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THE REGENERATION OF THE SIPHONS OF *CIONA INTESTINALIS* L.

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(Text-figs. 1–16)

The urochordates exhibit remarkable powers of asexual reproduction and regeneration, and have provided a fertile field for experiment along these lines. Much of this work has been performed upon ascidians such as *Diazona violacea* Savigny, *Archiascidia neapolitana* Julin, *Archidistoma aggregatum* Garstang and members of the Clavelinidae, etc., since they are relatively easy to obtain and their methods of budding are such as to lend themselves to experimental techniques. Species such as *Ciona intestinalis* (L.) and *Ascidiella aspersa* (Müller), etc., that do not reproduce asexually have, however, been little studied. A few experiments upon *Ciona intestinalis* have indicated that regenerative powers indeed exist, for they have shown that regrowth of the tunic, mantle, siphons, neuroglandular complex and much of the pharynx is possible. With the exception, however, of the regeneration of the tunic and neuroglandular complex, no detailed histological study of the processes involved has been undertaken. The present work attempts to remedy that deficiency with respect to the siphons and anterior end of the pharynx.

Historical

Hirschler (1914), using young *C. intestinalis*, discovered that the posterior region of the body could regenerate all the missing anterior organs (provided that a small portion of the pharynx were present). The anterior region could not, however, regenerate posterior organs. He noted that brown pigmented cells (the cells with orange pigment of Pérès, 1943) tended to accumulate at cut surfaces and attributed to them a phagocytic function. The numbers of regenerating 'ocelli' often varied considerably from those normal to the siphons.

Kammerer (1923) again demonstrated the ability of the siphons to regenerate, and added that the new growths were larger and longer than normal. He even stated that the offspring of the operated animals inherited the ability to grow longer siphons. This was disproved by Fox (1924) who showed that the length of the regenerating siphons was dependent upon nutrition and non-heritable.

Pérès (1948*a*) removed the tunic of *Ciona* and gave a very detailed account of the histology and chemistry of the growth of the new test. The epidermis

and three types of cell, said by him to be mesenchymatous, are involved: (i) cells with granules and acidophil 'bâtonnets' at first carrying carbohydrates, later to be replaced by proteins; (ii) cells with a single, large, refringent granule of a protein nature; and (iii) cells with a small acidophil vacuole containing a carbohydrate of high molecular weight, possibly glycogen. The epidermis secretes the ground substance of the tunic, formed as a result of the polymerization of carbohydrates. This is speedily invaded by the three types of cells just described and the chemical changes necessary for the full development of the tunic are then completed.

The regeneration of the neuroglandular complex of *C. intestinalis* has been said by Pérès (1943) to be the work of 'neoblasts'. The nature of these cells will be discussed later.

Much of the work on regeneration and asexual reproduction in ascidians has been performed on *Clavelina lepadiformis* (Müller), by Huxley (1926), Brien (1930) and Berrill & Cohen (1936). Brien has shown that, while the main sources of newly differentiating tissues and organs in the bud chambers are mesenchymatous and blood cells which he calls lymphoblasts, in the artificial regeneration of the thorax in adults the epicardium is largely responsible. Berrill & Cohen, who isolated pieces of stolon artificially, state that the cells forming the blastogenic mass are derived from a mesenchymatous septum which has disintegrated. They appear not to recognize the participation of the blood cells, noted by Brien.

The work of Brien (1927) on *Aplidium zostericola* Giard. indicates that the majority of the new tissues, cardiopericardiac vesicle, pharynx, peribranchial cavities, epicardium and oesophagus, of the blastozooid are constituted from the fragment of epicardium in the bud. This appears also to be true of *Diazona violacea* Savigny, and *Archidistoma aggregatum* Garstang (see Berrill, 1948 *a*, *b*), and of *Archiascidia neapolitana* Julin (see Brien, 1933).

Selys Longchamps (1916) has described the method of pallial budding in *Heterocarpa glomerata* Hartmeyer, and *Stolonica socialis* Hartmeyer, and Drach (1948) that of the Botryllidae. In the stolidobranchs it is apparently ectoderm and mesenchyme which are responsible for organogenesis and tissue formation.

