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ON THE ORGANIC BINDING OF IODINE IN THE TUNIC OF *CIONA INTESTINALIS* L.

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(Text-figs. 1–3)

It is now known that organic binding of iodine takes place in the endostyle of *Ciona* (Barrington, 1957) and *Amphioxus* (Thomas, 1956; Barrington, 1958), and there is evidence that at least di-iodotyrosine and thyroxine are formed as a result of this process in *Ciona* (Barrington, 1959; Robertson, personal communication. Since this biosynthesis appears to be a specialized property of a restricted group of cells, it has been suggested (Barrington, 1959) that it is not a chance by-product of endostylar secretion but that the thyroidal products are a biochemical necessity for the animals concerned. Attention has been drawn also to the occurrence of iodine binding at the surface of the tunic (or test) of *Ciona* (Barrington, 1957), and it is the purpose of the present paper to present some analysis of this latter situation and to enquire as to its possible relationship with the endostylar activity.

The whole of this work has been carried out at Nottingham on animals sent from the Plymouth Laboratory of the Marine Biological Association, and we are indebted to the Supply Department of that Laboratory for the trouble taken in despatching them.

In addition to routine fixation in Bouin's fluid in sea water, followed by staining with the Azan technique, a variety of histochemical procedures have been used. These include chiefly the periodic acid/Schiff (PAS) technique, the Millon reaction (Lison, 1953), the coupled tetrazonium technique of Barnard & Danielli (1956 and personal communication), the alcian blue technique (Steedman, 1950; Lison, 1954) the ferric-ferricyanide technique of Chèvremont and Frédéricq (Lison, 1953), and the DDD method for sulphydryl and disulphide groups (Barrnet, 1953; Barrnet & Seligman, 1954).

HISTOLOGICAL AND HISTOCHEMICAL OBSERVATIONS

The ascidian tunic is bounded on the outside by a thin surface layer which it is convenient to term the 'cuticle' (Saint-Hilaire, 1931). Pérès (1948*a*, *b*), whose studies have been of the greatest value to us in formulating our own interpretation, has concluded that this is composed of pure protein, in contrast with the remainder of the tunic which he describes as formed of cellulose (tunicin) with some glycoprotein.

The cuticle (Fig. 1), when well developed, is conspicuous in Azan preparations as a layer which stains sometimes red, sometimes blue, and which shows no obvious structure apart from a thin membrane-like boundary at its upper and lower surfaces. Saint-Hilaire (1931) referred to the fact that many cells with large granules lie near the cuticle and, in his words, appear to adhere to it. In fact, the cuticle (Fig. 1A) is continued inwards into numerous flask-shaped extensions, each of which encloses one of these cells with its characteristic granule, the latter always staining brilliantly with azocarmine. In this region it is often difficult to decide whether or not the granule is actually

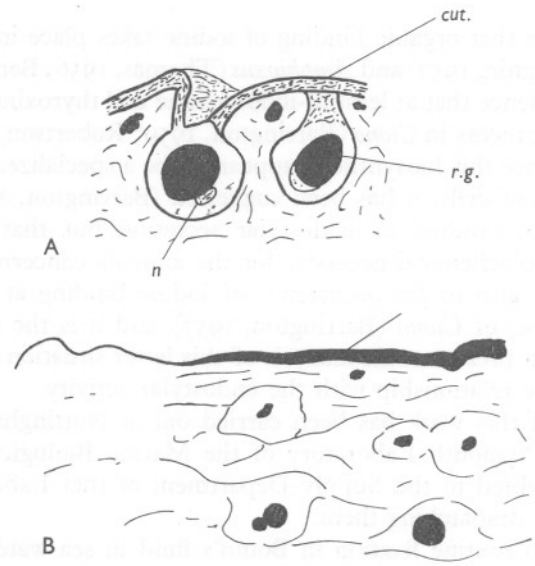

Fig. 1. A. Transverse section of the surface of the tunic of *Ciona*. B. Ditto, showing an early stage in the formation of the cuticle. *cut.*, cuticle; *n.*, nucleus of refringent granule; *r.g.*, refringent granule.

still enclosed in a cell, but similar granules are found in wandering cells throughout the tunic; in these the nucleus is clearly defined with the cytoplasm just distinguishable as a very thin bounding layer, and such features are also sometimes visible in the cuticular region (Fig. 1A). These are clearly the cells which Pérès describes as characterized by possessing a single large 'refringent granule', and we shall therefore use the latter term. According to him they are particularly concerned with the secretion of the cuticle, and our own observations support this view.

