which a variety of chemical reactions takes place which is probably greater than in the tunic scleroproteins. However, it is doubtful if the tunic is actively engaged in binding iodine directly from the external medium as Barrington's hypothesis requires. (see 3.8) Various enzyme systems must be present in the metabolically active endostyle and it is more likely that the endostyle, or perhaps the blood, was the initiator of iodine binding in ascidians.

Assimilation of Iodinated Mucins

The passage of the iodinated mucus can be traced through the length of the alimentary canal. Very little radioactive iodine is present in the faeces, but considerable amounts can be seen in the mid - intestine where the mucus rope from the oesophagus is still largely intact and wrapped around the food masses. If this iodine is protein bound, as it is assumed to be in autoradiographs, its presence in the intestinal mucus would indicate that no very effective endostylar protease is present.

Ciona treated with more than 1.0 μc/ml ^{131}I showed the passage of the tracer across the intestinal epithelium to the blood spaces around it (Fig. 5a).

3.5 Endostylar Appendix

The endostylar appendix is another structure which, like the pericardiac body, has been described only in *Ciona*, although Brien (1948) states that ascidians in general have both anterior and posterior 'culs - de - sac' to the endostyle. Berrill (personal communication) however considers the endostylar appendix to be absent from other ascidians and that it is one of the primitive features retained by *Ciona*, the only analogous organ being found in the Thaliacea (Berrill, 1950).

The structure of the endostylar appendix of *Ciona* was seen in most sections to be slightly different from that published by Millar (1953), (Fig. 6 .). The specimens investigated here have shown a band of zone 7 cells on both sides of the tube. Sections from the distal end of the tube however show that these bands of cells are absent from that part and the sections have the same appearance as in Millar's diagram. The sections of the endostylar appendix shown on Figs. 7a and 7b were taken from the distal third of the tube and contain no zone 7 cells between the ciliated roof cells and the zone 6 of that side. Alcian Blue shows very well the intracellular formation and secretion of the mucus into the lumen of the tube. The mucus gave the intense staining reaction typical of the mucus of the mid - gut epithelium, which was rarely seen in the endostyle except in a few instances in zone 1 cells. The major part, if not all, of the appendix mucus comes from the ciliated

columnar epithelium and none is seen in association with the cuboidal epithelium. Autoradiographs show that this mucus contains much ^{131}I (Fig.7a). The radioiodine does not appear to be intracellular at all but is associated only with the mucus lying in the lumen of the appendix. This iodinated mucus will be passed out of the tube onto the cilia of the retropharyngeal band and hence to the oesophagus. The oesophageal epithelium also secretes much mucus but it does not bind iodine.

The endostylar appendix, as seen in all sections, is a collapsed tube, spirally twisted dorsally, and closed at the distal end. It lies directly against the heart and its surrounding plexus of blood spaces is supplied by a small vessel from the pharyngeal end of the heart. In life the appendix is somewhat stretched by tension from external membranes, but the lumen is still small and the internal epithelia are closely opposed. The presence of iodinated mucins in the distal portion of the tube, even in the acute angle formed by the junction of the cuboidal and columnar epithelia, suggests that iodination os occurring in the tube through the agency of the roof cells. An alternative interpretation is that the iodinated components of the mucus are passed into the 'upper' region of the lumen from the zone 7 cells in a manner similar to that which is presumed to take place in the endostyle. However the ciliary action of the columnar epithelium must ensure that the mucus is removed from the lumen of the appendix and one would not therefore expect to find any ^{131}I in the least accessible parts of the lumen. Iodination of the mucus through the agency of either

or both halves of the roof of the appendix is therefore indicated. The source of the iodine could be from the coelomic fluid or the blood in the plexus around the appendix.

It is tempting to speculate whether this otherwise apparently functionless organ could be phylogenetically related to the thyroid gland. Its loss in the more specialised ascidians and other tunicates suggests that it is not necessary to the normal functioning of the adult animal, but it could be, in *Ciona*, a remnant of an embryonic potentiality which was transmitted by the most primitive larvae to their derivatives, the vertebrates, if neotony of ascidian larvae was indeed the origin of that class as suggested by Berrill (1955).

3.6 Blood

The presence of iodine in the blood was to be expected but that it should be revealed in the very small quantity of blood protein remaining in an histological section was considered unlikely. However, sections through the heart and adjacent blood vessels showed iodine to be present in the lumen and occasional images were seen in the sub - endostylar vessel. Radioiodine was also detected in the spaces of the spongy tissue around the gut in some species and was seen clearly to be present in the tissue surrounding the gut of Ciona (Fig.5a).

Protein bound iodine was also found in the cell - packed stolonic vessels of Podoclavella (Fig.8b). It did not appear to be closely associated with the cell clumps, but was distributed more or less evenly over the section of the vessels.

Fig. 9 shows ^{131}I associated with a blood clot in the heart of Ciona treated with 1.0 μc/ml for 50 hours at 20° C. The same section showed iodine to be present in, or closely adhering to, the walls of the heart. It was found that it was always the myocardium which accumulated iodine in the fibres. The pericardium also had some iodine associated with it, but never to the same extent as did the myocardium. Sections of the heart when stained with Alcian Blue (Fig.8a) gave a positive reaction in the short crosswise fibres in the walls, indicating the presence of an acid mucopolysaccharide. It would appear from autoradiographs that the ^{131}I is probably associated with these fibres.

Sections through the heart of Ciona also showed that the

pericardiac body accumulated much radioiodine (Fig.9). This body has not been seen in any other species and Berrill (1950) made no reference to it. It is thought to be composed of fibres derived from the walls of the major blood vessels (Millar,1953). That it contains metabolically active material is shown by the great density of the iodine uptake which penetrates to the centre of the mass. Since the pericardiac body only slowly increases in size due to the addition of more fibres during the life of the animal, the great quantity of iodine present cannot have been derived only from that already present in fibres added during the two days treatment.

The accumulation of radioiodine by the myocardium walls, which are derived embryologically from the pericardium, suggests that the myocardium has become a specialised iodine binding tissue. It is possible that the contractile tissues of the heart may be a site for the metabolism of protein bound iodine. In view of the continuity of the coelomic fluid in the epicardial sacs with the seawater in the pharynx it would not be surprising if such a source of iodine were utilised by an animal which had developed a metabolic dependence on the element. Injection of thyroxin in a suitable solvent into the blood stream might show whether the heart is affected by hormonal iodine.

3.7 Gonads

The first observations of iodine in the gonads were from the *Pyura* species and *Styela* where a faint, even image was noticed over the testicular lobes. The iodine was seen to spread inwards to the ovarian portion of the gonads through the parts of the testis layers. This distribution suggested that the penetration of iodine into the gonads might be by diffusion directly from the surrounding fluids. It was also noticed that only the younger oocytes accumulated iodine. These cells lie closest to the interstitial tissue so it is possible that iodine compounds are carried to the gonads by the blood. In view of the large amount of iodine found in the blood it seems quite likely that this source of iodine is in fact utilised. Further evidence that iodine is carried to the gonads by the blood was obtained from a section through *Styela* gonads which showed radioiodine to be present only in the interstitial tissue of the testis and some parts of the ovarian follicles. (Fig.10a). ^{131}I was also found in the developing eggs of a botryllid colony in which there was an unusually heavy overall uptake.

When radioactive iodine was injected into the coelom of *Ciona* the uptake by the gonads, particularly the ovary, was definite (Fig.5b,3c). The young oocytes showed the densest image, though iodine was present in all developmental stages, including ripe ova in the oviduct. The testis lobes showed some uptake also, although no marked zonation was seen. Follicles were sometimes seen in which the photographic image was denser over the spermatozoa and sperma-

tids than over the spermatogonial areas, (Fig 5b).

This distribution suggests that the iodine is accumulated only before the first meiotic division of the oocytes and that the quantity does not increase further with the growth of the egg. The slightly greater density of iodine in the spermatids than in the spermatocytes would be due to the concentration of the protein into a smaller volume during spermatogenesis.

3.8 Tunic

^{131}I was found in autoradiographs of all species examined (*Ciona intestinalis*, *Styela stolonifera*, *Ascidia aspersa*, *Pyura irregularis*, *Botrylloides leachii*, *Diplosoma macdonaldi*, *Podoclavella australis*). The extent of the binding varied greatly however, both in local concentrations and the area in which it occurred. Also individuals were often found in which the sections studied showed no uptake, although the animals were apparently quite healthy when fixed. Since many sections were cut in which iodine was detected only in small patches it is quite possible that the animals in which none was observed were not in fact completely devoid of iodine in the tunic.

In general iodine was metabolised in the younger or actively growing parts of the tunic. This was so both in the solitary and colonial forms. In the latter there was more likelihood of finding iodine towards the edges of the colony than in the centre and in the simple forms such as *Ciona* the accumulations of iodine were closer together and denser in the basal parts of the tunic than on the distal parts.

Although a thin image of radioiodine was often almost continuous over the surface of the tunic, this was frequently interrupted by denser accumulations which appeared to originate from very small or point sources. Sometimes several such sources would be located close together, giving an extensive dense image. This was usually found to be due to fragments of algae or serpulid worm tubes. Other-

wise these areas of relatively dense uptake could only be explained on the basis of activity greater than the general rate as might take place during regeneration of the tissues or replacement of worn off cuticle. This was seen particularly in those species which have a thin, almost brittle cuticle and a relatively non - fibrous matrix, such as *Ciona*, *Ascidia* and *Podoclavella*. In *Pyura* and *Styela* the picture was somewhat obscured by the thickened, rough cuticle.

The precise location of the iodine in the structure of the tunic was not easily determined but good evidence of iodine in the matrix of the tunic was found in *Pyura* spp., *Ascidia* and *Podoclavella* colonies. In some botryllid colonies the ^{131}I extended further into the matrix than was usually seen. The colonies were small and the iodine occurred at the periphery only. Sections through terminal ampullae of these colonies showed a faint photographic image of ^{131}I as would be expected if such iodine were present in the blood (Fig 4b). ^{131}I was definitely found in the fibrous layers around the blood vessels in *Pyura* tunic sections (Fig. 11a). It was observed to be most concentrated around the blood vessel, the density decreasing directly as the fibres and cellular components became more scattered in the matrix. The density of the ^{131}I increased again near the cuticular layers, which in *Pyura* are stratified and broken (Fig. 12b). There did not appear to be any greater concentration of iodine in the actual cuticle than in the sub - cuticular layers in any of the specimens examined. The cuticle is dark coloured in *Pyura* sections and thus may screen some of the photographic image of radioiodine

but there certainly is not enough iodine present, after three days treatment at 0.8 μc/ml, to radiate beyond the limits of the fragmenting cuticle. Also it was observed that fragments of cuticle which were almost detached from the main part gave no sign of iodine being present. This suggests that iodine is not taken up by the cuticle itself from the seawater but is supplied to it by the underlying tissues. The short fibres which were abundant in the matrix of Pyura tunic were not definitely associated with ^{131}I but many of them presented a beaded appearance which suggested that there was just enough ^{131}I present to affect the photographic emulsion. Certainly the densest areas of iodine also contained many fibres and cellular components, including refringent granules.

In Ascidia (Fig. 13a) the ^{131}I was seen to be closely associated with the large cells, containing refringent granules, which migrate across the tunic to the cuticle. Where such cells were grouped together in the sub - cuticular layers much iodine was present. It was also seen that these large cells, when incorporated into the cuticle, may be extruded from the tunic (Fig. 13b). It was noted that Ascidia treated for one day, whilst containing much iodine in the pharyngeal mucus and some in the muscles, had very little in the tunic, even in the cuticle. There was some evidence of it in the inner tunic layers adjacent to the muscles and blood vessels (Fig. 10b). After two days treatment the amount of iodine was greatly increased throughout the tunic, as described above. This observation was made on separate individuals and therefore subject to the variations

which might occur, but this does not invalidate the result entirely since there was plenty of evidence from other experiments to show that one day's treatment was insufficient to give definite results for ^{131}I in the tunic.

The association of ^{131}I with cellular components of the tunic was also observed in specimens of Styela, Pyura and Ciona.

Barrington and Barron (1961) have investigated the histochemical characteristics of the tunic of Ciona. Their results with Alcian Blue and P.A.S. stains agree with mine; the tunic contains many fibres, especially towards the surface, which give a positive reaction with these two stains, indicating that the fibres contain acid mucopolysaccharides. The reaction of the cuticle is negative for to Alcian Blue but positive to P.A.S. and to tests for tyrosine, indicating that the cuticle is composed of a mixture of carbohydrate and protein.

Barrington and Barron noted also that the refringent granules, which are found in close association with the cuticle and the fibres, gave the same reactions as the cuticle. The positive response of the granules to the tests for tyrosine and related amino acids is especially interesting in relation to the uneven distribution of radioactive iodine observed at the tunic surface of such species as Ciona, Podoclavella and the botryllids. The presence of tyrosine in the fibres and granules would tend to confirm the impression obtained from autoradiographs that iodine is associated with the fibres. The apparent absence of i dine from the matrix of relatively non - fibrous tunics could then be explained by the low density of

fibres in those tunics and the correspondingly decreased concentration of radioactive iodine. It was also observed in specimens of _Styela_, _Pyura_ and _Ciona_ that iodine was associated with the cellular components of the tunic but the density of the photographic image was not often sufficiently different from the background for any definite conclusions to be made about its precise origin. However specimens of _Ascidia_ which were examined (see above) showed conclusively that iodine is transported to the surface of the tunic by the large cells with refringent granules.

The important question of the direction of transport of the iodine can be studied most usefully from the results observed for _Pyura_ and _Ascidia_. From the evidence obtained by Barrington (1957) and the author about the distribution of radioactive iodine in the tunic of _Ciona_ it would appear that only the scleroproteins of the tunic cuticle contain iodine. Barrington apparently assumues that this iodine could have been accumulated directly from the seawater. However it is suggested by the evidence of iodine in the tunic of _Pyura_ and _Ascidia_ that it could be, and probably is, transported to the cuticle from the blood stream by the migration of the fibres and granules from the walls of the blood vessels and from the epidermis to the cuticle. This migration of fibres and cellular components was studied by Endean (1955, 1961).

Since the cuticle is sloughed off continuously it would be reasonable to suppose that there is a continuous passage of iodinated proteins through the tunic, the proteins being incorporated into

the substance of the fibres and granules during their formation and subsequently eliminated from the animal at the tunic surface. This supposition is in agreement with the presence of protein bound iodine in the seawater examined for the excretory products of <u>Ciona</u> (4.8). It would not be reasonable to suppose that the cuticle shedding contributes a major part to the excretory mechanisms of ascidians,although definite excretory organs are not present in most of them.

It seems probable then that the iodine in the cuticle originates partly,if not wholly,from within the animals. That iodine is not absorbed directly from the seawater by the cuticle was demonstrated,in <u>Pyura</u> for example,by the absence of radioiodine from pieces of cuticle partially detached from the underlying cuticle-forming layers,in which iodine was accumulated amongst the fibres and cell fragments. Therefore Barrington's hypothesis that the assimilation of iodinated fragments of ingested cuticle led to the development of a metabolic use for that element by ascidians must be re-examined,since the iodine must be present in the blood before it can be utilised in the formation of the cuticle. Undoubtedly fragments of iodinated cuticle will be amongst the ingested detritus upon which ascidians feed but the iodine in them is in a much less chemically active form than the inorganic iodine compounds present in the seawater. The presence of iodine in the cuticle can therefore be regarded as fortuitous,it being associated primarily with the blood plasma and thence the cells which carry iodinated

tyrosine compounds from the blood stream to the tissues.

3.9 Larvae

Larvae of *Ciona* were bred in the laboratory by mixing eggs and sperm stripped from several adults. The experimental procedure was taken from that published by Berrill (1937). It was found that very dim light conditions were necessary for the successful hatching of the larvae. Under laboratory conditions the larvae began metamorphosis about 12 - 20 hours after hatching. Free swimming larvae were taken from the culture and treated with 0.2 - 0.5 μc/ml ^{131}I in clean seawater for one to twelve hours. They were transferred to filtered seawater to remove excess radioiodide and then dropped individually into the fixative (hot formol-saline). After fixing, the larvae were stained lightly in bulk with borax carmine to make handling easier. Whole larvae were dehydrated and cleared before being placed on microscope slides in a drop of dilute collodion solution. When the collodion was dry the slides were prepared for autoradiography in the usual way. Some groups of larvae were embedded and sectioned at 5μ before autoradiography.

The autoradiographs revealed that the larvae take up iodine in several regions of the body. All the autoradiographs examined showed a fine, even distribution of radioiodine over the outer surface of the larvae, but it did not in all cases extend to the edges of the dorsal and ventral fins and rarely at all into the posterior extension of the fins (Fig.14a). This suggested that the ^{131}I was present in the epidermal cells and their secreted product, the cuticle, but it was not actively absorbed by the substance of the fins which are extensions of the cuticle. This observation was confirmed by

the fact that when the internal structures of the tail are resorbed during metamorphosis the tunic or cuticle is left behind with little or no iodine in it whilst the dedifferentiated cell clumps contain much iodine (Fig.14b).

Internal uptake of iodine was varied. In only two larvae was there any concentration associated with the notochord,and that appeared to be in the walls or sheath. Apart from this no uptake associated with internal structures was seen in the tail region.

In the trunk region some larvae showed a slight concentration in the area dorso-posteriad to the sensory organs which is presumptive neural complex (Willey,1893). This concentration was more marked in the metamorphosing larvae,where the iodine present in the resorbed cells passed into the trunk (Fig.15b).

No larvae,of any age or duration of treatment with radioiodine ,showed any uptake by the otolith or the ocellus.

In the ventral pharynx the most common condition was a patchy dense uptake at or near the mid-ventral line,but a few larvae were seen in which there was a definite concentration in the pharynx, which may have been due to accumulation by the cells lining the pharynx,that is the presumptive endostyle. This was more noticeable in attached larvae,during metamorphosis. In several such larvae some accumulation by the anterior part of the trunk was noticed. This part of the larva is not directly connected with the definitive tissues of the adult,being mostly concerned with the adhesion of the larva prior to metamorphosis. During metamorphosis the structures of the tail are resorbed into the trunk in what appear to be large

clumps of cells, though the regular arrangement of these clumps may be due to the shrinking of the epidermis, as suggested by Berrill (1937). Almost all of the tail structures are utilised in the production of the ascidiozooid in this way; only the tunic or cuticle is left behind. The larva in Fig.15a shows this process nearly completed; the stump of the notochord is seen protruding from the trunk and the anterior quarter of the tail contains many large clumps of dedifferentiated cells with which is associated a considerable quantity of radioiodine. There is no iodine in the vacated posterior portion of the tail except in a few scattered cells. In the same larva an increased concentration of radioiodine is seen in the posterior part of the trunk and an accumulation of it is seen in the pharynx (Fig.15b).