Berrill (1951), in a review of regeneration and budding in tunicates, states that in ascidians 'while the external tunic of cellulose is readily regenerated by exposed or renewed epidermis... regeneration of other tissues and structures may be accomplished by a variety of internal tissues depending upon the level of the zooid from which regeneration takes place.... Siphons reform from epidermis and atrial epithelium.... At post abdominal levels all structures other than epidermis are reconstituted from epicardial tissue alone when it is present, or from septal mesenchyme.'

Finally, Berrill (1950) is of the opinion that the epicardium is probably mesodermal and not endodermal as has hitherto been thought, but does not pursue the subject.

METHODS

The *Ciona intestinalis* upon which operations for siphon removal were performed were adults varying in length from 1·9 to 7 cm., averaging 3·7 cm.

The specimens were first anaesthetized in menthol in sea water for 1 hr. to avoid siphon retraction and then put into fresh sea water from Plymouth Sound. The water was changed daily and kept at a temperature varying between 15 and 16° C. The siphons were removed in such a way that the cut edges were flush with the body surface (Fig. 1). In several specimens this involved the removal of a small part of the anterior end of the pharynx.

Thirty-nine specimens were used for observations upon the living animal during the period of regeneration. In nine of these, both siphons were removed, in sixteen the inhalant, and in fourteen the exhalant siphons alone. In these experiments the animals were kept alive for a minimum of 6 days to cover the period of growth of siphons normal in all respects other than length. Two animals in which both mouth and atriopore were removed were kept alive for a fortnight for study of the further development of the newly regenerated organs. In five of the above operations the neuroglandular complex was also removed. In three further experiments, in which both siphons were removed the neuroglandular complex was damaged but not excised.

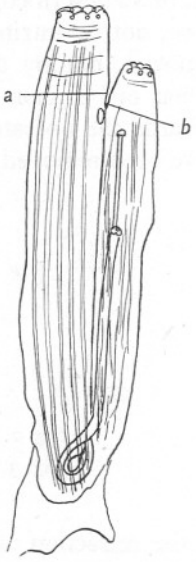

Fig. 1. *Ciona intestinalis*, showing level of cut for removal of inhalant siphon (*a*) and exhalant siphon (*b*).

Ninety-nine animals were operated upon for the removal of both siphons and then preserved for observations upon the histological changes involved in regeneration. The animals were killed at regular intervals, hourly up to 6 hr., two-hourly from 6 to 24 hr., and thereafter at four-hourly periods up to 96 hr., three being killed at each stage. The anterior thirds of the animals were fixed, either in Bouin-Dubosq or Benoit. The sectioned material was cut at 4 or 6μ and stained in Unna's pappenheim, or toluidine blue and eosin after Bouin-Dubosq, or with iron haematoxylin counterstained with light green after Benoit. The Feulgen test was carried out in many cases.

In addition, sixteen animals from which both siphons had been removed were used for experiments in *intra-vitam* staining. The material was immersed either in neutral red or janus green B. The concentration for each was 1 ml. of a 0·1 % solution of the stain in 9 ml. of sea water. Neutral red was used as an indicator of the presence of intracellular neutral red vacuoles, regarded by Baker (1945, 1949) as a component of the Golgi apparatus, janus green B as

an indication of the distribution of mitochondria. Six injured and five uninjured animals were immersed in neutral red, and two injured and two uninjured in janus green B for a period of 24 hr. in each case.

Observations on the Living Material

For half an hour to 1 hr. after operation the effect of the anaesthetic is still evident; the reduced siphons, if the two apertures remaining after operation can be so described, still gape. From 1 to $2\frac{1}{2}$ hr. later, however, the 'siphons' close, not by intrinsic muscular movements, since the sphincters have been removed, but by differential movements of the anterior end of the body. When one siphon alone has been removed, the anterior end bends over towards the operated side, thus effectively closing the aperture; when both have been removed, differential contractions of the mantle musculature result

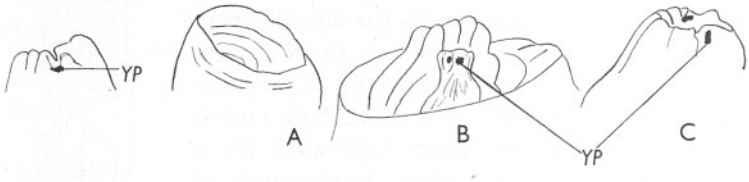

Fig. 2. Fig. 3.

Fig. 2. Mouth, 1 day after operation. *YP*, yellow pigment.