For example, the cuticle varies greatly in development from specimen to specimen. It may appear as no more than a very thin membrane, but in sections of such material (Fig. 1B) areas can be found where it is becoming much thicker, and at such points there is an accumulation of refringent

granules which look as though they are undergoing reduction or fragmentation. It seems evident that these are the source of the increased cuticular deposit, which they resemble in their staining reactions, and that this is why the granules and their cells become enclosed in extensions of the cuticle.

Pérès found that the cuticle and the refringent granules reacted positively to the Millon test, and in confirmation of this we have obtained a definite pink colour in the granules and a fainter one in the surface layer. The reaction has, however, proved rather weak, and we have found the coupled tetrazonium technique of Barnard & Danielli (1956) to give a more precise response, with the added advantage of permanence. With this method, we find that a positive red coloration is given by the refringent granules (more especially at their surface), as also by the cuticle. The remainder of the tunic is predominantly unstained, but the fibres are positive, as are also numerous fine particles, which may perhaps be fibre material. Blocking by benzoylation for 9 h almost completely eliminates this reaction, apart from an occasional weak response at restricted points of the cuticle. We therefore infer that the cuticle and refringent granules include protein, containing tyrosine and perhaps also histidine and tryptophan, and that this is also true of the fibres. Saint-Hilaire (1931) noted the presence of protein in the ascidian tunic, but, using the Millon test, found a good deal of variation between different genera, with a positive reaction from the whole of the tunic of *Halocynthia papillosa*, but none from the tunic of *Tethyum gyrosum*. The significance of such variations is not at present clear, and it will be desirable to investigate them further, particularly in relation to their possible influence upon the degree of iodine binding in different genera.

The results of the alcian blue test are in sharp contrast with the above, for the cuticle and the refringent granules are negative, while the remainder of the tunic, together with the fibres, are positive. This implies that acid mucopolysaccharides are present in the main body of the tunic but are absent from the cuticle and refringent granules, a result which is in agreement with Pérès's description of the former as differing from the latter in staining metachromatically with toluidine blue (presumably gamma metachromasia). He also uses the term 'glycoprotein' in this context, but in current terminology a positive alcian blue response and gamma metachromasia are held to be an indication specifically of acid mucopolysaccharides (Lison, 1953; Pearse, 1954).

With the PAS test, which was not used by Pérès, a positive reaction is given by the cuticle and by the refringent granules; in the rest of the tunic the fibres are positive, and there is a good deal of fine granulation which reacts in the same way, while the general matrix gives a very faint coloration. It seems clear from this response of the cuticle that it cannot be composed of pure protein, as Pérès supposed. Both it and the granules are negative to Sudan black, so that there is no evidence for the presence of lipoidal compounds

which might account for the response, and in view of this, and of the absence of acid mucopolysaccharides, it seems likely that the cuticle and granules must consist of protein associated with carbohydrate in some form of glycoprotein complex.

Because of this, and of the recent demonstration by Brunet & Carlisle (1958) of the presence of chitin in the tubes of the Pogonophora, it seemed necessary to re-examine the possibility of chitin being also responsible for the PAS-positive reaction in the cuticle of *Ciona*. For this purpose we have used the standard colour and solubility tests for chitosan as defined by Campbell (1929), using as test material the cuticle of *Ciona*, after removal of as much as possible of the underlying jelly, and comparing the reactions of this with those of *Flustra* (Hyman, 1958) and the cuticle of the locust, and also with samples of bacterial cellulose and of filter-paper.