According to Berrill (1955) the tadpole larva of the ascidians is a creation of their own, especially suited to their needs for a motile, habitat seeking phase in the life-cycle, which by neotony gave rise to the ancestral vertebrate stock. Ciona is thought by Berrill to be the most primitive of the modern ascidians and its larva likewise. The physiological phenomena of the cionid larva are therefore more likely to be allied to those of the vertebrates than are those present in other tunicates, or even in Amphioxus, which is considered to be an offshoot of the evolutionary line.

Several important questions arise from the foregoing observations; (1) has the iodine any metabolic significance for metamorphosis? (2) did this metabolic use, if any, originate with the

ascidian larva? (3) is the accumulation of iodine inherited from a pre-ascidian ancestor, regardless of its metabolic significance?

The very extensive and rapid accumulation of so much iodine suggests that it may have a metabolic significance. If the binding of iodine by the epidermis of the tadpole larva did not originate in the ascidians then an inherited metabolic necessity for binding iodine could be implied, the larva utilising its major absorbtive area, the epidermis, for this purpose since no functional pharynx and gut are present. It could also be that the ability to accumulate iodine in the epidermis was inherited from a pre-ascidian form and that it assumued metabolic significance when combined with the phenomenon of metamorphosis found in the ascidian larva. In this case it would not be surprising to find that the epidermis of the adult retained some of its primitive capacity to bind iodine. Barrington (1956) mentioned that some occasional traces of iodine had been seen in association with the epidermis of adult Ciona and this has also been the case with the work reported here. The occurrence of iodine in the epidermal glands of some Hemichordates (Gorbman, 1955; Thomas, 1964) merits attention in this respect, since it demonstrated that iodine can be bound by external tissues other than scleroproteins.

If the iodine compounds present in these larvae can be shown to exert a definite metabolic effect, most probably on the rate of metamorphosis, then it would be safe to conclude that the ascidians, if not other tunicates, are closely allied to the main vertebrate stock. It has already been shown that thyroxin, amongst various

other substances,exerts a considerable effect on the rate of metamorphosis in ascidian larvae (Grave and Nicoll,1940;Berrill,1947; Weiss,1928). Grave (1944),from experiments on <u>Styela partita</u> concluded that the larvae produce a substance which accelerates metamorphosis and its concentration increases throughout the larval life until a lethal concentration may be reached. Could it not be that the iodine bound by the larval epidermis is in a compound which so affects the metabolic rate,and hence the rate of metamorphosis,that a lethal concentration of the compound might arise under laboratory conditions and even in some individuals in nature? A dependence on a minimum concentration of such a compound for the completion of metamorphosis would necessitate a sufficient supply of iodine. Once the dependence was established then metamorphosis would be delayed if insufficient iodine was available,as might happen in the less saline environment of the estuaries in which the evolution of the vertebrates is supposed to have occurred.

3.10 The Effect of Goitrogens

Some macroscopic effects of the antithyroid drugs used were noted. In the intact animal it was seen that the muscle tone was very poor and the nerve reflexes slow after treatment. Also the tunic was visibly swollen in such species as Ciona and Ascidia. On opening the tunic it was seen that the heart and blood vessels were distended. The gonoducts contained a great accumulation of gametes which had not been discharged normally. The endostyle was also seen to protrude further into the pharynx than was usual, but whether that was due to hyperplasia of the endostyle or to the distension of the sub-endostylar blood vessel was not immediately clear.

Sections of ascidians treated with goitrogens showed that the histological change which they caused was an accumulation of fluid in cells with secretory functions. Such cells had greatly enlarged vacuoles. This was very obvious in the mucus secreting cells of the endostyle, that is zones 1,2,4,6 and to a smaller extent zone 7. (Fig 16) The cuboidal epithelium of the pharynx was also generally enlarged by swelling of the vacuoles. The gut epithelium was likewise affected and also the follicle cells of the ovary. There was some thickening of the collagenous walls of the sub-endostylar blood vessel in Ciona and also of the tissue supporting the lips of the endostyle. In the endostylar appendix the epithelia of the roof showed some hyperplasia, which was more noticeable in the columnar, ciliated epithelium than in the cuboidal epithelium.

The binding of radioactive iodine into proteins was completely

suppressed both by KCNS and by methyl thiouracil. No activity was recorded by the stripping film technique of autoradiography from histological sections. Although chromatographical analysis of similarly treated ascidians showed that the uptake and binding of radioactive iodine was not completely suppressed by these agents (4.3 - 4.6) it is quite understandable that the very small amounts of activity present in a pooled homogenate of several goitrogen treated *Ciona* would not be detectable on 7μ sections from individual animals.

The absence of radioactive iodine from sections of thiouracil treated ascidians is a confirmation of the claim by Leblond and Gross (see 3.3) that only P.B.I. remains in histological sections since iodinated amino acids and much iodide were detected by chromatography of similarly treated animals (Part 4).

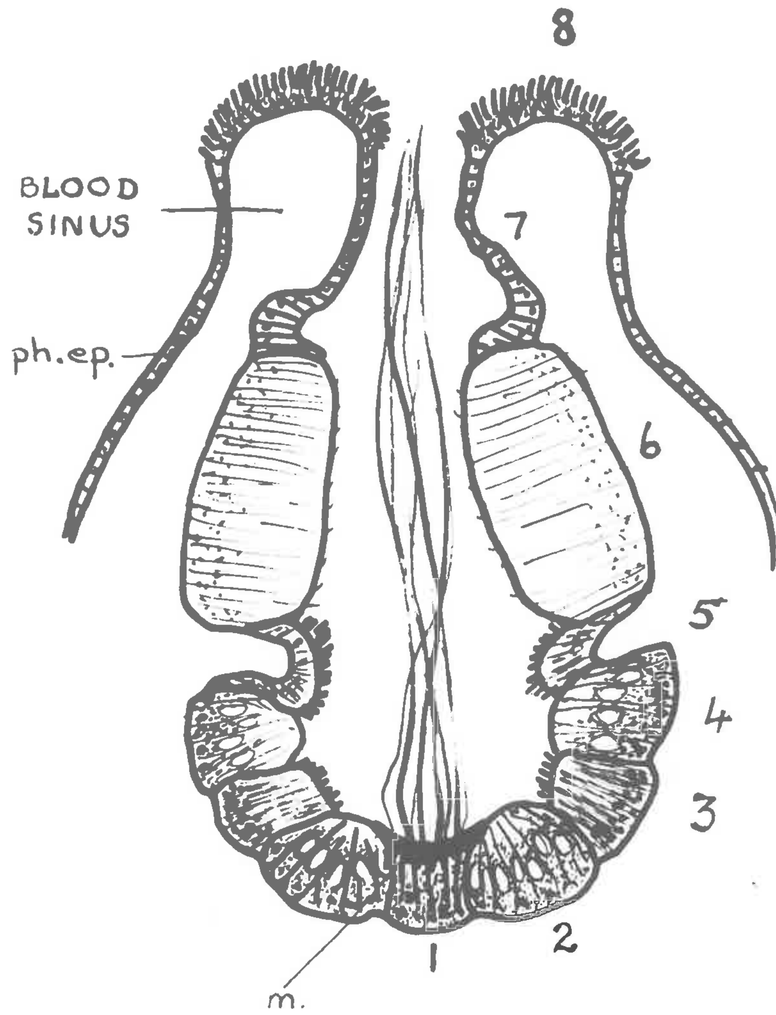


FIG. 3a. Diagrammatic drawing of an ascidian endostyle to show the zones as numbered by Barrington.

m = mucus, ph.ep. = pharyngeal epithelium

FIG. 3b. Autoradiograph of T.S. endostyle of *Ciona*. ^{131}I is present at zones 7 and 8. Both intra- and extra-cellular ^{131}I is seen in both zones.

FIG. 3c. Autoradiograph of a section through the ovary of *Ciona*. treated with ^{131}I for three days. ^{131}I is present in all stages of oogenesis;it is seen in both cytoplasm and nuclei.

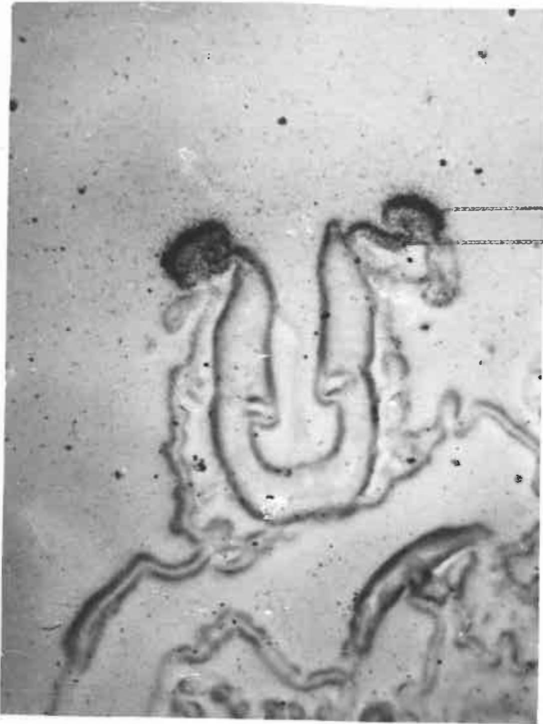


FIG.3b.

zone 8 with ^{131}I
zone 7

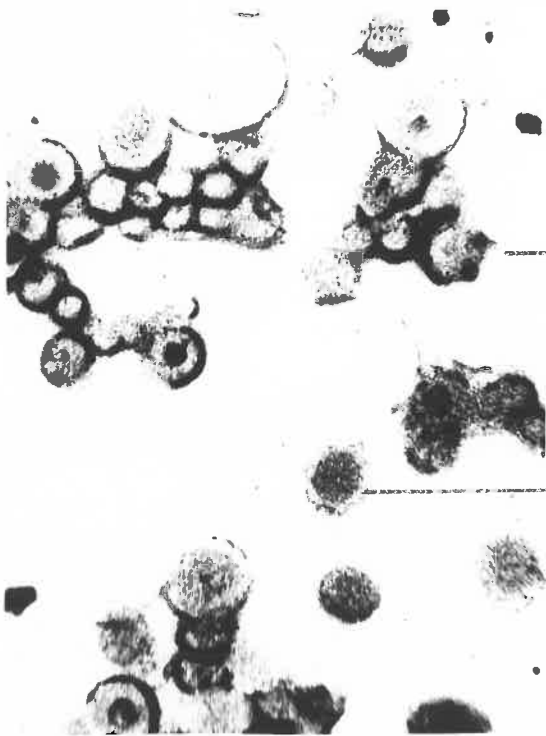


FIG. 3c.

oocytes

free ovum

FIG. 4a. T.S. endostyle of _Botrylloides leachii_. Autoradiograph. ^{131}I is accumulated over zones 7 and 8; also on the pharyngeal epithelium.

FIG. 4b. Autoradiograph of section of tunic of _B. leachii_ from edge of colony. Section includes an ampulla. ^{131}I present in the cuticle and matrix of tunic and epithelial lining of ampulla.

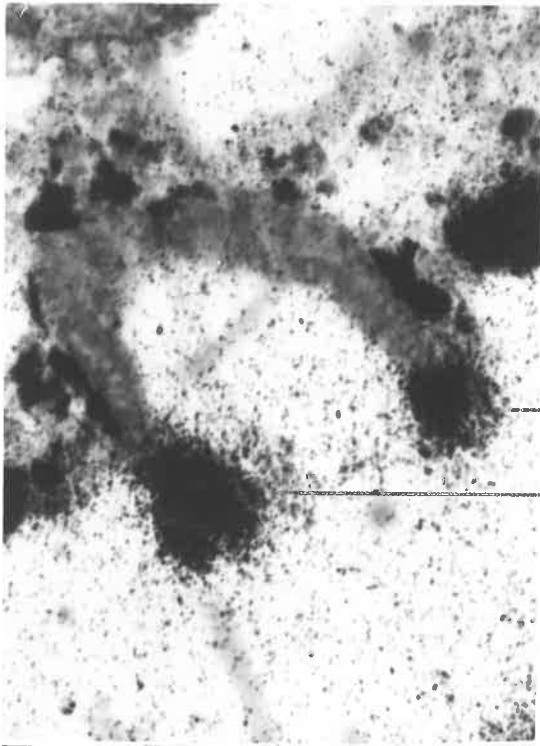


FIG. 4a.

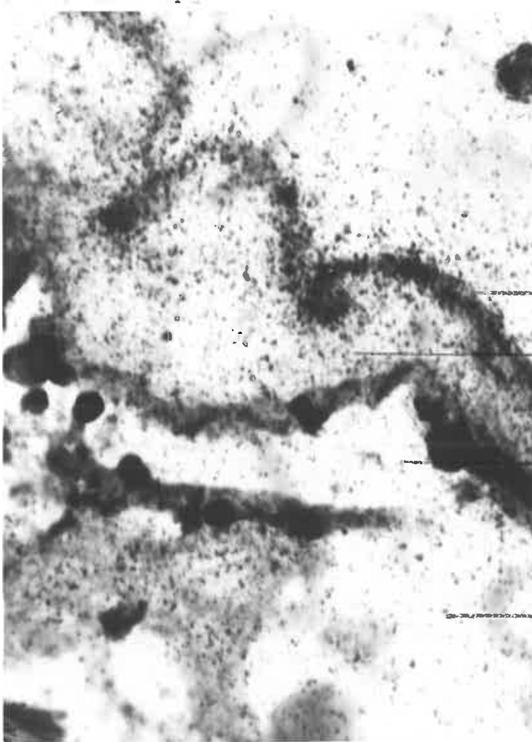


FIG. 4b.

FIG. 5a. Autoradiograph of T.S. abdominal region of *Ciona* showing passage of ^{131}I through the intestinal epithelium and some accumulation in the surrounding testis follicles.

FIG. 5b. Autoradiograph of T.S. testis of *Ciona*. Uptake of ^{131}I denser in the centre of the follicles, i.e. in the spermatids.

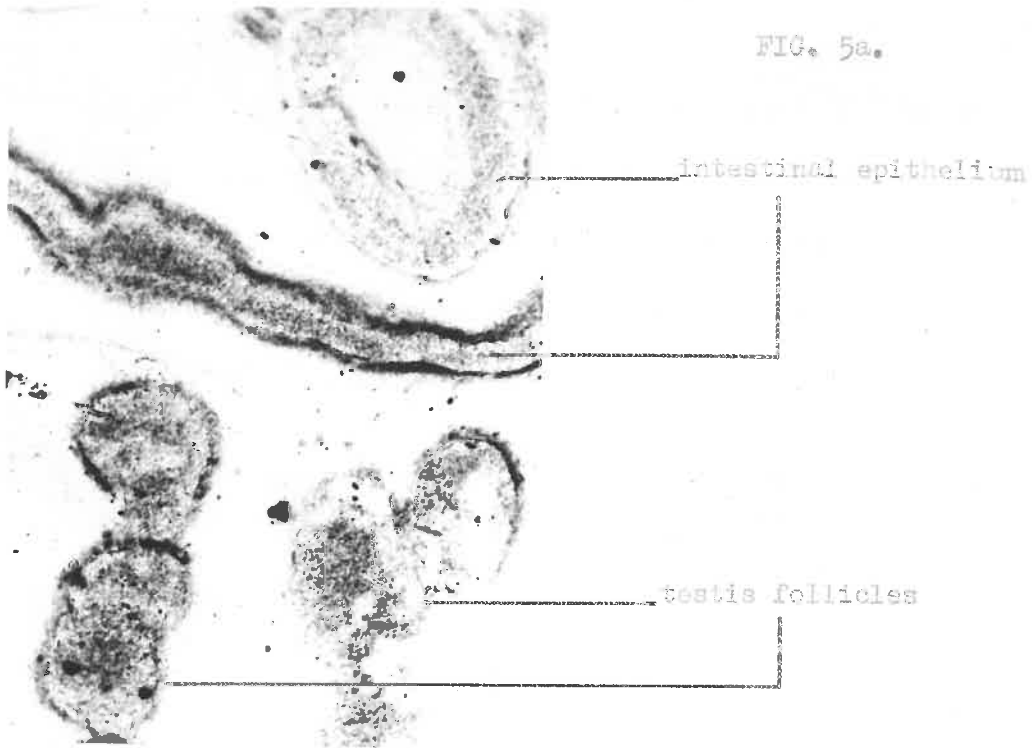


FIG. 5a.

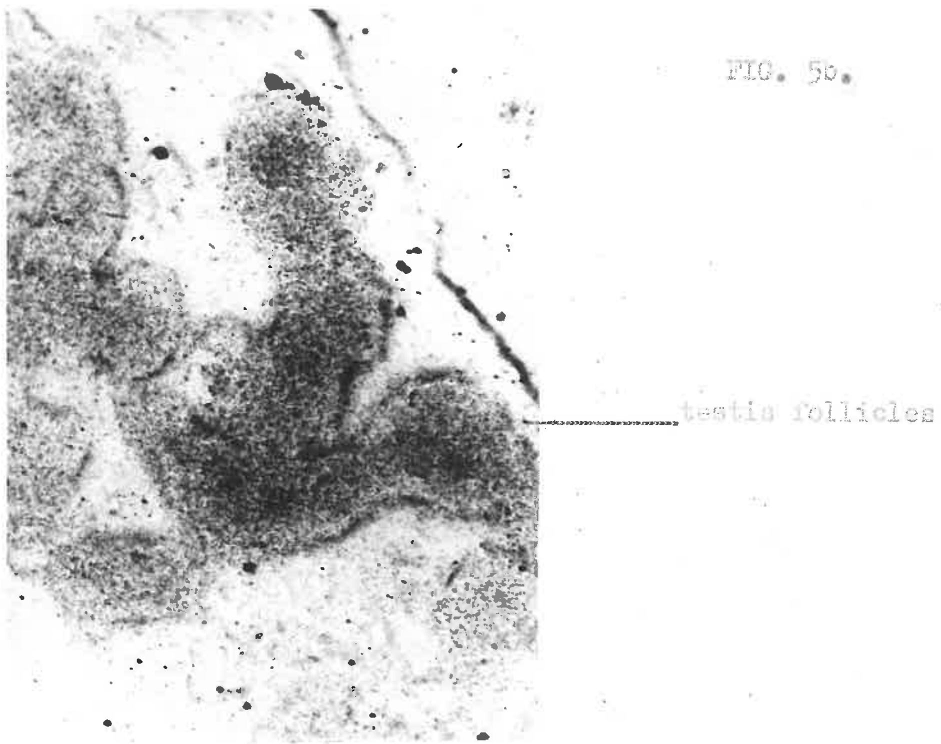
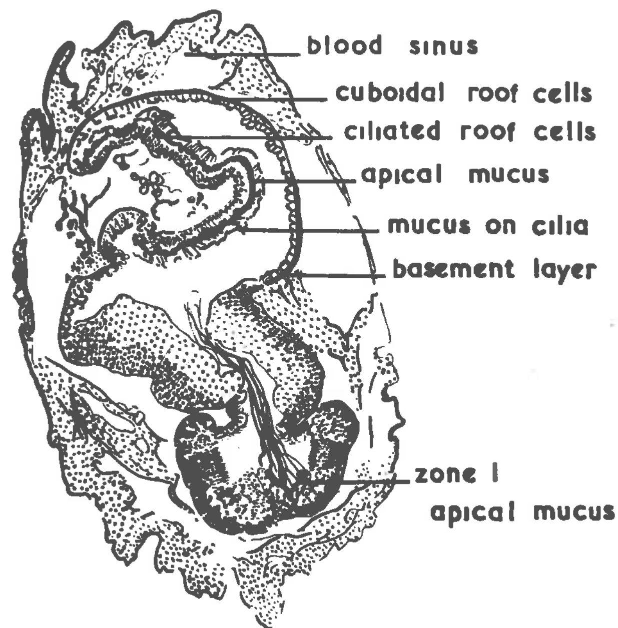


FIG. 5b.