After treatment for 20 min at 160° C in saturated potassium hydroxide the reactions of *Flustra* and of the locust cuticle were as expected; the material was coloured dark brown on the addition of the iodine solution, on adding 1% sulphuric acid it became dark blue by reflected light and violet by transmitted light, while on adding 75% sulphuric acid it became reddish-brown and dissolved. The material also dissolved rapidly in 3% acetic acid and the solution gave a conspicuous white precipitate on the addition of 1% sulphuric acid. The *Ciona* cuticle was coloured light brown by the iodine solution, on adding 1% sulphuric acid it became dark blue by reflected light but blue with a greenish tinge (and definitely not violet) by transmitted light, and on adding 75% sulphuric acid it became blue and dissolved. It would appear that these reactions must be largely due to the presence of cellulose (tunicin), the presence of this in the tunic being very well-established and readily demonstrable by the Schulze and cuprammonia tests. It is said (Campbell, 1929) that cellulose gives no colour either with the iodine solution or with 1% sulphuric acid, but we find that while filter-paper and bacterial cellulose, after alkali treatment, certainly give no colour with iodine they can develop a variable degree of blue-black colour on the addition of the acid. Thus the only positive indication of the presence of a substance other than cellulose in the *Ciona* cuticle after alkali treatment is the brown colour reaction which it gives in the presence of the iodine solution; it may be that this indicates the presence of some stable glycoprotein complex, but there is no evidence that it is chitin. This negative conclusion is reinforced by two additional observations. First, the cuticle gives no obvious response to treatment with 3% acetic acid and no white precipitate can be obtained from the latter by the addition of sulphuric acid. Secondly, Dr S. Wallwork of the Department of Chemistry of this University has been kind enough to carry out an X-ray diffraction study of the cuticle and finds no evidence for the presence of chitin in it. It may be added that earlier workers have obtained negative results from tests for chitin applied to the tunics of *Halocynthia papillosa*

(Saint-Hilaire, 1931) and *Phallusia mammillata* (Saint-Hilaire, 1931; Wester, 1910), and Rudall (1955) found no evidence for its presence in *Rhabdopleura* and the Enteropneusta.

Pérès obtained a positive response from the refringent granules with the Chèvremont and Frédéricq (ferric-ferricyanide) test, and concluded that they perhaps contained sulphydryl groups. We have ourselves obtained with this test a positive response both from the cuticle and the granules, but we have been unable to secure satisfactory blocking in the control preparations. In consequence of this we have preferred to rely upon the more recently developed DDD methods (Barrnett, 1953; Barrnet & Seligman, 1954), and have obtained positive results with them. The procedure for disulphide groups evoked a very faint but definite positive reaction in the cuticle and granules, although the cuticular ingrowths around the latter seem mostly negative. With the procedure for sulphydryl groups a positive response is given by the cuticle, granules and cuticular ingrowths. This reaction, which can be satisfactorily blocked by the method recommended by Barrnet & Seligman (1954), is slightly stronger than that obtained with the disulphide procedure, but the difference is too slight to justify much emphasis. Using the procedure for demonstrating simultaneously both sulphydryl and disulphide groups, with sodium thioglycollate as the reducing agent, a well-defined purple colour is obtained from the cuticle granules and ingrowths, this reaction being decidedly stronger than either of the previous ones. The fibres in the rest of the tunic are very faintly coloured, but it is doubtful whether this could be regarded as a positive response. Slides of sections of the skin of the mouse were processed simultaneously with the tunic sections, and it was apparent that the reactions in the cuticle and granules of the latter, although quite definitely positive, were substantially weaker than those given by regions of relatively high sulphur content such as are found in the stratum corneum and hair follicles of the mouse.

CHROMATOGRAPHY

For the identification of the products of iodination we have made use of radioactive iodine. Animals were immersed for 48 h in sea-water containing 200 μC of ^{131}I per litre, and paper chromatography of the tunics was then carried out according to the procedure of Bowden, Maclagen & Wilkinson (1955), with *tert.*-amyl alcohol/2N ammonia as solvent (Gleason, 1955). Potassium iodide, di-iodotyrosine, tri-iodothyronine and thyroxine were usually added as carriers to the tissue extracts either before distillation or when the drops were placed on the paper, but in some cases a mixture of these was run side by side with the extracts and none was added to the latter. When the run had proceeded sufficiently far the chromatogram was dried and the distribution of the radioactive iodinated compounds recorded by leaving the paper in contact with X-ray film; the latter was developed and fixed and then

superimposed on the chromatogram after the distribution on this of the carrier spots had been visualized with the ceric sulphate-arsenious acid reaction (Bowden *et al.*, 1955). Permanent records of these spots were made either by tracing or by ultra-violet exposure.