Endostyle Appendix x150 P.A.S.

FIG. 6. Drawing of T.S. endostylar appendix of _Ciona_, stained with Periodic Acid - Schiff reagent. Section typical of proximal half of the appendix and includes a group of zone 7 cells on both sides between zone 6 and the roof cells. The zone 7 cells are absent from the distal third of the appendix. Reduced during photography to x 50.

FIG.7a. Autoradiograph of T.S. endostylar appendix of _Ciona,_ showing ^{131}I accumulated along the surface of the ciliated columnar epithelium. There is a dense accumulation in the apex of the roof.

FIG. 7b. T.S. of an adjacent section to Fig. 7a stained with Alcian Blue, which shows the mucus on the surface of the columnar epithelium.

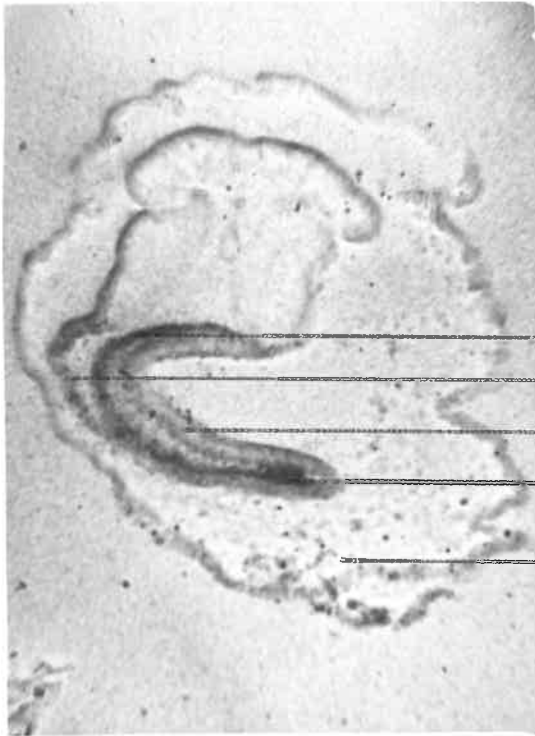


FIG. 7a.

FIG. 7b.

FIG. 8a. Section through the heart of *Ciona* stained with Alcian Blue. The myocardium shows cross fibres which stain blue. The pericardium contains a smaller number of these fibres. The clump of cells present also stained deeply wbth Alcian Blue.

FIG. 8b. Autoradiograph of section through stolonic bud of *Podoclavella* containing undifferentiated cells. ^{131}I is distributed throughout the bud. H.P.

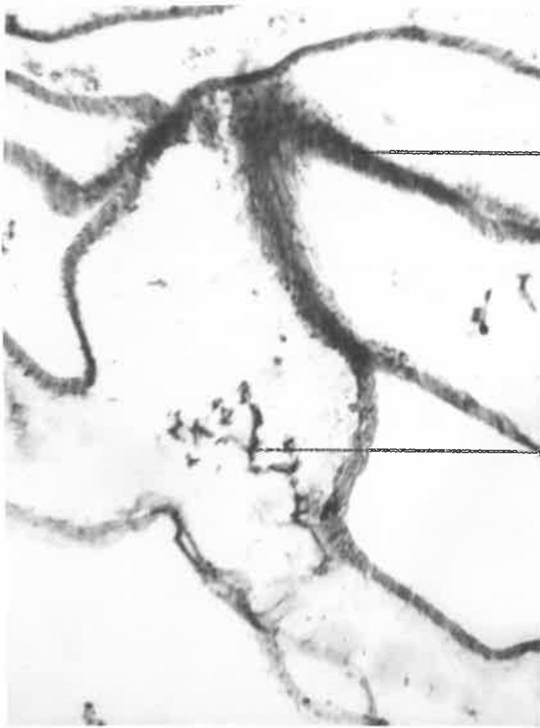


FIG. 8a.

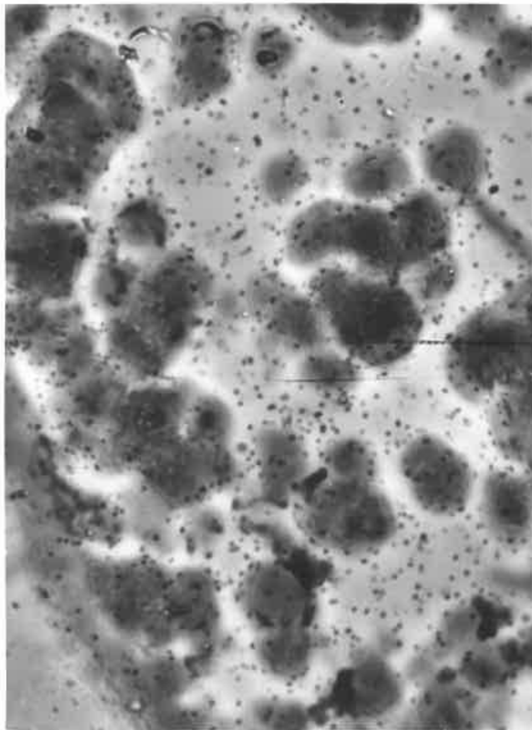


FIG. 8b.

FIG. 9. Autoradiograph of section through the heart of *Ciona* treated with 1.0 μc/ml for three days. Dense uptake by the pericardiac body and myocardium; uptake also by the pericardium and blood clots.

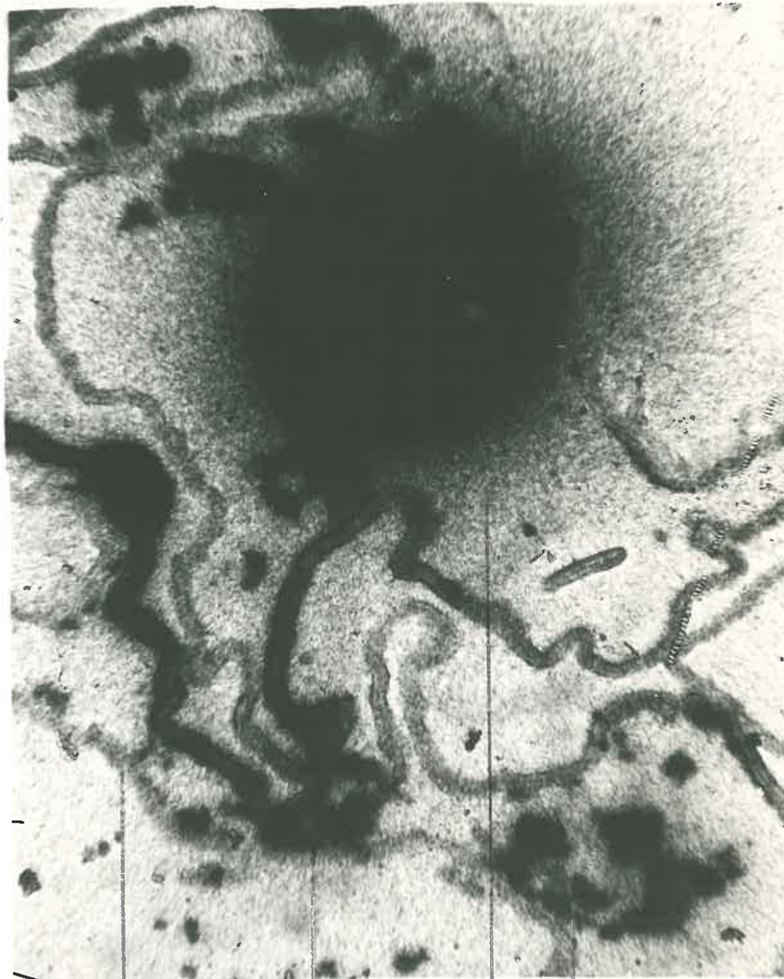


FIG. 9

FIG. 10a. Autoradiograph. T.S. *Styela* testis follicles showing interstitial ^{131}I.

FIG. 10b. Autoradiograph. T.S. *Ascidia* body wall and tunic, showing ^{131}I associated with muscle blocks and the matrix of the tunic.

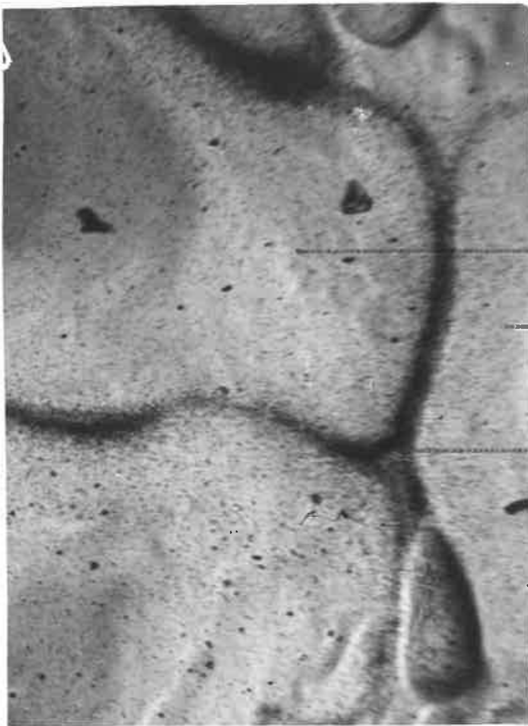


FIG. 10a.

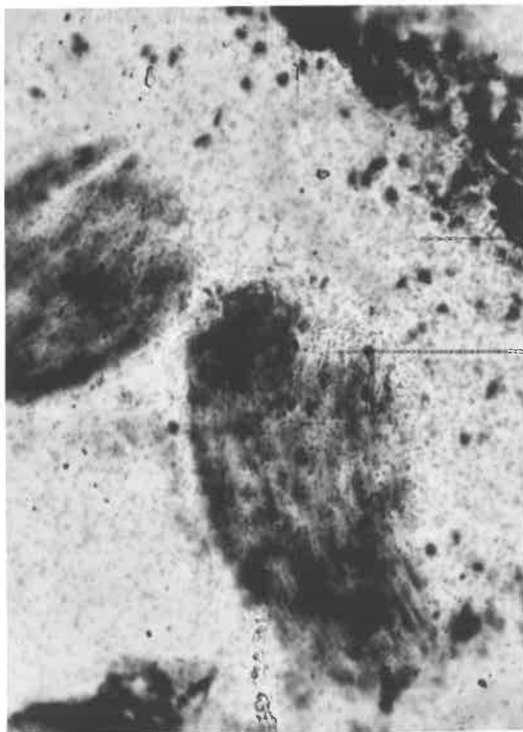


FIG. 10b.

FIG. 11a. Autoraiograph of section through blood vessel in the tunic of _Pyura_ showing decreasing density of ^{131}I away from the walls of the vessel.

FIG. 11b. Autoradiograph of section through the tunic of _Podoclavella_ showing ^{131}I accumulated at walls of vessel.

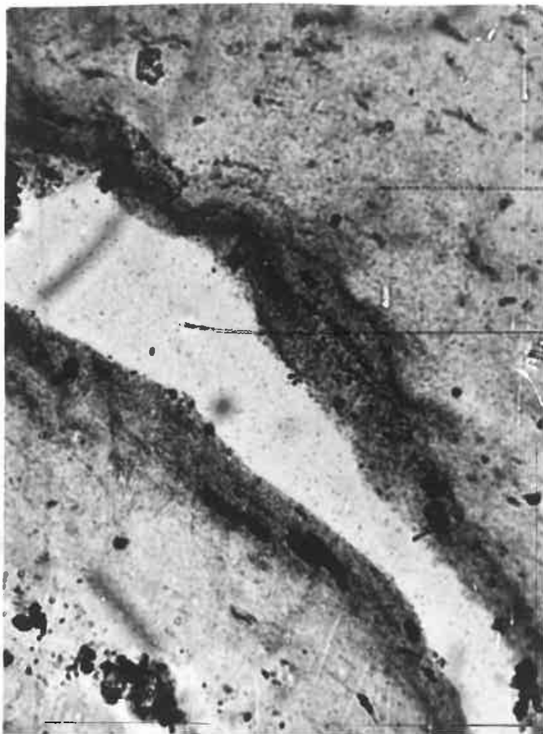


FIG. 11a.

matrix

blood vessel

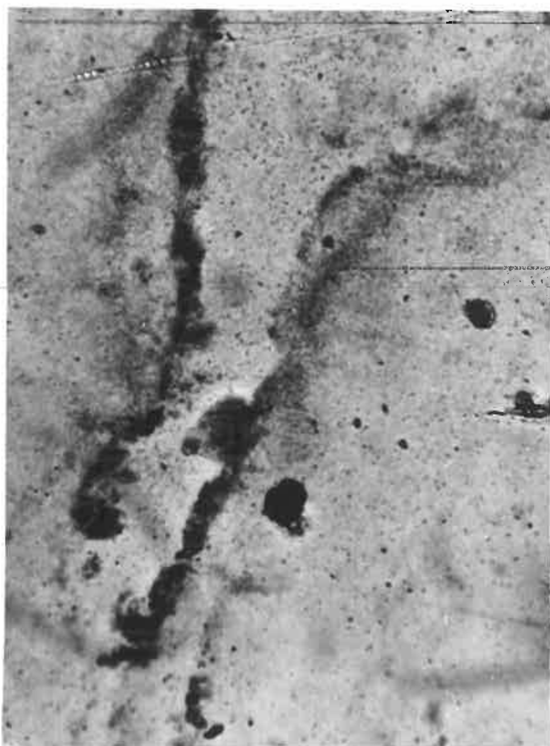


FIG. 11b.

^{131}I in walls of vessel

FIG. 12a. Autoradiograph of a section through the tunic of *Pyura* with a piece of cuticle almost detached which contains no ^{131}I. Photograph taken immediately adjacent to lower figure, which contains much ^{131}I in the cuticle. Section also shows ^{131}I associated with cellular and fibrous elements in tunic matrix.

FIG. 12b. As above, showing low density of ^{131}I in the inner matrix and greatly increased density in the sub-cuticular layers and cuticle.

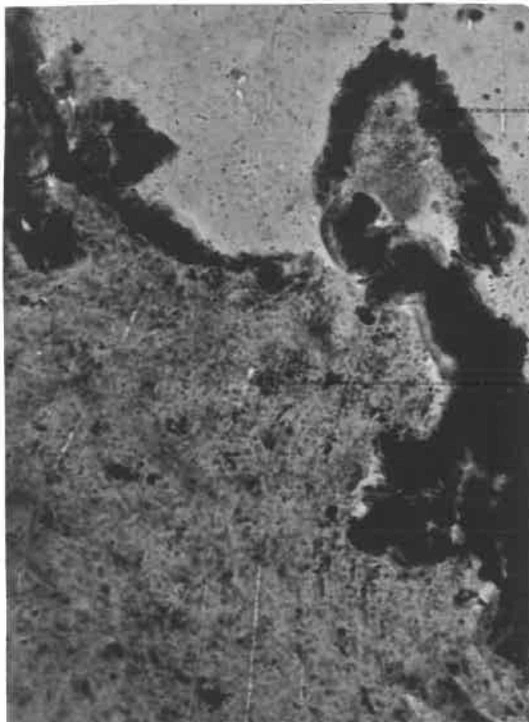


FIG. 12a.

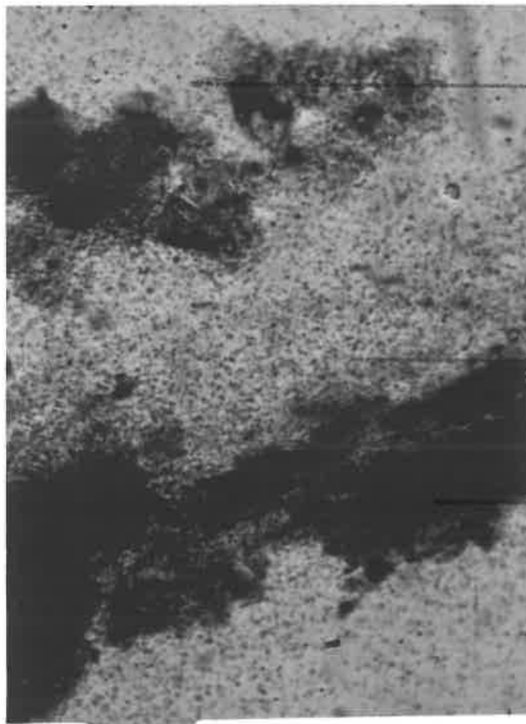


FIG. 12b.

FIG. 13a. Autoradiograph of T.S. _Ascidia_ tunic, showing the association of ^{131}I with large cells, chains of which pass from the inner tunic to the cuticle.

FIG. 13b. Autoradiograph of T.S. _Ascidia_ tunic, showing the incorporation of the cells containing ^{131}I into the cuticle.

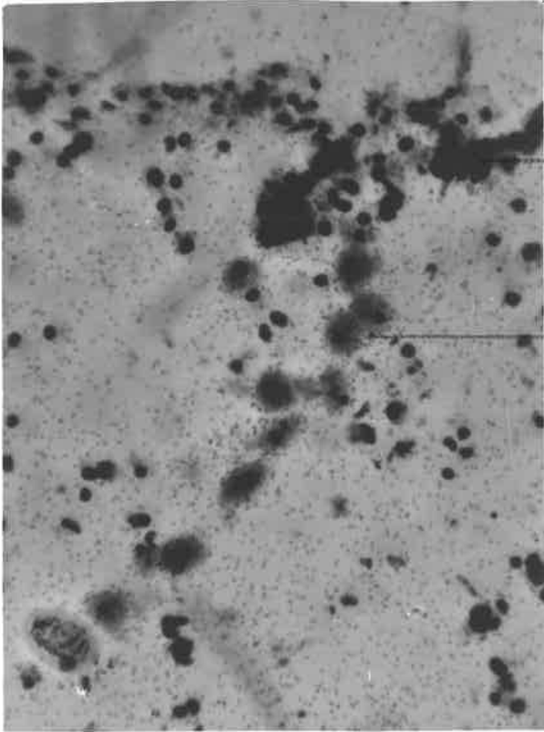


FIG. 13a.

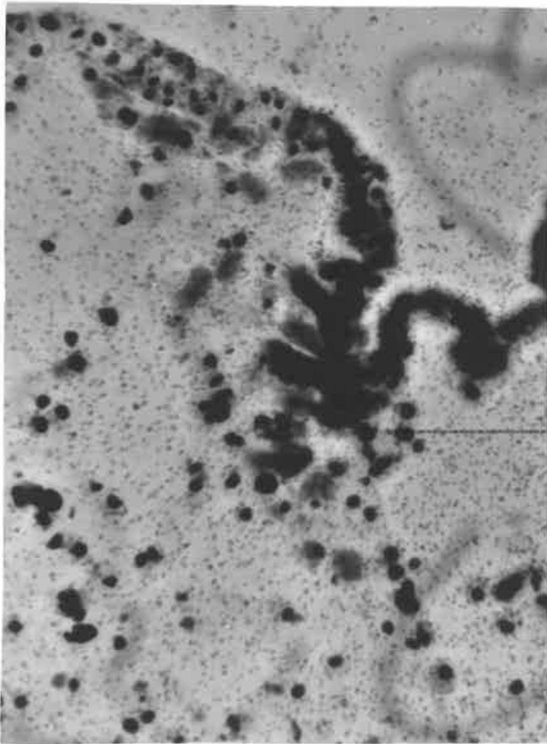


FIG. 13b.

FIG. 14a. Autoradiograph of whole mount of a free-swimming larva of *Ciona*. ^{131}I is present over the surface of the body but does not extend into the region of the fins. There is no concentration of ^{131}I in the sensory vesicle.

FIG. 14b. Autoradiograph of whole mount of *Ciona* larva at commencement of metamorphosis. Most of the tail has been resorbed into the trunk. The anterior part of the tail contains ^{131}I in association with the cell clumps. There is much ^{131}I in the trunk.

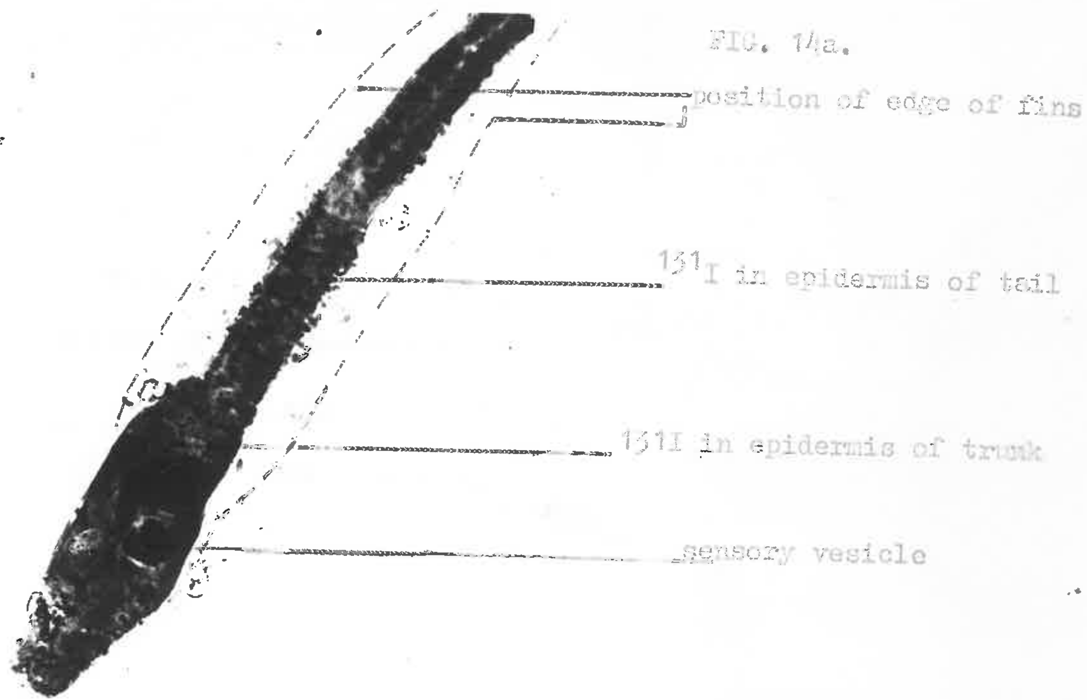


FIG. 14a.

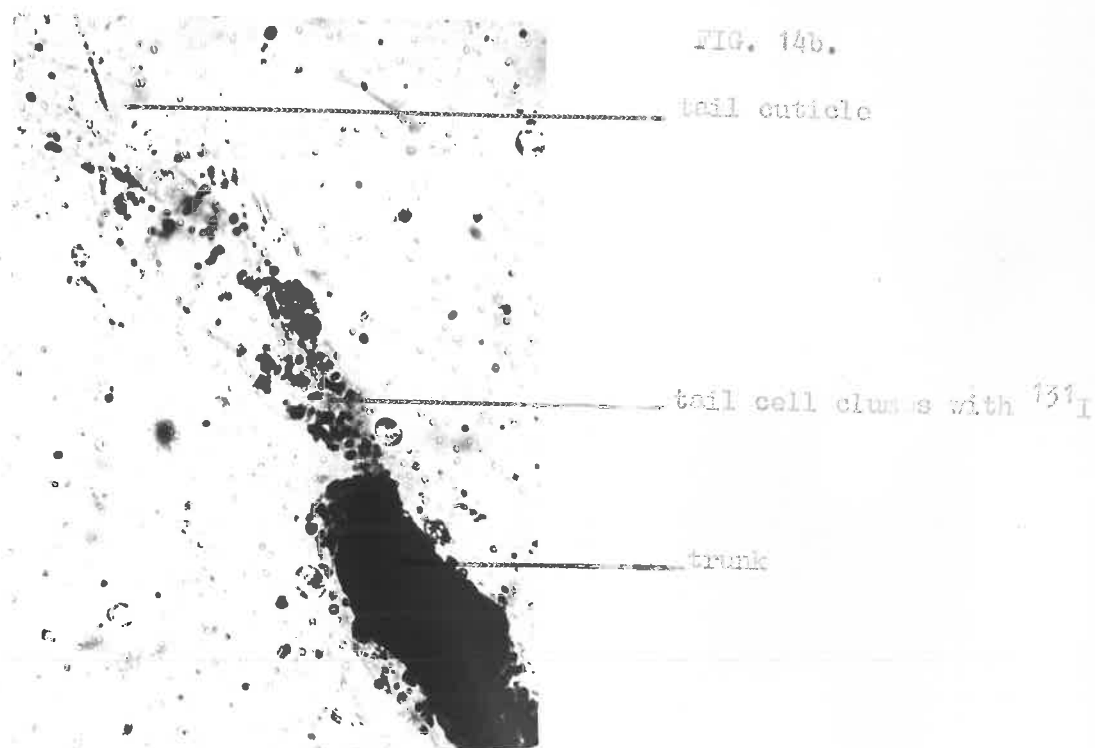


FIG. 14b.

FIG. 15a. H.P. of Fig. 14b showing the passage of ^{131}I into the trunk with the resorbed tail cells.

FIG. 15b. Autoradiograph of whole mount of metamorphosing larva, showing very dense uptake of ^{131}I in posterior trunk and accumulation of ^{131}I in the pharynx and anterior trunk region.

FIG. 15a.

FIG. 15b.

FIG. 16. T.S. endostyle of *Ciona* treated with methyl thio-uracil. Alcian Blue / Chlorantine Red stain. Shows the enlarged vacuoles in zones 2,4,6,7 and 8 and in the pharyngeal epithelium; distortion of the zones and distension of the collagen around the sub-endostylar blood vessel.

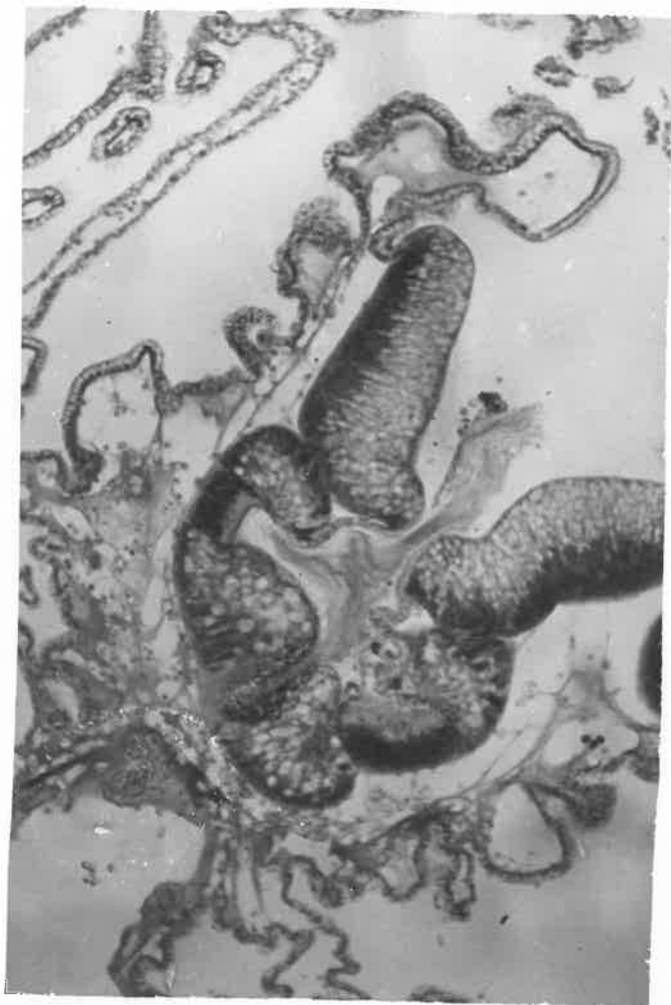


FIG. 16

Part 4. IODINE COMPOUNDS PRESENT IN *CIONA*

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4.2 Hydrolysis page 55

4.3 The blood iodine compounds page 62

4.4 The endostyle iodine compounds page 68

4.5 The gonadal iodine compounds page 73

4.6 The tunic iodine compounds page 74

Tables 1 to 5

Figures 17 to 24

4.1 Material and methods

The simple ascidian *Ciona intestinalis* was used almost exclusively for this work. *Pyura stolonifera* gonads were used for some experiments. *Ciona* was chosen because it was easier to dissect out the various organs required from it than from other species and it was particularly easy to obtain the blood. Also it was obtainable for several months of the year.

Ciona were collected as required for experiments. When less than 5 cm. long about ten were used for experiments, or parts thereof. Less than ten of the larger animals were needed in order to obtain adequate concentrations of ^{131}I in the samples but the number used was as large as possible in order to eliminate the effects of individual variations in the compounds. Immature zooids were not mixed with mature ones in these experiments.

The *Ciona* were treated with Na^{131}I at an initial concentration of 0.8 - 1.0 μc/ml for varying times according to the object of the experiment. 1.0 μc/ml represents an increase of 0.015 per cent in the iodide content of the local seawater, at chlorinity 19.38 $^{o}/_{oo}$. The addition of this much iodine would not therefore cause an abnormal increase in the iodine content of the medium, inspite of the range of salinities experienced in the locality where *Ciona* was collected.

For qualitative examination of the ^{131}I compounds before and after hydrolysis a period of two or three days was allowed. During this time a little extra food was added (as dried liver powder). Five or six animals of 7 - 10 cm length were treated in each liter.

of seawater with constant aeration. The water level was kept constant as evaporation occurred. The temperature was not controlled unless the air temperature exceeded 20°C; then a water bath was used to keep the seawater at this temperature. The temperature of the water in which Ciona is found locally regularly exceeds 20°C during the summer months so experimental temperatures up to that were not considered to be detrimental to the animals.

After treatment the Ciona were allowed to expand in normal seawater; they were then drained as thoroughly aspossible. No narcotising agent was added in ase it interfered with the iodine metabolism and because excretion of iodine compounds would have occurred during the period of narcotisation, which usually required several hours.

The tunic was removed and the inner gelatinous layer scraped off it, leaving only the tough outerpart, which included the scleroproteins. All embedded particles were removed before it was cut into pieces p rior to homogenisation.

The body wall was cut transversely across the abdominal region. This released the coelomic fliud which was washed away with seawater, after which the animal was blotted dry with Kleenex tissue. The heart was carefully freed from its surrounding membranes and the animal arranged on a tissue so that the heart projected away from the other organs. The tip of the heart was cut off and the blood drained into a receiving tube. Any fluid coming from the body cavities was prevented from reaching the heart of the tube by the

tissue. It was found that far more blood could be collected from each animal in this way than by using a hypodermic syringe introduced into the heart as the very thin walls of the heart invariably collapsed and blocked the mouth of the needle. Cutting the heart released the fluid from the pericardial cavity and this was therefore included in the fluid termed 'whole blood'. However there was no detectable difference in the iodine compounds obtained by the two methods.

The pharynx was then opened by a lateral,longitudinal incision. The endostyle was removed with as little adherent tissue as possible. The endostylar appendix was also excised and pooled with the endostyles.

The ovary was taken out and the gut cut at the beginning and end of the region covered by the diffuse white testis. The gut was carefully freed of any contents. Attempts to free the testis from the gut wall were not successful. The gonads were combined after early experiments had shown that both testis and ovary had the same iodine compounds.

The endostyle,gut,ovary and tunic were dipped into distilled water before being collected in ice-cold phosphate buffer at pH 8.2. As little buffer as possible was used to avoid diluting the samples. The blood was diluted with the same amount of buffer. About 100 mg of methyl thiouracil were added to the collections of organs and the blood to prevent artefact formation in the presence of any free iodine which might be liberated (Taurog et al.,1955;Gleason,1955).

Some fine glass powder was then added also and the organs homogenised finely by an electric homogeniser. The homogenates were then coarsely filtered through glass wool and thefiltrates ,containing fine particles,were collected in graduated tubes. The volume of filtrate was between two and three milliliters. The filtrates were divided into two equal parts if hydrolyses were to be done. The bl blood was well stirred then divided likewise. An extra 50 mg methyl thiouracil was added to the portions to be hydrolysed to prevent the utilisation of any free iodine released during hydrolysis. The other halves of the filtrates were frozen until used for chromatography as unhydrolysed samples,in future referred to as the 'homogenates'.

Chromatographs were prepared of equal aliquots (0.3 mls) of the homogenates and hydrolysates. The aliquots were measured with an Agla micrometer syringe fitted with changeable L -shaped glass capillary tubes,intstead of the usual hypodermic needle. The samples were applied as 2 cm strips across the origin line of the chromatographs. In intial experiments the samples were applied to a complete sheet of paper. It was often noticed that variable degrees of spreading of the samples occurred as they were carried down the paper. This made quantitative estimation of the iodine compounds in a strip cut from the sheet inaccurate. To overcome this the p aper was divided into the necessary number of 2 cm strips before development by cutting out 1 cm strips from just above the origin line to within two cms of the foot of the paper. This meant

that all the applied sample was contained in the 2 cm strip. The major drawback to this method was that the solvent front was not at the same level right across the paper because of interference by various agents in the samples. Chromatography on whole sheets of paper eliminated these variations by the lateral pull of the solvent front on adjacent areas. The variation in the solvent front in the pre-cut strip method was only significant in the gonad samples developed in B.D.A. and initially led to some misinterpretation of the results. The effect was eliminated by extracting the homogenates with chloroform before applying aliquots of both the aqueous and chloroform layers to the paper.

Whatman No.1 paper was used for all experiments. Standards of mono- and di-iodotyrosine, triiodothyronine and thyroxin (Na salts) dissolved in n-butanol were applied to the same sheet of paper as the samples. In this thesis the abbreviations used for these iodo-aminoacids are those proposed by Harrington and Pitt-Rivers (1957), as follows -

monoiodotyrosine - MIT	thyroxin - T_4
diiodotyrosine - DIT	iodide - I^-
triiodothyronine - T_3	protein bound iodine - P.B.I.

Duplicate chromatographs were developed with two solvents systems to check the identification of compounds. One system was the diphasic n-butanol - dioxan - ammonia /4 - 1 - 5 (Gross, Leblond Franklin and Quastel, 1950), which is abbreviated to B.D.A. The other solvent was the monophasic n-butanol - acetic acid - water /78 - 7 - 15, abbreviated to B.Ac. The latter gives good separation

of MIT and DIT but differentiates poorly between the thyronines, which are carries close tot the solvent front. The B.D.A. separates T_3 and T_4 well but does not differentiate so clearly between MIT and DIT. Iodide is carried to different Rf values by these solvents.

The chromatographs were developed in ascending solvent for 12 - 15 hours at 25°C to about 30 cm length. The papers were then thoroughly air dried before being sprayed with a solution of ninhydrin (0.5 per cent in 95 percent ethyl alcohol) to locate the standards.

In some experiments the relative amounts of the various iodine compounds were determined by passing the 2 cm wide strips of paper containing the chromatographed samples below the window of a GM4 tube. The strips were pulled through a special lead holder to prevent radiation from the remainder of the strip being counted as well as that passing through the slit in the lead holder. The slit was 0.5 x 2.0 cm. The strips were then placed against Kodak Medical X-ray film for autoradiography and exposed for at least ten days. The films were developed in D 19b.

The activity of the consecutive sections was plotted against the distance of each section from the origin,for each sample. In many experiments the radioactive peaks were quite distinct and there was almost no activity recorded from the intervals between the peaks. In such cases the percentage compostion was easily calculated from the summation of the activity for each peak. Some experiments however, and particularly the blood samples,gave chromato graphs which had a variable but continuous residual activity

along the strip, which usually ceased about 10 cm or less behind t the front. In such cases it was sometimes difficult to be certain just where the limits of the peaks were. The limits were therefore determined from the subsequent autoradiographs, since autoradiography was much more sensitive to low activities than the scanning equipment used and the true peaks were better defined. The activity of such peaks was still calculated from the scanning data and gave results in good agreement with those from clear chromatographs. It is considered though that the data derived from the scanning of the chromatographs, whilst giving a good indication of the composition of the samples, should not be taken as absolute. Errors due to the residual activity would cause an increase in the relative amounts calcultaed for the compounds with the least activity which may therefore be smaller than the amounts given.