The material consisted either of the whole tunic, or of the surface layer remaining after as much as possible of the gelatinous tunicin had been removed, and the method of preparing extracts of this for chromatography was varied in the following ways:

(*a*) Material, cut into small pieces, was treated at 37° C for 48 h with buffered trypsin solution. After centrifuging and ether extraction, the supernatant was extracted with butanol/HCl. Chromatography of this extract would reveal iodinated amino acids released by the tryptic hydrolysis of protein present in the tunic, together with any free amino acids.

(*b*) Material, cut into small pieces, was extracted at 4° C for 24 h in saline, a few drops of toluene being added. After centrifuging and ether extraction, the supernatant was extracted with butanol/HCl for chromatography; this would reveal any free iodinated amino acids present in the tunic, together, of course, with any which might have been liberated by autolytic action.

(*c*) The fluid residue left after the butanol/HCl extraction in procedure '*b*' was hydrolysed at 37° C for 48 h by the addition of trypsin solution buffered to pH 8·5, and was then extracted with butanol/HCl. Chromatography of the extract would reveal iodinated amino acids released by hydrolysis of any saline-soluble proteins or polypeptides which were present in the original saline extract.

(*d*) The solid centrifugate from procedure '*b*' was treated with N-NaOH for 5 h at 50° C; after centrifuging and ether extraction, the supernatant was extracted with butanol/HCl. Chromatography of the extract would reveal iodinated amino acids arising from the alkaline hydrolysis of any saline-insoluble protein present in the tunic.

Results

A number of preliminary runs were made with procedure '*a*' in order to standardize the technique, and the resultant chromatograms gave from the beginning clear evidence for the presence of di-iodotyrosine and thyroxine, in addition to iodide. In a series of four definitive runs, each from a different batch of animals, di-iodotyrosine was clearly present in all, while thyroxine was detectible in three (Fig. 2). There was a suggestion also of the presence of tri-iodothyronine, but the separation in this terminal region of the chromatogram was unsatisfactory and the matter needs further study before any conclusion can be drawn. It has been common in our chromatograms for an additional substance to be closely associated with the iodide, and this, too, needs further study.

As regards the saline extracts (procedure '*b*'), a definitive set of three runs (from three separate batches of animals) showed that iodide was clearly demonstrable, as would be expected (Fig. 3). Iodinated amino acids were, however, absent from two, and there was no more than a doubtful trace of di-iodotyrosine in the third. After tryptic hydrolysis of these extracts

(procedure '*c*'), iodinated amino acids were still absent from the two mentioned (Fig. 3), while the third showed some di-iodotyrosine and a doubtful trace of thyroxine.