Where the relative amounts of the iodine compounds are given the figures refer only to the activity of the compounds. No attempt has been made to translate these activities into the amounts of the actual compounds, since it is not certain how many of the iodine atoms in say, T_4, are radioactive, because the thyronines may not be composed entirely of radioiodinated tyrosines.

For convenience of reproduction the autoradiographs of the chromatographs have been traced. The cross-hatching is intended only as a guide to the density of the activities of the various peaks on the same chromatograph.

4.2 Hydrolysis

To determine which iodo- amino acids were incorporated into proteins hydrolyses of those tissues known to contain protein boundiodine were done. These hydrolysates were compared with unhydrolysed samples of the same tissues and differences in the amino acid composition were determined by scanning the radioactive chromatographs and by subsequent autoradiography of those chromatographs. Technical and theoretical information for this work was obtained from Block, Durrum and Zweig (1958), Fieser and Fieser (1950), Schmidt (1938), Cowgill and Pardee (1957) and Meister (1957).

This work was not attempted until a number of unhydrolysed samples had been studied so that the variations in tissue iodine content had been observed. The effects of ^{131}I concentration in the medium and the duration of treatment were also studied before hydrolyses were done.

The duration of treatment was an important factor. Since there is a progressive incorporation of iodine, firstly from iodide into amino acids, thence into protein, the maximum amount of protein bound iodine would not necessarily coincide with the equilibrium stage between uptake and excretion of iodine. The rate of incorporation of ^{131}I into protein was studied in the blood and some other tissues (4.3 et seq.). From there results it was decided that treatment for three days before hydrolysis of the tissues would give the best results. Prolonging the treatment would have given a higher P.B.I. content but a considerably lower total activity which would have made scanning, and even autoradiography,

inefficient.

Several methods of hydrolysis were tried so that the results could be compared. Enzymatic hydrolysis was the first to be considered since it is the most natural way of breaking down substances which pass through the alimentary canal, such as the endostylar secretions. The preparations of trypsin and pancreatin which were immediately available proved to be largely inactive, having been stored for some years in uncontrolled atmospheric conditions. Some fresh pancreatin (Light's 3x U.S.P.) was ordered by airmail and stored in a refrigerator on arrival. It was used over a period of several months and gave apparently similar results all the time. An experiment done when it had been stored for more than one year however showed that its hydrolytic powers were not as strong as they had been.

Samples which were to be hydrolysed with pancreatin were collected in veronal or phosphate buffer at pH 8.2. One half of the prepared homogenate was frozen at once, whilst to the other half was added approximately 100 mg pancreatin. The hydrolyses were carried out in 15 ml centrifuge tubes which were thoroughly cleaned and oven dried at 100°C. They were stored inverted until used. The usual expedient of covering the hydrolysis mixture with a thin layer of toluene was abandoned when it was observed that some of the radioactive iodine passed into the toluene, or into any other oil used to prevent the entry of airborne bacteria into the mixture. Instead the clean, dry tubes were not turned mouth upwards until the final mixture was poured in; they were then sealed immediately

with Parafilm. No more noticeable putrifaction occurred with this method than with the toluene method. The tubes were then placed in an incubating oven at 38°C for three days. The hydrolysates were centrifuged to settle the sediment before aliqots were taken of the supernatants for chromatography.

Hydrolyses were also tried with HCl, $Ba(OH)_2$ and NaOH. Only the NaOH gave really consistent results. The various amino acids were sometimes detected by autoradiography but it was evident that deiodination was occurring in HCl and $Ba(OH)_2$ hydrolysates. There was usually much evidence of iodine towards and at the front of chromatographs which was interpreted as being due to artefacts and elemental iodine formed during the hydrolyses. Hydrolyses done with HCl always showed iodine at the front of chromatographs developed with B.Ac., which was considered to be elemental iodine released from the tissues by the acid medium. Other artefacts were not prominent in HCl hydrolysates. Much iodine was probably lost from the hydrolysates during the removal of excess HCl by evaporation. Attempts to overcome this by neutralising the excess acid resulted in 'salting' troubles on the chromatographs. The best results were obtained by taking N-butanol extracts of the acid hydrolysates and reducing them <u>in vacuo</u> at room temperature.

The hydrolyses done with $Ba(OH)_2$ (by adaptation of the method of Kendall, 1914) resulted in heavy oxidation to iodide and iodine. Some amino acids were detected but the method was not persued further.

Some success was obtained with NaOH as the hydrolytic agent. The use of methyl thiouracil to prevent artefact formation (Gleason, 1955; Taurog, 1955; Kennedy, 1958) greatly enhanced the clarity of the chromatographs although some anomalous results were obtained in some experiments. Iodine compounds which did not correspond to the standards used were located on several chromatographs, but no consistency was observed in the location (Rf) of these unknowns. Mostly they were in the middle region of B.D.A. chromatographs, forward of the iodide bandRf 0.43) but not far enough forward to be identified with the unknowns studied by Kennedy (1958). Some of the faint iodine bands located between about 0.6 and 0.7 in B.D.A. could have been due to iodothyronines other then T_3 and T_4. The most persistent artefacts formed were subdivisions of the iodide band. These are discussed more fully in the section on artefact formation.

The method used for hydrolysis with NaOH was as follows. The tissues were homogenised in distilled water with methyl thiouracil to prevent the further utilisation of iodide. After extraction of the homogenates with $CHCl_3$ and filtering, half of each filtrate was frozen. The other part(1 - 2mls) was made up to 2N by the addition of 5N NaOH. An extra 50 mg of methyl thiouracil was also added. This mixture was placed in a centrifuge tube and a bubble stopper placed in the mouth of the tube. Hydrolysis was carried out in a steam bath for at least 5 hours. The resultant hydrolysate was neutralised by H_2SO_4 and excess acid added to give a pH 3 - 4. After centrifugation to remove any precipitates

present, either butanol extracts were taken and reduced in vacuo at room temperature, or the aqueous hydrolysates were applied directly to papers for chromatography. The unhydrolysed homogenates were thawed and centrifuged and samples of the supernatants applied to the papers alongside the hydrolysates. Since P.B.I. is not soluble in butanol it was not feasible to extract the homogenates with butanol before chromatographing the samples. Butanol extraction, whilst giving clearer chromatographs was not necessary so long as the homogenates were well filtered and not too much was applied to the papers. Choosing the optimum conditions for treatment with ^{131}I greatlyincreased the activity of the samples and hence made smaller aliquots necessary for successful scanning and autoradiography.

Butanol extraction of hydrolysates was done since all the products of hydrolysis of iodinated proteins should be soluble in n-butanol.wheras many inorganic compounds are comparatively insoluble. However a direct comparison of homogenates and their hydrolysates was not possible unless both were in aqueous solutions since the inorganic iodide compounds and P.B.I. were only partially soluble in butanol.

Artefact Formation

To test the possibilty that compounds of pancreatic origin might become iodinated during hydrolysis some pancreatin was incubated with a few microcuries of $Na^{131}I$ in phosphate buffer with and without methyl thiouracil. A sample of boiled pancreatin was also

tested. The hydrolysates were chromatographed in both developing solvents. The double 'iodide' band was the only artefact formed and was suppressed by methyl thiouracil. This inorganic iodine artefact was not consistent in appearance during these experiments nor in the general experiments on hydrolysis. It was occasionally seen in unhydrolysed tissue samples. $Na^{131}I$ also gave a double band when chromatographed in B.Ac. and the same sample had a diffuse lower border when developed in B.D.A. The sample had been stored for at least two weeks in the laboratory since its dispatch from the Commonwealth X-ray and Radium Laboratory. The formation of this double band was not usually noticed in the $Na^{131}I$ used as a standard during experiments, which ^{131}I would have been stored under laboratory conditions for a much shorter time. These results suggest that the formation of the inorganic iodine compounds is brought about by the developing solvents and not by the effects of hydrolysis. The double iodide band also appeared sometimes on chromatographs of NaOH hydrolysates.

The formation of an oxidised form of iodine has been investigated by Taurog(1961). The use of methyl thiouracil to prevent artefact formation was proposed by Gleason (1955) and Taurog(1955). Gleason stated that radioactive iodide does not exchange with the stable atoms in iodothyronines in conditions which ensure complete absence of free iodine but will do so readily in synthetic solutions in physiological pH range. Alexander (1961) however asserts that <u>in vitro</u> there is no exchange of ^{131}I with ^{127}I in thyronines

in homogenates of human and rat thyroid glands under conditions where iodination of tyrosines takes place readily. Alexander also claims that, *in vivo*, only MIT will exchange ^{127}I or H^+ for ^{131}I in the presence of iodide peroxidases and that thyronines are not affected under the same conditions.

Kennedy (1957) investigated the formation of an artefact formed during NaOH hydrolysis of iodinated proteins. He found it was also supressed by methyl thiouracil.

4.3 The Blood Iodine Compounds

The blood of *Ciona* contains a larger amount of iodine than is found in terrestrial vertebrates. Typical figures are 126 µg/100 mls for *Ciona* and an average value of 6.3 µg/100 mls for human blood (total plasma iodine). For the purposes of comparison it is stated here that the iodine content of the local seawater was found to be about 6 µg/100 mls. The proportion of the ascidian blood iodine which is organically bound is considerably less than in human blood but still accounts for the larger part of the total blood iodine. Half or more of the organically bound iodine in *Ciona* blood was found to be firmly protein bound and could not be separated by butanol extraction, as can be done with the human serum protein-thyroxin system. Of the remaining organically bound iodine in *Ciona* blood, thyroxin was a consistent component, and its precursors MIT and DIT were also found. T_3 was sometimes present.

There were marked variations in the percentage composition of the blood ^{131}I compounds after similar periods of treatment. The data for three such experiments are given in Table 1. After two days treatment at 0.8 - 1.0 µc/ml ^{131}I the equilibrium stage should have been reached, and it should have been possible to determine the proportions of organically bound ^{131}I and inorganic ^{131}I at that stage. The variations in the data obtained however made it necessary for some other method to be employed. Therefore the percentage composition of the blood iodine compounds at various times was investigated and average values for the equilibrium composition derived from the histogram obtained. (Fig. 17). The ratio of organic to

inorganic ^{131}I at 50 hours was found to be approximately equal. This means that the organic iodine content of Ciona blood is about 60 µg/100 mls. Therefore Ciona blood contains ten times the amount of organically bound iodine which is found in human blood.

About 15 percent of the blood ^{131}I is contained in MIT, DIT, T_3 and T_4. The remaining 35 percent of the organically bound ^{131}I is firmly protein bound and although the proteins are partially soluble in n-butanol, thyroxin is not released from the proteins by extraction with n-butanol, except perhaps that which is contained in the 15 percent of free amino acids. This indicated that the blood of Ciona is not iodinated in the same way as is vertebrate blood.

The proteins of Ciona blood were therefore precipitated by T.C.A. and separated from the plasma by centrifugation. The precipitate was hydrolysed with 2N HCl in sealed tubes at 90°C. Chromatographs of whole blood, hydrolysed blood proteins and the deproteinised supernatant were prepared. Fig.18 shows that only MIT was produced from the protein by hydrolysis. A small amount of unhydrolysed protein, a little iodide and much free iodine were also present. Only the iodine was absent from the unhydrolysed samples. Both the whole blood and the supernatant contained iodo-thyronines. Subsequent hydrolyses with NaOH or Pancreatin confirmed these results. (Fig.19-20). There was sometimes an indication of a very small increase in the thyroxin content but it was quite definite that MIT was the major organic iodine component of hydrolysed blood. By treating the animals for at least three days with ^{131}I the amount of P.B.^{131}I. in the blood was increased to about 70 percent of the total activity,

and little or no ^{131}I remained as iodo-tyrosines. Hence any MIT found in the hydrolysates could only have come from the proteins. No attempt was made to determine which type of plasma protein bound the iodine in Ciona because very little is known about the composition of ascidian blood. Macroscopic examination of the blood of Pyura and of Ciona showed that considerable 'clotting' took place in the former but none was evident in Ciona, indicating that the blood is not of the same composition in the two genera. The Director of Rutgers Serological Museum was unable to give any information about the proteins of ascidian blood. It was felt that an electrophoretic examination of Ciona blood would be of little value without a knowledge of the basic constituents of the blood.

The effect of goitrogens on Ciona blood was not easy to interpret (Table 2). Both KCNS and methyl thiouracil(m-TU) reduced the total activity of ^{131}I in the blood to less than one fifth of the control value. The residual activity however was still distributed more or less normally between organic and inorganic fractions, although the amount of protein bound iodine was much less than in the controls, there being an accumulation of the iodo-aminoacids instead. This would indicate that these goitrogens affected not only the iodide accumulating mechanism but also the formation of proteins from amino acids. This was seen to be so in the other tissues investigated as well. The most inexplicable feature of the effect of these goitrogens was that in the blood (but not in the endostyle and tunic) m-TU did not block the formation of iodo-amino acids to the extent which might be expected from its effect on mammalian blood iodine.

The inorganic iodine content was less than 40 percent of the total with both the higher and lower concentrations of the goitrogens which were used. The higher concentration of m-TU (3.4×10^{-4}M) caused definite blockage of iodo-aminoa acid formation in the other tissues. The lower concentration (2.8×10^{-9}M) did not. The formation of organically bound iodine compounds in the presence of KCNS would be explicable since iodide which entered the blood by diffusion would be bound in the normal way. Methyl thiouracil however should prevent the binding of iodide which diffused into the blood, unless some other binding mechanism, not involving a very specific enzyme system, is present in ascidians. Such a primitive mechanism has been postulated (Pitt-Rivers et al., 1959) to explain the presence of small quantities of protein bound MIT remaining in rats treated with thiouracil.

It was thought possible that some physiological and environmental factors might influence the organic iodine compounds of the tissues, particularly those in the circulating system, the blood. The most likely environmental factors to affect the iodine compounds are the supply of iodine and the temperature of the seawater. The effect of temperature on the relative composition of the blood iodine compounds was investigated by treating the animals with ^{131}I at temperatures of 8°C (low winter temperature in boreal waters) and 20°C (moderately high summer temperature in South Australian waters). The low temperature was maintained in a special refrigerator, the animals being constantly aerated and receiving periodic illumination. The experiment was done in summer when the normal

water temperature was about 20°C, so those animals treated at 20°C were in fact a control set whilst the others were exposed to a temperature which was unnaturally low for them. There were no marked differences in the composition of the blood iodine compounds of the two groups. The ratio of inorganic to organic ^{131}I was the same at both temperatures (14.5 percent iodide). There was however an increase of about 30 percent in the total activity in the 8°C group over that of the control group, but this result would need to be verified by a larger number of experiments before any significance can be attached to it.

The most important internal factor which might alter the pattern of the iodine compounds was thought to be the state of maturity of the gonads. Therefore some very young ones, less than three centimetr es long with undeveloped gonads, were treated with some fully reproductive animals. The blood of the young ones was found to contain 19 percent inorganic iodine and 81 percent organic ^{131}I whilst the mature animals contained 14.5 percent and 85.5 percent respectively. Whilst these differences are very slight a great discrepancy appeared between the total activities for the two samples; the younger blood contained nearly twice as much radioiodine as did the older blood. This can best be explained by assuming that the increased amount of tunic substance and the maturation of the gonads depleted the blood of its iodine compounds in the fully mature animals. Other wise it can be inferred that the metabolic rate of the young animals influences the total iodine content of the blood and possibly of the other tissues.

Included in the experiments on artefact formation (4.2) was a series to test the ability of the blood to utilise radio-active iodine *in vitro*. It was found that whole blood formed P.B.I. when incubated in phosphate buffer at pH 8.0 (the natural pH as determined by pH meter). Boiled blood and blood with methyl thiouracil added did not form P.B.I. Although this latter result is contradictory to that of the experiments done to ascertain the effect of goitrogens on the tissues of intact animals (in which methyl thiouracil did not entirely suppress the formation of P.B.I.) it is possible that the quantity of blood used for the *in vitro* experiment would not permit the amount of P.B.I. formed,in the presence of a relatively large amount of m-TU,to be detected by autoradiography.

4.4 The Endostyle Iodine Compounds

Homogenates of the endostyles with associated mucus and the endostylar appendices gave a generally consistent analysis with P.B.I. and iodide as the major components. The proportion of radioactivity in the protein and iodide fractions naturally varied with the length of treatment with radioiodine but also varied in experimetns using the same conditions of treatment. The most likely cause of such variations would be the amount of inorganic iodine adsorbed on the endostylar mucus, and not the concentration of P.B.I. present. The latter will vary between individuals, and may also be affected by the rate of production of mucus for feeding purposes. As noted in Part 3.4 there may be variation iniodine binding in the endostyle but the entire endostylemmay, if there is any control iodine metabolism, bind iodine at a more or less steady rate whilst normal feeding takes place. Extreme variation amongst individuals in a pooled group would easily affect the ratios of the compounds however. Perhaps the best guide to the cause of these variations is the relative amount of the compounds in the other tissues of the same group of animals. Some figures are given in Table / to illustrate the variation in percentage composition of the blood, endostyle and tunic.

From autoradiographs of chromatographs it was evident that the amount of P.B.I. increased continuously over the period investigated, that is, up to three days. The amount of P.B.I. at three days was, by visual examination, at least twice that present at one day. Scanning the chromatographs showed that at three days there was

often four or five times as much P.B.I as at one day. However, as the one and three day samples were not taken from the same animals these figures are not an absolute value for the increase of P.B.I. with time. They indicate that some storage of organic iodine compounds must have occurred in the endostyle;alternatively the endostyle was not binding iodine directly from the seawater, but was supplied by the blood stream and therefore could form P.B.I. only at the rate governed by the supply of iodo-amino acids from the blood.

Free iodo-amino acids were occasionally detected in the endostylar homogenates,but only in very small quantities. A trace of T_4 was found in about fifty percent of homogenates,but its identification was often doubtful because of the diffuseness of the autoradiographic image. Also it was noted that it occurred only on chromatographs developed in B.D.A. This may have been due to the inefficiency of B.Ac. as a developing solvent for thyronines or perhaps to deiodination by that solvent. The presence of methyl thiouracil in the homogenates should have prevented the artefact formation during chromatogrpahy,so presumably the traces of iodo-amino acids were genuine. Whether they were due to endostylar iodine binding activity alone or also to the small traces of blood left in the sinuses around the endostyle is not known.

Hydrolysis of the endostyle and mucus,either by NaOH or by pancreatin produced MIT as the most abundant iodo-amino acid,(Fig 20). Traces of DIT and T_4 were found in one or two hydrolysates;their presence in the control homogenates of these hydrolyses was negative

or doubtful. Hydrolysis by pancreatin produced a very small amount of an unknown iodine compound which ran at Rf 0.35 in B.D.A. Chromatographs developed in B.Ac. gave a definite artefact which was not separated completely from the iodide band, which it slightly preceded (Rf 0.23 for I^-). There was no other distinct radioactive area on B.Ac. chromatographs which could have corresponded to the 'unknown' seen on B.D.A. chromatographs. The same compound was present in the blood and tunic hydrolysates and gonad homogenates. A similar compound was present in NaOH hydrolysates of the blood, but was not detected on the corresponding endostyle hydrolysates. The possibility of iodinated histidines being formed was considered, but the location of the compound on the chromatographs did not correspond with the Rf values for MIH and DIH in the solvents used.

The Effect of Goitrogens on Endostylar ^{131}I

If the endostyle binds iodine in a truly thyroidal manner (taking up iodine from the internal medium, or perhaps the external medium, and converting it to iodo-amino acids and proteins) then similar effects would be expected from higher vertebrates and ascidians treated with goitrogens. Thus thiocyanate would be expected to eliminate both bound iodine and iodide from the body since it blocks the iodide accumulating mechanism and methyl thiouracil would be expected to reduce greatly the organically bound iodine with a consequent accumulation of iodide above the normal level.

The two goitrogens had a similar effect on the total activity of the endostyle in that they reduced the activity to approximately fifty percent of the control value in all experiments. The results of two typical experiments are shown in Table 3 a and b. In (a) the goitrogen concentration was relatively low (M^{-3}, i.e. 10^{-8} KCNS and 2.8×10^{-9}M methyl thiouracil). In (b) the concentration of goitrogens was higher (2.0×10^{-5}M KCNS and 3.5×10^{-4}M m-TU). Unfortunately the concentration of ^{131}I was not the same for the two experiments but by increasing the time of treatment with the lower concentration the controls of the two experiments had almost equal amounts of inorganic and organically bound iodine.

The KCNS did not have the effect expected of a thyroidal system since it permitted the accumulation of about fifty percent of the control total activity with both the high and the low concentrations used. In both cases the proportion of organically

bound to inorganic iodine was the same,and only differed slightly from the control ratios. Thus it would seem that iodide is not accumulated by the endostyle entirely in the same manner as in the thyroid gland of vertebrates.

With methyl thiouracil the lower concentration made only a small effect on the proportion of organic to inorganic iodine,but the higher concentration exerted a definite effect,reducing the organically bound iodine from 58 to 14 percent. The total activity of the inorganic iodine remained the same in both control and treated animals. The fact that both concentrations of methyl thiouracil reduced the total activity of the samples by fifty percent is best explained by assumuing that it has some side effect on the general metabolism of the animals. This may,or may not,indicate that the organic iodine produced by the endostyle exerts an hormonal effect on the metabolic rate,since the behaviour of the endostyle may be governed by the blood iodine content.

The results from these experiments show that although the iodine binding mechanisms of the endostyle show some affinities with those of the thyroid gland of vertebrates,it is not entirely similar and cannot,on the basis of these results,be regarded as physiologically homologous with the thyroid gland.

4.5 The Gonadal Iodine Compounds

In the great majority of these experiments only P.B.I. and iodide were found, the relative activity of the fractions varying considerably. On hydrolysis the protein was usually completely broken down, in contrast to the hydrolytic products in the other ti tissues where a small amount of iodinated protein remained even after the acid or alkaline hydrolyses, (Fig 21).

The acitivity counted for the gonadal P.B.I. was not markedly less than (and sometimes more than) that in the endostyle or tunic samples of the same experiment. One would therefore expect a similar degree of hydrolysis to be effected under similar conditions unless the type of protein concerned was different from that in the other tissues. If the iodine - protein relationship were different then the hydrolytic products might also be different. The only certain difference detected was in the amounts of MIT and inorganic iodine produced, ther being little of the amino acid released and an increase in the iodide fraction. In the other tissues there was little or no increase in the iodide fraction on hydrolysis.

Because some of the gut of Ciona was in the gonads sample there was the possibility that the ^{131}I compounds detected were not solely derived from the gonads. Some experiments were therefore done using Pyura gonads, which are discrete organs except for their blood supply. Protein bound ^{131}I and $^{131}I^-$ were found in Pyura gonads as in Ciona, (Fig 22, 23)

The goitrogens used eliminated organically bound iodine.

4.6 The Tunic Iodine Compounds

Barrington and Barron (1960) treated the outer layer of the tunic of Ciona in various ways to determine its composition. They found that the hydrolysed scleroproteins contained DIT and sometimes T_4, with possible traces of T_3. The experiments reported here, whilst not contradicting their results, do not entirely agree with them.

The most important difference was that in all my experiments MIT was present, usually as the dominant iodo-amino acid, in terms of percentage of contained radioactive iodine. Barrington and Barron did not find MIT at all. Also, less than half of my experiments yielded any thyroxin and DIT occurred in only half of them. These results were compiled from experiments using either homogenates only or hydrolysates and control homogenates. The results of these experiments are given in Table 4. Earlier experiments in which the analytical procedure was not standardised were not included in the table, but many gave similar results.

The month of the year in which the experiments were done was included because the age of the zooids is possibly relevant to the presence of thyroxin in the tunic. Those experiments in which thyroxin was definitely found were done with very large animals about six months old. The local population of Ciona is characterised by a gradual discoloration of the tunic both by detritus and mud deposits and by the growth of micro-organisms. This discoloration, particularly by algae, is not usually noticeable until the animals are at least four months old. Although the loose detritus

and macroscopic algae and serpulid tubes were removed from the tunic before starting experiments it was inevitable that microscopic particles would remain. This suggests that the presence of thyroxin could be due to extraneous matter, particularly to microscopic algae. Until some method of removing or preventing such growths is invented the part played by the ageing of the tunic itself cannot be properly investigated. It is evident to anyone handling *Ciona* that the scleroprotein layer becomes thickened during the first few months of growth and it is therefore likely that the protein-thyroxin content increases during this time.

The absence of MIT from Barrington's analyses could be due to the low concentration of tracer iodine used, the MIT which was formed with ^{131}I being completely incorporated into DIT and thyronines after two days. Barrington and Barron did not specify the quantity of seawater in which their *Ciona* were treated, but unless it was about 5 liters to 20g wet weight of animals it would be surprising if they detected much radioactivity as M^{131}IT.

The Effect of Goitrogens on the Tunic.

The effect of goitrogens on the tunic iodine compounds was similar to that found in the endostyle. From Tables 5 a and b it is seen that both KCNS and methyl thiouracil reduced the total activity of the samples to half or less of the controll sample. Methyl thiouracil had the more severe effect on the amount of P.B.I. The weaker concentration used reduced the P.B.^{131}I. to 1.8 percent of the total activity but the total organically bound ^{131}I was almost equal to that of the control. The higher concentration, whilst it did not have such a marked effect on the P.B.^{131}I. caused a great increase in the inorganic iodine fraction.

KCNS in the higher concentration did not affect the inorganic ^{131}I content, which would suggest that the iodide can be absorbed directly from the medium and utilised by the cuticle forming layers. This result was not corroborated by the experiment using the lower concentration. The effect of the stronger concentration appears to be anomalous.

TABLE 1

Tissue and Exp.No.	P.B.I.	iodo-amino acids	total organically bound ^{131}I	inorganic iodide	unknown
BLOOD 1	23.75	1.60	25.35	74.40	0.25
2	51.70	20.50	72.20	16.50	11.30 Rf pre-iodide
3	48.20	37.30	85.50	14.50	--
ENDOS. 1	12.94	2.47	15.41	84.20	0.39
2	58.70	--	58.70	41.30	--
3	21.90	44.80	66.70	27.20	--
TUNIC 1	17.70	15.72	32.42	59.60	2.38
2	64.50	14.15	78.65	21.40	--
3	36.60	42.30	78.90	21.10	--

Percentage of total ^{131}I activity in iodo-compounds of the Blood, Endostyle and Tunic. Results of three experiments in which the animals were treated for two days at 0.8 - 1.0 μc/ml ^{131}I.

TABLE 2a

Exp.	Total activity c.p.m.	Ratio to control activity	P.B.I.	iodo-amino acids	organically bound ^{131}I	inorganic iodide	ratio to control activity	unknown
Control	14635	1.0	51.70	20.50	72.20	16.50	1.0	11.30
KCNS 2.0 x 10^{-5}M	2185	0.149	17.15	32.05	49.20	38.20	0.345	12.60
m-TU 3.4 x 10^{-4}M	2855	0.195	12.00	38.50	50.50	35.50	0.420	14.00

The effect of goitrogens on the blood iodine compounds.

Experiment - 24 hours in goitrogen solution, followed by 50 hours in fresh goitrogen solution plus 0.9 $\mu c/ml$ ^{131}I.

TABLE 2b

Exp.	Total activity	Ratio to control activity	P.B.I.	Iodo-amino acids	Organically bound ^{131}I	Inorganic iodide	Ratio to control activity	unknown ? inorg iodine
CONTROL	19122	1.0	22.80	34.50	57.30	26.80	1.0	15.90
KCNS $10^{-8}M$	2028	0.108	9.70	53.00	62.70	28.20	0.113	9.10
m-TU $2.8 \times 10^{-9}M$	2770	0.141	8.10	43.80	51.90	38.70	0.21	9.40

The effect of goitrogens on the blood iodine compounds.

Experiment - 24 hours in goitrogen solution, followed by 70 hours in fresh goitrogen solution plus 0.66 μc/ml ^{131}I.

The results are expressed as percentages of the total activity.

TABLE 3a

Exp.	Total activity c.p.m.	Ratio to control activity	P.B.I.	Iodo-amino acids	Total organically bound ^{131}I	Inorganic iodine	Ratio to control activity	Unknown
CONTROL	1611	1.0	29.00	29.20	58.20	39.10	1.0	2.70 (Rf 0.98)
KCNS $10^{-8}M$	673	0.418	5.05	43.65	48.70	51.30	0.55	--
m-TU $2.8 \times 10^{-9}M$	709	0.440	1.05	49.60	51.10	48.90	0.56	--

The effect of goitrogens on the endostylar iodine compounds.

Experiment - 24 hours in goitrogen solution, followed by 70 hours in fresh goitrogen solution plus 0.66 μc/ml ^{131}I.

The results are expressed as percentages of the total activity.

TABLE 3b

Exp.	Total activity c.p.m.	Ratio to control activity	P.B.I.	Iodo-amino acids	Total organically bound ^{131}I	Inorganic iodine	Ratio to control activity
CONTROL	1065	1.0	58.70	--	58.70	41.30	1.0
KCNS 2.0 x 10^{-5}M	775	0.728	24.00	24.00	48.00	52.00	0.92
m-TU 3.5 x 10^{-4}M	520	0.488	10.50	3.80	14.30	85.70	1.0

The effect of goitrogens on the endostylar iodine compounds.

Experiment - 24 hours in goitrogen solution, followed by 50 hours in fresh goitrogen plus 0.9 µc/ml ^{131}I.

The results are expressed as percentages of the total activity.

TABLE 4

The occurrence of iodo-amino acids in homogenates and hydrolysates of the tunic at various times of the year.

+ = definite, – = negative, ? = probable but very faint.

Developing solvent	Sample treatment	MIT	DIT	T_3	T_4	Month
B.Ac.	Hydrol.	+	–	–	–	June
B.Ac.	Homog.	+	–	–	–	"
B.D.A.	Homog.	–	–	?	–	"
B.D.A.	Hydrol.	+	+	+	–	"
B.Ac.	Hydrol. $Ba(OH)_2$	+	?	–	–	July
B.Ac.	Hydrol. pancreatin	+	+	–	?	July
B.Ac.	Homog. control	?	?	–	–	"
B.D.A.	Homog.	+	?	–	–	August
B.Ac.	Homog.	+	–	–	–	"
B.Ac.	Hydrol.	+	?	–	–	"
B.Ac.	Homog.	+	+	–	–	November
B.D.A.	Homog.	+	–	–	+	"
B.D.A.	Hydrol.	+	+	+	+	"
B.D.A.	Homog. 8°C	?	?	–	–	November
B.D.A.	Homog. 20°C	?	?	–	–	"
B.Ac.	Homog.	?	?	?	–	December
B.D.A.	Homog.	+	+	?	+	"

TABLE 5a

Exp.	Total activity c.p.m.	Ratio to control activity	P.B.I.	Iodo-amino acids	Total organically bound ^{131}I	Inorganic iodine	Ratio to control activity
CONTROL	2105	1.0	64.50	14.10	78.60	21.40	1.0
KCNS 2.0 x 10^{-5}M	1095	0.52	22.00	35.50	57.50	42.50	1.03
m-TU 3.5 x 10^{-4}M	590	0.28	12.00	24.50	36.50	63.50	0.835

The effect of goitrogens on the tunic iodine compounds.

Experiment - 24 hours in goitrogen solution, followed by 50 hours in fresh goitrogen solution plus 0.9 μc/ml ^{131}I.

The results are expressed as percentages of the total activity of ^{131}I.

TABLE 5b

Exp.	Total activity c.p.m.	Ratio to control activity	P.B.I.	Iodo-amino acids	Total organically bound ^{131}I	Inorganic iodine	Ratio to control activity
CONTROL	2280	1.0	28.20	25.00	53.20	46.80	1.0
KCNS $10^{-3}M$	907	0.4	4.40	37.00	41.40	58.60	0.5
m-TU $2.8 \times 10^{-9}M$	1126	0.495	1.80	47.00	48.80	51.20	0.535

The effect of goitrogens on the tunic iodine compounds.

Experiment - 24 hours in goitrogen solution, followed by 70 hours in fresh goitrogen solution plus 0.66 μc/ml ^{131}I.

Results are expressed as percentages of the total activity of ^{131}I.

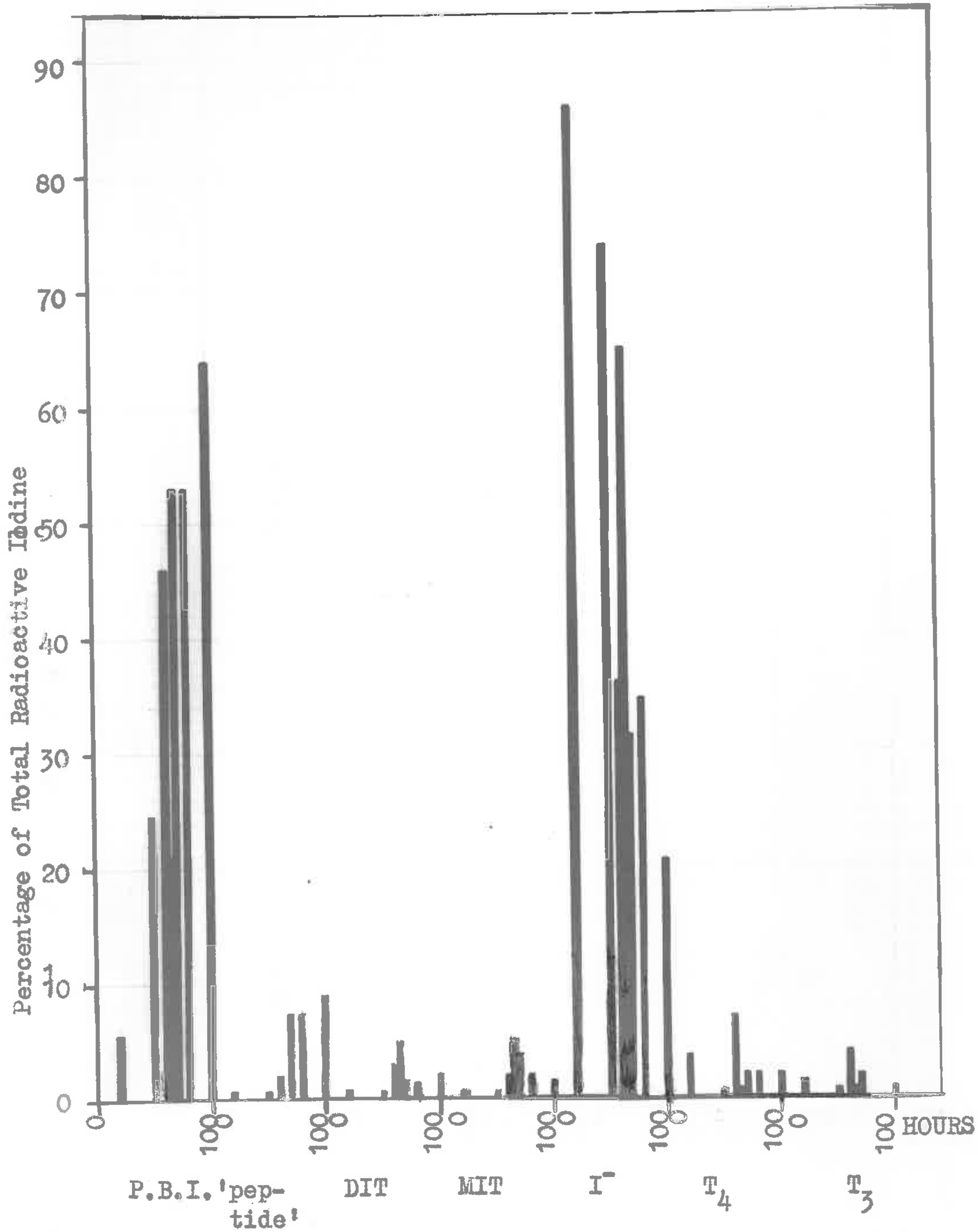

FIG.17. Histogram showing the percentage composition of the blood iodine compounds at various times from 0 to 100 hours of treatment. Initial concentrations of ^{131}I,0.7-1.0 μc/ml.

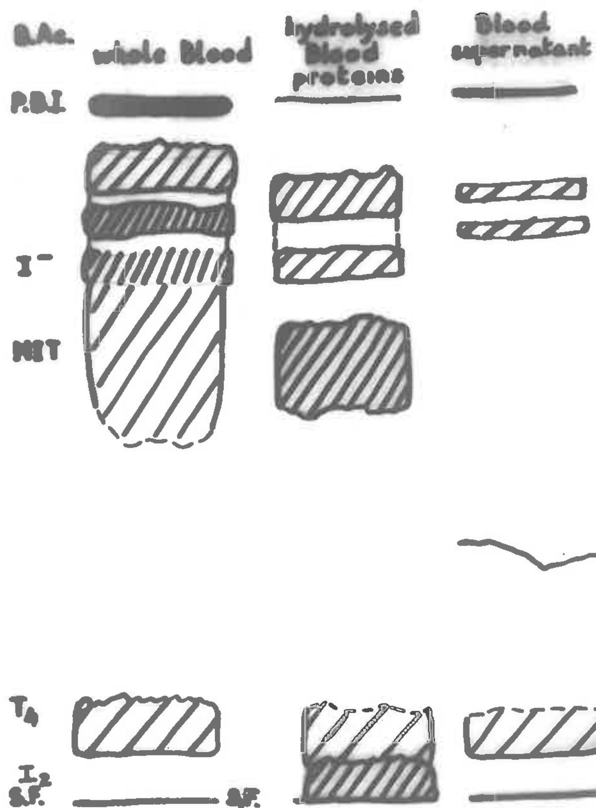

FIG.18. Products of hydrolysis by HCl of the blood proteins.
From autoradiograph of chromatograph.