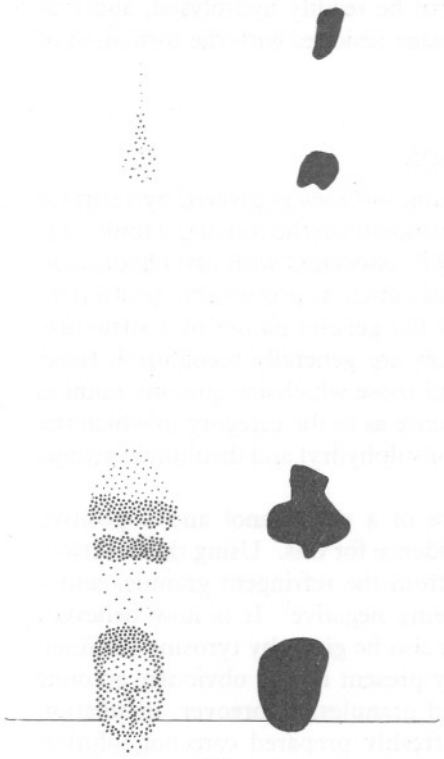

Fig. 2

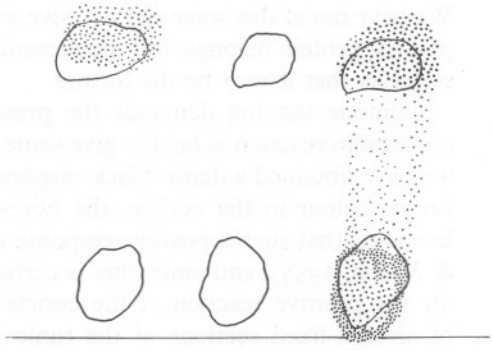

Fig. 3

Fig. 2. Tracings of chromatograms of (to the right) a carrier mixture, showing (reading from the bottom upwards), di-iodotyrosine, iodide, thyroxine and tri-iodothyronine, and (to the left) an extract of the tunic of *Ciona* (procedure '*a*'). Both drops were run in parallel on the same paper. The chromatogram of the carrier mixture was recorded by ultra-violet exposure and that of the tunic by exposure on X-ray film of the sites of radioactive iodine. For further explanation, see text.

Fig. 3. Tracings of chromatograms of extracts of the tunic of *Ciona*, procedure '*b*' to the left, procedure '*c*' in the centre and procedure '*d*' to the right. A drop of carrier mixture was added to each drop on the paper. The sites of radioactive iodine are shown by stippling and of the carrier substances by simple outline (di-iodotyrosine, iodide and thyroxine, reading from the bottom upwards). For further explanation, see text.

By contrast with this, NaOH hydrolysis of the saline-insoluble residue left after these extractions (procedure '*d*') gave clear evidence of the presence of iodide, di-iodotyrosine and thyroxine (Fig. 3), and the last of these seemed a little more readily detectible after this procedure than after tryptic hydrolysis (procedure '*a*'). The tracings shown in Fig. 3 illustrate the results obtained

with parallel runs of each of the procedures '*b*', '*c*' and '*d*', using extracts derived from the same batch of material as a starting point. We conclude from the results as a whole that iodine is bound in the surface layer of the tunic mainly to saline-insoluble protein which can be readily hydrolysed, and that the binding involves the iodination of tyrosine residues with the formation of at least di-iodotyrosine and thyroxine.

DISCUSSION

Our observations clearly confirm that the tunic of *Ciona* is covered by a surface layer, or cuticle, which is distinct in composition from the rest of the tunic; it is certainly rich in protein, but this is probably associated with carbohydrate in a glycoprotein complex. The function of this cuticle is presumably protective, and one would expect its protein to be of the general nature of a structural protein. Two categories of such substances are generally recognized, those which are bonded by disulphide groups and those which are quinone-tanned. We have not at this stage any decisive evidence as to the category to which the present protein belongs, but the presence of sulphydryl and disulphide groups suggests that it may be the former.

Quinone tanning demands the presence of a polyphenol and a positive argentaffin reaction is held to give some evidence for this. Using this reaction, we have obtained a dense black response from the refringent granules and a brown colour in the cuticle, the fibres being negative. It is now believed, however, that such a positive response can also be given by tyrosine (Dennell & Malek, 1955), and since this is certainly present it may obviously account for the positive reaction of the cuticle and granules. Moreover, incubation of alcohol-fixed sections of the tunic in freshly prepared catechol solution (Smyth, 1954) has given no evidence for the presence of polyphenol oxidase, while the pale colour of the surface does not in itself suggest that quinone tanning is involved in its formation. While, therefore, a final decision on this point must await the study of a wider range of ascidians, the present evidence certainly favours the view that the surface protein is a keratin-like substance.

Since it appears to follow from the chromatography results that the iodine binding at the surface is associated with this protein, it becomes possible to relate the situation with that which has been noted in many invertebrates. The binding of iodine by scleroproteins has been recorded in a number of groups (Gorbman, 1955; Roche & Michel, 1951). It is certainly true of some of these, and probably of all, that the iodine is bound by the tyrosine residues, for the formation of mono-iodotyrosine and di-iodotyrosine has been clearly demonstrated. Thyroxine can also be formed, and it has been suggested that the yield of this is peculiar to each protein, being determined by the position of its tyrosine residues and the consequent facility for polymerization. In the gorgonids and sponges, for example, where the proteins concerned are

pseudo-keratins (Roche & Michel, 1951), only traces of thyroxine have been demonstrated; presumably, therefore, the architecture of the protein molecules of the cuticle of *Ciona* is somewhat more favourable for the formation of this product than is that of the scleroproteins of those groups.