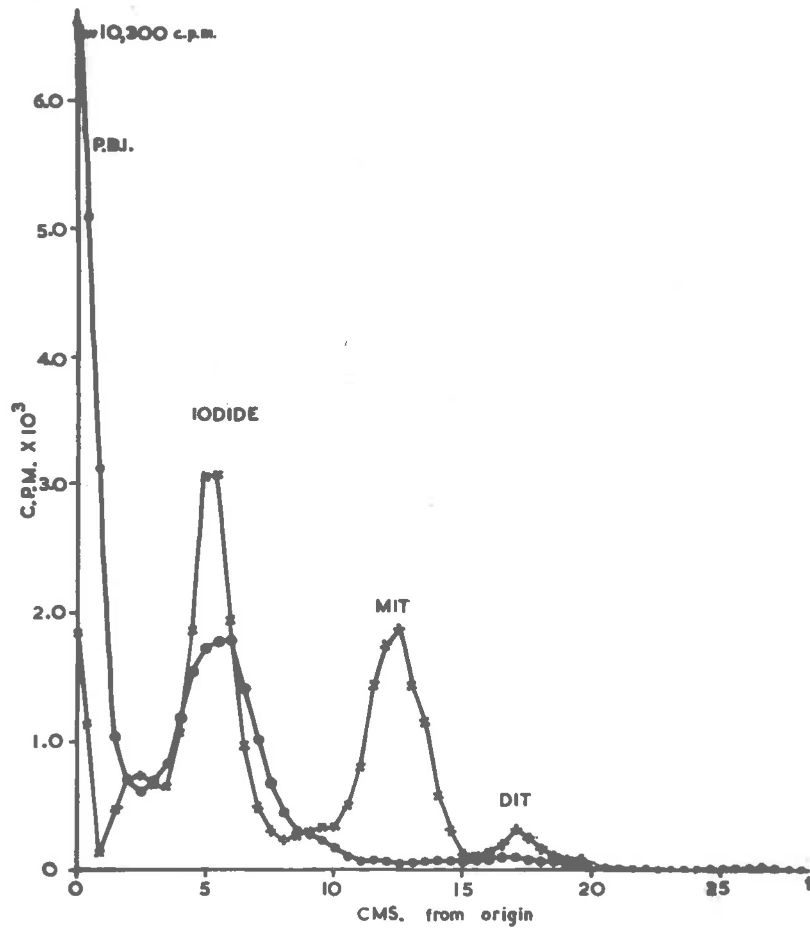

FIG. 19. Products of hydrolysis of the blood by NaOH. Graph of radioactivity of chromatograph.

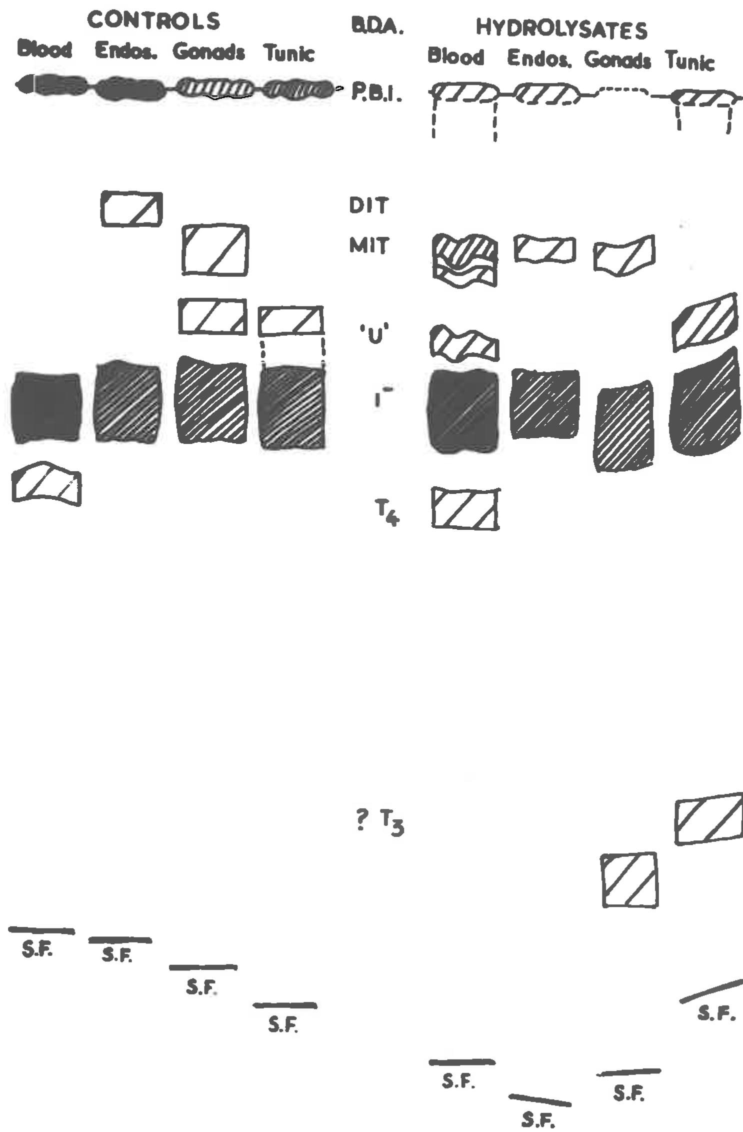

FIG. 20. Products of hydrolysis by pancreatin of the blood, endostyle, gonads and tunic. From autoradiograph of chromatograph.

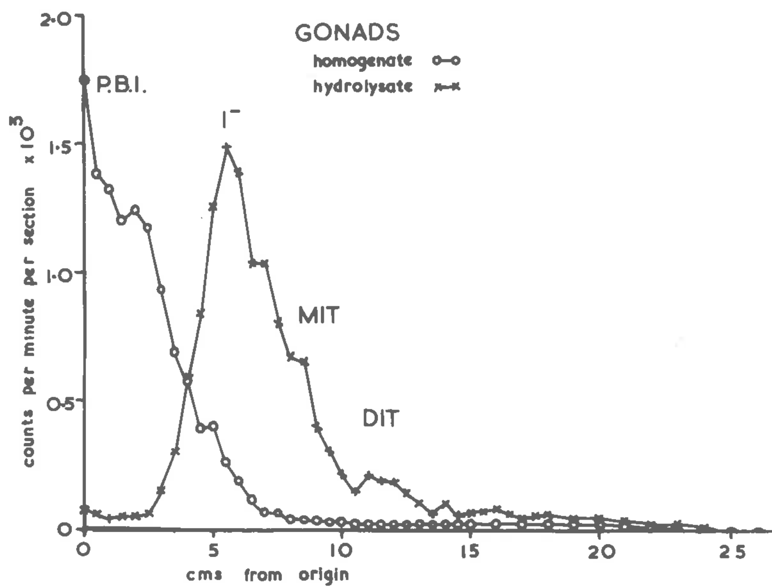

FIG.21. Gonadal ^{131}I compounds in homogenate and NaOH hydrolysate. Graph of radioactivity of chromatograph.

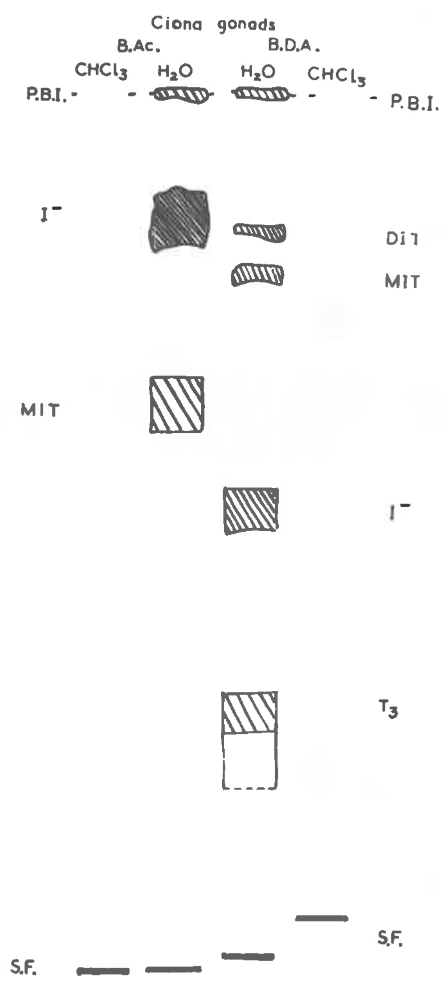

FIG. 22. *Ciona* gonadal ^{131}I compounds.

Pyura gonads

B.Ac. B.D.A.

$CHCl_3$ H_2O H_2O $CHCl_3$

P.B.I. P.B.I.

pept. ?

I^-

?

I^-

T_3

S.F. S.F.

FIG. 23. *Pyura* gohadal ^{131}I compounds.

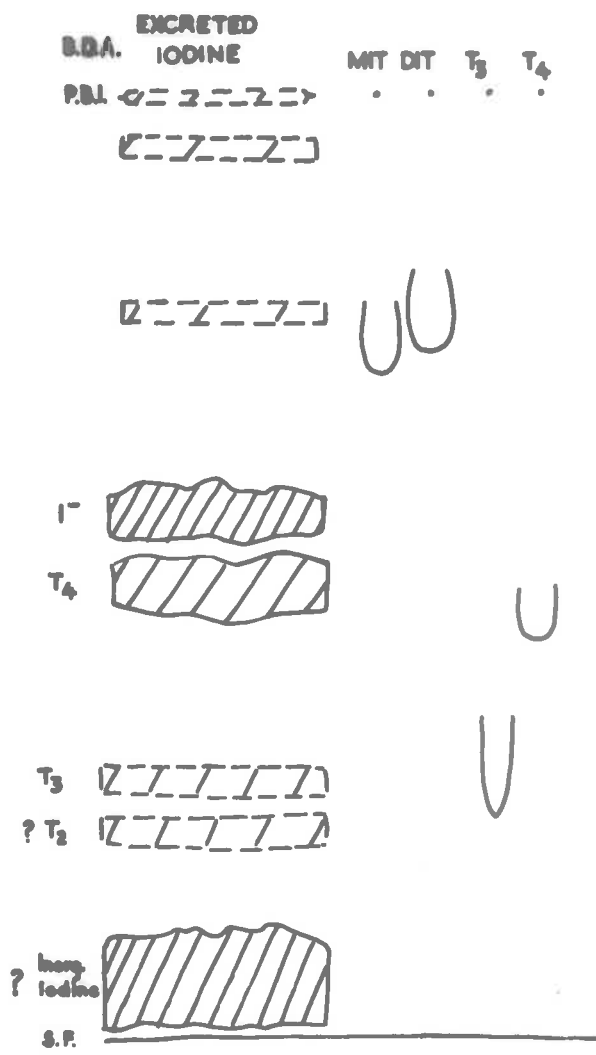

FIG. 24. ^{131}I compounds excreted by Ciona into the medium during three days treatment. Butanol extract of medium.

Part 5. THE INORGANIC ANALYSIS OF IODINE

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5.1 The Iodine Content of Local Seawater.

In calculating the percentage amount of radioiodine present in the seawater used for these experiments one important approximation was made - that of the normal iodine content of the local seawater, which was taken to be 50γ/1. This was an average value given by Harvey (1945) and Sverdup, Johnson & Fleming (1942). It was derived from measurements done in the earlier part of this century on waters from various oceans. It is now recognised that these measurements may be a little inaccurate due to inadequacies in the assay methods used before the discovery of the catalytic action of iodine on the reduction of Ceric salts by arsenious acid (Chaney, 1940). This is the most sensitive method generally available for determining very small amounts of iodine. There is still scope for improvement in the methods of releasing the iodine from its various compounds for the final assay. Inorganic analyses, such as natural waters, do not

present great problems such as are encountered in analyses of organically bound iodine.

The method finally used was that of Barkely and Thompson (1960). Previously the method given by Dubravcic (1953, 1955) was tried. Although it gave some results which agreed fairly well with those obtained by Barkely and Thompson's method, there was considerable inconsistency even among duplicate samples in the same batch and the method was abandoned in favour of the later one. It is worth noting however that the two methods did give comparable results. Had the fault, probably technical, in Dubravcic's method been corrected the two methods would have been an excellent check for one another since they are very different, both in the manner of releasing the iodine from its compounds and in the colour complex whose optical density is measured.

All the samples were diluted to standard chlorinity (19.38 $^{o}/oo$) for the estimations. The iodine content of the sea

water from the upper basin of the Port River was found to be 53.2 γ/1. The water from the Outer Harbour at the same season of the year (autumn) contained from 60 - 69 γ/1. The salinity of the original samples was 20.75 ‰ at the Outer Harbour and 20.27 ‰ in the upper basin. The high value of 69 γ/1 was from an offshore water sample which had been in a sealed bottle for some time. Dubravcic's method gave results of 70 - 75 γ/1 for water from the Outer Harbour.

It was concluded from these experiments that the assumption of an iodine value of 50 γ/1 was valid and would not affect the total iodine concentration ($I^{127} + I^{131}$) significantly in the experiments done.

5.2 The Determination of Iodine in Ascidians

Some estimations of the iodine content of ascidians were done by Cameron (1915). He used an analytical method which he found convenient rather than accurate (Kendall, 1914) and which was able to detect amounts of iodine over 0.005mg. He consequently had to use large amounts of ash in order to detect quantities as low as 0.001 %. It is not surprising that he failed to detect iodine in samples of the endostyle weighing less than 0.01g. He placed considerable emphasis on the relatively large amount present in the outer tunic and the apparent absence of the element from the soft tissues. He noted that the inner tunic of Pyura contains iodine, unlike his findings with other species. This is probably due to the larger blood supply to the tunic in that genus than in many other species. He gave no details of the growth of algae etc. on the tunic of the species he used, but since he was aware of the large amount of iodine contained in algae he presumably removed any

such growth. His results showed that iodine in the tunic was generally of the order of n x 10^{-2} %, increasing to n x 10^{-1} % in the Pyuridae and Styelidae, which species have a thick, well vascularised tunic. He made no estimation of the amount of iodine in the animals as a whole, but a value of n x 10^{-4} % can be deduced from his results.

Vinogradov (1953) reviewed the many iodine and bromine estimations for animals and seawater, including Cameron's papers in which 'test' is confused with 'testes'. The values for seawater he gives as I=n x 10^{-6} %, Br=n x 10^{-3} %. He concluded, presumably from Cameron's work, that ascidians accumulate iodine preferentially to n x 10^{-4} % and that the bromine content was generally of the same order as in seawater. The degree of concentration of these elements by invertebrates in general and by fishes he gives as I=n x 10^{-4}, Br=n x 10^{-3} and I=n x 10^{-3}, Br - n x 10^{-2} % dryweight, in invertebrates and fishes respectively. Some sponges and corals

accumulate these to a higher degree, with iodine still being concentrated more effectively than bromine. The differences in the amounts of these elements in the various types of animals is apparently due to the relative amounts of skeletal protein they contain.

When this work was started it was hoped that the iodine content of the various tissues, particularly the blood, would be determined for several species, under various conditions of external iodine concentration. Although it would have been difficult to reduce the iodine content of seawater without upsetting the natural balance of the elements (as would happen if artificial seawater were used) it was hoped that the great affinity for iodine which the brown algae exhibit could have been used to substantially reduce the iodine content of some seawater before ascidians were put into it. In Vinogradov's review it is stated that most marine organisms accumulate iodine in proportion to the

amount of the element present in the water. The vertebrates alone, having a metabolic use for iodine, maintain certain levels of the element in their bodies, although the level may change at different times in the life cycle. If it could be shown that ascidians maintain a definite iodine concentration in spite of fluctuating external conditions it would add greatly to the evidence for the argument that ascidians are closely allied to the vertebrates.

Unfortunately it was not possible to complete such a large program of work and iodine determinations were therefore restricted to the ash of some of the local species. With exception of _Pyura_ and _Styela_ they represent families not assayed by Cameron.

In choosing a method for iodine determination certain factors have to be considered. These are (a) the method of release of iodine from the tissues and (b) the method of determining the iodine released. The various methods are reviewed by Salter & Johnston (1948), Riggs and Mann (1940) and Acland (1957).

There are several ways of releasing iodine from organic compounds - by hydrolysis or oxidation with strong acids or alkaline incineration or oxidation in $KMnO_4$ solution. The iodine must then be reduced to a volatile form and distilled off. It can then be estimated by titration with thiosulphate and starch indicator or colorimetrically by its effect as a catalyst on the reduction of ceric salts by arsenious acid. The latter method is the one now usually employed, being very sensitive and convenient when a large number of samples have to be handled. It has been argued by some (e.g. Acland 1957) that acid hydrolysis always results in loss of iodine and that alkaline incineration is the only reliable method. However the cost of the apparatus for Acland's method was prohibitive and it had to be ruled out for such short term research activity. This method is used by the Department of Medicine at the Queen Elizabeth Hospital Woodville, S.A. for research into iodine metabolism and Mr. B. Good of that department kindly

determined the iodine content of the blood of <u>Ciona</u>. The result of this estimation, by alkaline incineration, was 126 µg % $^{w}/v$ (total iodine). Taking the iodine content of the local seawater to be 60 µg/1 the blood of <u>Ciona</u> is found to accumulate iodine to twenty-one times the level in the external medium. Only the one estimation was done, the blood sample having been pooled from several animals.

The other methods of releasing iodine from organic compounds were surveyed with a view to choosing one which could cope with an unknown quantity of iodine in a relatively large amount of organic matter. The method finally chosen was that of Taurog et al. (1946) in which digestion was done with chromic and sulphuric acids. The oxidised iodine was held in solution as non-volatile iodic acid until released by reduction with phosphorous acid as the volatile iodine, or possibly iodide. The iodine was distilled off and collected in a sodium hydroxide trap. An aliquot of the hydroxide solution was analysed for iodine by the ceric - cerous reduction method. The

distillation apparatus was adapted from Chaney (1940), the proportions of the apparatus being changed from those in his diagram and a stopcock added to facilitate removal of the distillate, as was described by Talbot et al. (1944).

The method was checked for possible loss of iodine during the digestion phase by digesting known amounts of iodine as KI and as DIT. Both these substances were digested and distilled without any loss of iodine. The DIT was used (at 0.1 γ/ml) to test the release of iodine from an organic compound. The accuracy of the colorimetric technique was tested by adding known amounts of iodine to the sample distillates before measuring the cerio-cerous reaction. The reaction rate was found to be unaffected by additional iodide in the range of concentrations used (0.0 to 0.3 γ/ml).

Because of the relatively high iodine content of ascidian ash the size of the aliquots of distillate had to be reduced from 4 mls (as used by Taurog) to 0.5 mls. The blank distillate aliquots were

likewise reduced. The standard curve, obtained by using blank distillate and known amounts of iodine, as KI, covered the range of 0.00 to 0.30 γ/ml. The distilled water used was prepared by passing ordinary distilled water through an "Elgastat" ion exchange column, the effluent having a resistance of more than 8×10^5 ohms. This water had no detectable iodine content.

The samples were prepared by homogenising thoroughly cleaned and drained animals in iodine free water, using a "TOSCO" blendor. Two colonies of *Podoclavella*, each containing 30-50 zooids, were combined. Three or more specimens of the solitary ascidians were pooled, and the *Didemnum* samples were from three separate colonies. The *Styela*, *Ciona* and *Didemnum* were free of any macroscopic growths, but *Styela* had a thin layer of microscopic organisms which was lightly scrubbed off. *Pyura* was more difficult to clean, but scraping and scrubbing removed most of the adherent matter. Where necessary the surface of the tunic was cut off in order to remove

large embedded particles.

The homogenates were spread on petri dishes previously cleaned in chromic acid and rinsed with iodine-free water. The dishes were placed in an oven at 95°C for several hours. The ash was then scraped out and thoroughly ground up before being placed in weighing bottles. It was then redried to constant weight and stored in a desiccator. When used for experiments 0.1g was weighed out and digested as previously described. At least three samples of the ash of each species were analysed. The results are shown in Table below.

Genus	Iodine as % dry weight
Didemnum	5.45×10^{-4}
Ciona	3.6×10^{-4}
Pyura	1.32×10^{-3}
Styela	2.28×10^{-3}
Podoclavella	2.4×10^{-3}
Seawater	5.86×10^{-6}

The amount of iodine in these ascidians is thus seen to be

more than that found by Cameron, there being ten times as much in *Pyura* and *Styela* than estimated by him. In view of the relative thickness of the tunic and the ramifications of the stolonic vessels in *Podoclavella* it is not surprising that the iodine content of that species should be of a higher order than in the other genera of the order Enterogona, although the scleroprotein layer of the tunic is very thin.

A preliminary estimation of the iodine content of the blood of *Pyura* gave a figure far in excess of that for *Ciona*. The blood was obtained by slicing through the tunic and allowing the exuded fluid to drip into a container, as described by Endean (1960). *Pyura* blood is quite different from that of *Ciona* in that it is slightly viscous and cloudy and it 'clots' into a very viscous form. These properties are not exhibited by *Ciona*. The clotting of ascidian blood is not fully understood but it is thought to be a different mechanism from that in the vertebrates. The disparity between

Pyura and *Ciona* blood is probably due to differences in the serum protein level; this in turn could have a significant effect on the iodine content of the blood, which could partially account for the difference in the iodine content of the whole animals. Determinations of the serum protein - bound iodine would have to be done, as well as total blood iodine, before comparisons of the blood iodine levels of various species could be made. It does not seem likely though that animals whose blood is very different from that of vertebrates, and which blood even differs within the same class, could have a physiology comparable with that of the higher chordates.

Part 6. DISCUSSION

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6.1 The Significance of the Results of Experiments and Observations Recorded in Part 3.

In the species investigated, which represent all the orders and many of the families of the Tunicata, it is seen that radioactive iodine is incorporated in a protein component of the mucus of the endostyle. Whether or not this iodine is taken directly from the incurrent seawater, or is passed to the endostyle from the bloodstream, as other metabolites must be, is not certain. The injection of radioiodinated thyroxin into the blood, followed by histological and chromatographical analyses might elucidate this important problem. Fluorescent antibody techniques could also be used to ascertain the relationships of the iodo-proteins of the various tissues.

The use of radioiodinated thyroxin would also help in analysing the formation of the iodinated scleroproteins in the tunic. It seems fairly clear, from the evidence presented here,

that the cuticular iodine compounds are formed from compounds carried to it from the blood stream and not directly from the seawater. It is not impossible however that iodide and other ions could pass through the protective, waterproof cuticle, especially where it is broken. Such ions could then be metabolised by the underlying cuticle-forming layers. This apparently happens in the larvae since their cuticle does not appear to contain iodine, the element being present in the underlying tissues. It must reach those tissues by penetrating the cuticle as the larvae do not have a functional pharynx. Barrington (1957, 1960) does not mention what he considers to be the path taken by the iodine to the cuticle, but he presumably believes it to be obtained directly from the sea, since he considers the tunic scleroproteins to be the primitive source of iodinated proteins which ascidians utilised during their evolution. The results presented in Part 3.8 however necessitate a re-examination of his hypothesis.

The tunic and the endostyle almost always showed some uptake of ^{131}I - if either lacked it entirely then the other usually did too, which would indicate that uptake by both tissues is dependent on the metabolic state of the entire animal. That such a correlation does exist was shown in Part 4 in which it was found that the proportions of organic ^{131}I and inorganic ^{131}I in the endostyle and tunic varies with the amount of organically bound ^{131}I in the blood. (Table /).

The other tissues which were found to contain ^{131}I gave less consistent results. In the blood this was almost certainly due to the low concentration of serum proteins or their loss from the sections during histological processing. That they were found mostly in the heart cavities, and their association with the walls of those cavities, suggests that they have a metabolic significance in that organ. Physiological experiments on the intact animals and biochemical assays with the relevant tissues could be done to

elucidate this problem.

The variations in uptake by the gonads cannot be explained by the loss of protein during fixation. However the amount of ^{131}I in the reproductive system of Ciona was only of the same order or less than that in the endostyle (Part 4) so it would be very sparsely distributed through the gonads. Also, from Ciona, it was seen that ^{131}I was accumulated only during the early stages of oogenesis and therefore animals whose oocytes were not in an accumulative phase would not take up iodine during treatment. Even so some zooids were found in compound ascidians and Podoclavella colonies in which ^{131}I was evident on autoradiographs of the few oocytes present.

The presence of iodine in the gonads, particularly in the ovary, was to be expected since there is chemical evidence of its presence in the ovaries of various chordates, and autoradiographic evidence of its presence in some of them. Leloup and Fontaine (1960), summarising the extra-thyroidal occurrence of iodine in the

lower vertebrates, state that the lamprey can accumulate up to 70 percent of a dose of tracer iodine, most of which remains inorganic but some of which is protein bound by the ovary. The testis does not take up as much as does the ovary. In selachians and dipnoi the developing oocytes concentrate radioiodine (as was observed in _Ciona_). The amphibian ovary also accumulates iodine - _Rana_ will take up 43 percent of an injected dose into the ovary. Birds concentrate iodine in the developing egg to a considerable extent, the element being taken up by the egg during yolk formation (Roche, Michel, Michel, Maurois, 1951). Mammals are also known to accumulate iodine (Roche and Desruisseau, 1950) but to a lesser extent than do the birds; the difference being due to the mode of development of the embryo.

In a review of the extra-thyroidal metabolism of iodine Brown-Grant (1961) comments on the relatively large amount of iodine in the ovaries of vertebrates compared with the amount present in

other non-thyroidal tissues. The presence of iodine in the eggs and its accumulation by the larval or foetal stages is thus seen to be common to both ascidians and vertebrates and might be taken as an indication of close phylogenetic relationship. Some knowledge of the occurrence of iodine in echinoderms and their larvae would be of interest in view of the supposed evolutionary connection between the two phyla.

In spite of the individual variations the histological survey shows that ascidians generally accumulate iodine from the seawater and form protein bound iodine in the endostyle, tunic, gonads and blood. That they accumulate iodine to a level above its concentration in the seawater is shown in Part 5. The two levels of iodine concentration ($n \times 10^{-3}$ and $n \times 10^{-4}$ percent) can best be explained by comparing the volume and surface area of the tunic of those species with the greater iodine content (*Pyura*, *Styela*, *Podoclavella*) with the structure of the tunic of the other species (*Ciona*, *Didemnum*).

6.2 The Significance of the Results of Part 4.

Although all the chromatographical analyses were done on *Ciona* there is no reason to suppose that the results are not valid for other species also. Any significant differences between the iodine compounds of various species which may be found will tend to support the theory that the compounds concerned are not part of a well organised hormonal system.

Perhaps the most interesting feature of the results of these analyses is the demonstration of the large amount of iodine present in the blood as protein bound iodine. One factor which must be borne in mind when discussing the blood iodine compounds is that the blood of ascidians differs in many respects from that of vertebrates. Unfortunately so little is known about the proteins of ascidian blood that a useful comparison of their physiology with that of vertebrate blood cannot yet be made. The most significant thing about the ascidian blood iodine is that it is firmly protein

bound and is present mostly, if not wholly, as MIT. This is in contrast to the condition found in those vertebrates which have a thyroid gland with a definite endocrine function; in these the blood iodine is almost entirely contained in iodothyronines which are loosely bound to certain serum proteins. This latter system is primarily one specialised for the transport of the hormonal compounds. In *Ciona* it would seem that such a system has not been developed, either because there are no iodo-compounds with an hormonal function or because the metabolic pathways involved are quite different from those in the higher vertebrates. If it is true that the vertebrates evolved from a stock closely related to the ascidians then the former condition would seem to be the more likely, since the thyroid hormone system of the vertebrates would have evolved from the primitive physiological conditions found in the lower chordates.

The presence of small quantities of iodo-thyronines in the blood is interesting. It could indicate that, in spite of the

dominance of MIT in the P.B.I. fraction, a less primitive system, with hormonal significance, is also present. These thyronines could also be transported in the vertebrate manner but during chromatography in butanol solvents they would have become detached from the protein components and so considered to be 'free' iodo-amino acids. The blood was the only tissue in which the presence of iodo-thyronines was consistent, although they were occasionally found in homogenates of the endostyle and tunic also. If the endostyle is to be considered the more important iodine binding tissue then a more consistent occurrence of these compounds might be expected.

What is the origin of the iodo-organic compounds present in the blood? Are they all formed by the blood itself or do they enter the blood stream through the intestinal wall as iodo-tyrosines (from the endostylar mucus) which are then utilised to form the blood proteins. My results give some indication that there may

be dual origin. A small number of histological autoradiographs showed that some ^{131}I was transported across the intestinal epithelium in a form which was precipitated by the fixative. There is no reason to suppose that that portion of the iodine, presumably in peptide combination, is the only part to be transported across these cells since amino acids and inorganic compounds will not be precipitated. It is quite possible that the blood itself, being a complex tissue, can also form organic iodine compounds from inorganic iodine. It has been shown that the blood can form P.B.^{131}I. when incubated in phosphate buffer with radioactive iodide. This formation of P.B.I. was suppressed by the addition of methyl thiouracil. Although no quantitative results were obtained from this experiment (which was one of a series on artefact formation, Part 4.2) it shows that the endostyle is not the only primary iodine binding tissue in ascidians. It has not yet been determined whether the tunic can bind inorganic iodine _in vitro_.

Whatever the origin of its organic iodine compounds, the blood certainly contains more of them than any other tissue in Ciona. Whether this is true of the species with thick fibrous tunics (e.g. Pyura) is not certain, but the iodine in the cuticle of such tunics is no longer available for distribution to other tissues (unless the tunic is resorbed during a period of starvation) and therefore cannot be included in an assessment of the iodine which is available for general metabolism.

The large amount of P.B.I. in the blood suggests that the blood is the 'storehouse' of iodine in ascidians, as the thyroid gland is in vertebrates. The presence of a store of easily transportable organically bound iodine in the blood also suggests that it may have some physiological significance, but it seems doubtful if the metabolic pathways involved in its utilisation can be closely similar to those in the vertebrates. Here again the fluorescent antibody techniques might be used to determine the destination of

the blood proteins which contain iodine. It might be found that the sub-neural gland is affected by the circulating blood iodine compounds. No definite evidence was found from my autoradiographs that ^{131}I was located in the neural complex of the species studied but a number of specimens examined (mostly _Ciona_ and _Pyura_, but also _Ascidia_ and _Styela_) gave a doubtful positive autoradiograph from the ganglion and some (_Ciona_) from the duct of the gland where it was juxtaposed to the ganglion. Radioiodinated thyroxin, injected into the blood stream and into the neural gland, might be a good way of ascertaining whether there is any normal supply of thyroxin to the neural complex and particularly if it passes between the two components of the complex. Positive results would suggest that the complex does have a 'pituitary like' function, as suggested by Carlisle (1951) and earlier authors (Bacq and Florkin, 1935; Butcher, 1930). Recent work has tended to discount this evidence that the complex has such a function (Dodd, 1955, 1959).

6.3 The Significance of the Effects of Goitrogens

The P.B.I. of the blood exhibits a greater susceptibility to the action of goitrogens than that of the other iodine binding tissues. This suggests that some part of the iodine binding potential of the blood is governed by a different mechanism to that which regulates the binding by the other tissues and a part of the blood. The great decrease in the blood P.B.I. content effected by goitrogens may be due to a more specialised mechanism allied to that found in higher vertebrates. The remaining organically bound iodine in the blood may represent a more primitive system of iodine binding and its regulation. The term "primitive" in this context is used by Pitt-Rivers et al. (1958) as meaning a system different from that which is dominant in mammals. In recent years, especially since radio-active iodine compounds became generally available, workers studying the effects of goitrogens have discovered that these substances do not entirely suppress the formation of protein bound iodine from the

vertebrate body. A very small amount of iodine is still bound in spite of heavy or continuous doses of these agents, combined or alone.

Taurog, Potter and Chaikoff (1955) found that rat thyroid homogenate formed MIT from iodide without any additional substrate and it was firmly protein bound. Taurog, Tong and Chaikoff (1958) also discovered that untreated, hypophysectomised rats continued to bind iodine at a very low level, mainly as MIT. Evans et al (1960) gave young, hypophysectomised rats large doses of iodide and discovered that it exerted a marked effect on the growth rate although no extra-thyroidal thyroxin was formed. These results influenced Pitt-Rivers and her associates (Pitt-Rivers, Galston and Halmi, 1958; Pitt-Rivers and Tata, 1959; Pitt-Rivers, 1960) in the formation of the idea that there are two iodine binding mechanisms acting in vertebrates - a highly specialised one which needs special enzymes to convert MIT to DIT and the thyronines, which enzymes are very sensitive to the action of goitrogens; and a primitive iodine binding mechanism which utilises

a less specialised and less goitrogen sensitive oxidising enzyme for the formation of MIT, which is ultimately used in protein synthesis. Pitt-Rivers (1960) claims that this system is very resistant to drugs and to cell damage.

Some very recent work on the mechanism of iodination has yielded some interesting results. Alexander (1961), investigating the mechanisms of iodination in the thyroid and salivary glands, discovered that the peroxidases of the latter will oxidise bromide as well as iodide - that is, the salivary gland has not such a specific peroxidase system as has the thyroid gland. Also these peroxidases can iodinate histidine although if tyrosine is also present it will be iodinated preferentially. Galton and Ingbar (1961), investigating peripheral thyroid hormone metabolism, discovered that some tissues could form an oxidised form of iodine which could re-iodinate organic compounds to give new P.B.I. from the serum thyroxin supplied to the tissues. They did some experiments with animals of vertebrate orders other than mammals and discovered that

tadpole liver was the least specialised tissue of the series investigated. Tadpole liver could de-iodinate T_4, T_3, TriAc, TetrAc, DIT and MIT and was the most active in adverse conditions in forming P.B.I. from liberated iodine. Mouse liver however could not form similar P.B.I. from MIT and DIT. This suggests that amphibia have a less specialised system for metabolising organic iodine than the mouse has.

This evidence does suggest that two systems of iodine binding are present in the chordates and that the balance between them has changed during the evolution of the vertebrates. It is quite likely then that the ascidians may have only the beginnings of the more specialised system, which may be the only one linked with hormonal iodine.

6.4 The Significance of Larval Protein Bound Iodine

In view of the dominance of MIT in the protein bound iodine system of the adult ascidians it would be very interesting to know which iodine compounds are present in the larval proteins. The larval P.B.I. is apparently located mostly in the epidermis, which is consistent with evidence of its location in the Hemichordata and Larvacea (e.g. in ~~*Balanoglossus*,~~ *Dolichoglossus* as found by Gorbman et al, 1942; and in *Saccoglossus*, as found by Thomas, 1962; also Thomas (1962) has some evidence of ^{131}I binding by the epidermis of *Oikopleura*). Possibly it may be bound by the underlying muscles in the tail and in the undifferentiated cell clumps in the trunk. It does not seem to be in the actual cuticle - that is, it is not bound in scleroproteins as in the adult cuticle. The extensive presence of organically bound iodine in these larvae, whose free swimming period is a matter of hours, implies some metabolic significance for the iodine accumulated during that time. This is corroborated by the presence of iodine in the eggs

and sperm and hence in the embryos before hatching as larvae. If any appreciable amount of iodo-thyronines are present in the larval proteins then it would be reasonable to suppose that they may exert some influence on the growth rate and on metamorphosis. If only MIT is found then it seems unlikely that hormonal action of the vertebrate type will be found to exist in the larval ascidians. The work of Galton and Ingbar (1961) must be borne in mind however, since it indicates the possibility of the existence in lower vertebrates of a much less specialised system than is found in the higher vertebrates for the binding and transport of iodine which may still have an hormonal function.

6.5 Conclusion

It has been shown in this thesis that protein bound iodine is distributed in the same tissues as those in which it is present in the vertebrates (with the exception of the tunic which is an anomalous tissue). The organisation of the iodine compounds in the adult tissues in a system which is less sensitive to the action of goitrogens than that of vertebrates suggests that the utilisation of organic iodine in the ascidians is not as specialised as in higher vertebrates. The dominance of MIT in the ascidian iodo-proteins corroborates this and suggests that ascidians may have two systems of iodine metabolism, the predominant one being concerned with the formation and utilisation of MIT, the other like that of the higher vertebrates being concerned with the more complex iodo-amino acids. Whether or not either of these systems has any hormonal activity in ascidians has yet to be demonstrated.

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