Such considerations bring us to the question as to what relationship, if any, exists between the iodine binding in the cuticle of *Ciona* and that which has been shown (Barrington, 1957) to be associated in the endostyle with a particular group of acid mucopolysaccharide-secreting cells. It may well be, of course, that the co-existence of these two types of iodine binding is entirely fortuitous, but there is another line of thought which bears on this problem. Hecht (1918) has given some reason for supposing that the surface of the tunic is being continuously worn away and replaced. Our observations provide a basis for such a process, for the accumulation of the refringent granules at the surface, and their enclosure by cuticular material, show that continuous deposition of the protein could readily occur. It is worth noting here, incidentally, that according to Roche & Michel (1951) iodination in invertebrate pseudo-keratins occurs most abundantly in the young and rapidly growing parts, so that continued deposition of new protein at the surface of the tunic might well favour the occurrence of iodination there. Now it has been suggested (Barrington, 1959) that the wearing away of the cuticle would result in the release into the water of an iodinated protein which could easily enter the alimentary canal with the food particles carried in by the ciliary currents, particularly since the animals concerned are sedentary and gregarious (while, of course, other ascidian genera are colonial), and that in this way the tunicates might have come to utilize the products of iodination and to have become dependent upon them. Theoretically, this could have been a starting point for the subsequent evolution in the endostyle of a group of cells specialized for the more efficient production of these iodinated products and so, ultimately, for the origin of the thyroid gland as a derivative of that organ.

This argument requires that the animals should be able to digest the protein and so release the iodinated amino acids, and our present results show that this would, in fact, be possible. Scleroproteins are in general thought of as being proteins which are especially resistant to enzymic digestion, but the softer pseudo-keratins are much less resistant than are the hard eukeratins, as much as 25–60% being dissolved by treatment with pepsin and trypsin (Block & Bolling, 1939). Our chromatographic results show that di-iodotyrosine and thyroxine are readily released from the cuticle by tryptic digestion as well as by treatment with N-NaOH. This suggests that we may here be dealing with a soft pseudo-keratin, although it must be emphasized that information as to the amino-acid composition of the cuticle is necessary before this can be established with any assurance. What is clear, however, is that the protein could be digested under physiological conditions, and that its iodinated

residues could be taken up by the animal. Here, then, is a possibility, and at present it is no more than that, of visualizing how the iodination of tyrosine, beginning as a chance by-product of the organization of the surface of the tunic, might have become incorporated into the normal biochemical processes of the tunicates. Even if this possibility is accepted, however, the extension of it to provide an explanation of the origin of the thyroid gland could only be justified if the tunicates are regarded as a basal group from which the vertebrates were derived. Such a view has been widely favoured, but it is not the only one (Bone, 1960), and the matter is raised here primarily because it is at least certain that the facts relating to the distribution of iodine-binding capacity in the Protochordata must be incorporated into any general theory of the origin of vertebrates.

We are greatly indebted to Dr Hamish Robertson, of the Department of Agricultural Chemistry, University of Aberdeen, for guidance in the application of paper chromatography to the identification of thyroidal compounds, and to Dr S. Wallwork, of the Department of Chemistry, University of Nottingham, for carrying out an X-ray diffraction study of the cuticle.

SUMMARY

Organic binding of iodine occurs at the surface of the tunic of *Ciona* with the formation of at least di-iodotyrosine and thyroxine. This process is associated with the presence of a cuticle which is rich in protein, and which contains —SH and S—S groups and probably also some carbohydrate. The protein is largely insoluble in saline, but is readily hydrolysed by trypsin or by N-NaOH with the consequent release of the iodinated amino acids. Attention is drawn to the possible bearing of this situation on the evolution of thyroidal biosynthesis in the Chordata.

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