Fig. 3. Two days after operation. A, mouth (specimen IV); B, mouth (specimen I); C, atriophore (specimen II).

in the retraction of the siphon region into the anterior end of pharynx and atrium. However, in 3 hr. time the apertures are again open. Momentary stimulation with a dissecting needle fails to produce any closure of the actual apertures, and only prolonged stimulation provokes a repetition of the bending or retraction movements described above.

During the first day (Fig. 2) certain reactions to the operation are observed. The cut edges bend inwards, and in 60% of the specimens are the site of the irregular deposition of yellow pigment, a phenomenon noted by Hirschler (1914).

In the course of the second day (Fig. 3) very slight growth of the siphons occurs, sufficient to restore a regular, but not characteristically lobed shape. The new growth is extremely delicate and there is no visible evidence of regeneration of a new tunic by the new tissue. In a few animals the yellow pigmentation becomes more intense, but in most it was noticeably paler, and from one had entirely disappeared.

The changes initiated during the second day continue into the third. As a result of continued but differential growth, the siphons acquire the characteristic lobes. Further, during this period, secretion of tunicin by the new

tissues begins. The siphons now open and close normally, an indication of the presence of sphincter muscles. By the end of the fourth day the 'ocelli' have differentiated (Fig. 4).

Thus, in 4 days, siphons complete in all respects other than length are regenerated. There were only two exceptions. In one specimen the pigment spots appeared on the third day, and in another they were not visible until the fifth. The removal of only one instead of both siphons does not expedite the repair process for the stages and speed of regeneration are identical in each.

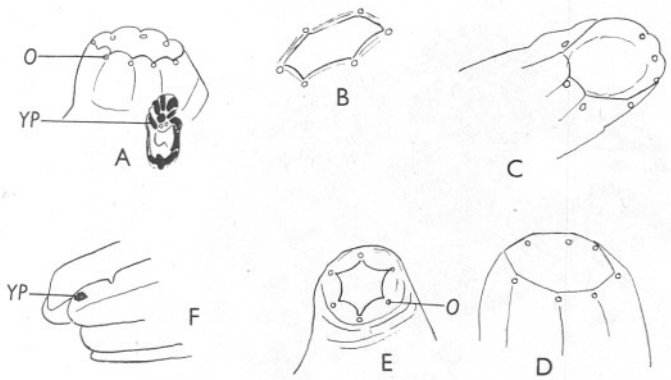

Fig. 4. Four days after operation. A, mouth (specimen I); B, atriophore (specimen II); C, mouth (specimen III); D, mouth (specimen IV); E, exhalant siphon: neuroglandular complex injured but left *in situ*; F, exhalant siphon: neuroglandular complex removed. *O*, ocellus (pigment spot); *YP*, yellow pigment.

However, a profound effect is produced by the removal of the neuro-glandular complex together with the siphons (Fig. 5). Regeneration of the siphons is much slower, not being completed until 13 or 14 days have elapsed. If the complex is damaged by maceration with a needle, but is not excised, the regeneration rate is normal.

The process of regeneration subsequent to the fourth day involves a thickening of the new tunic and continued growth in length of the siphons, the normal size being regained in 2–3 weeks after operation.

Neutral red staining was used to indicate the presence of intracellular 'neutral red vacuoles' regarded by Hirsch (1939) as the Golgi pre-substance, and by Baker (1945, 1949) as part of the Golgi apparatus. This cell organelle is generally regarded as being connected with secretory activity. It was therefore hoped that this technique would give evidence of increased secretory activity by the cells of the injured region. Observations over a period of 2 days in fact indicated that the injured region took up neutral red to a greater extent than the undamaged tissues. Microscopic examination after 24 hr. showed, however, that cells other than those with yellow pigment granules, muscle and test cells, betrayed a much greater affinity for the stain that had been expected.

Both cytoplasm and nucleus of haemoblasts, of cells with many refringent granules or with a single granule, of lymphocytes, and of amoebocytes took up the stain. The granules where present coloured more intensely than the cytoplasm. In phagocytes and in senescent cells in the tunic, the contents of the vacuoles took up the stain intensely; the cytoplasm and nucleus were coloured to a much smaller degree. As the senescent cells moved towards the edge of the tunic the intensity of the neutral red within the growing vacuoles steadily increased.

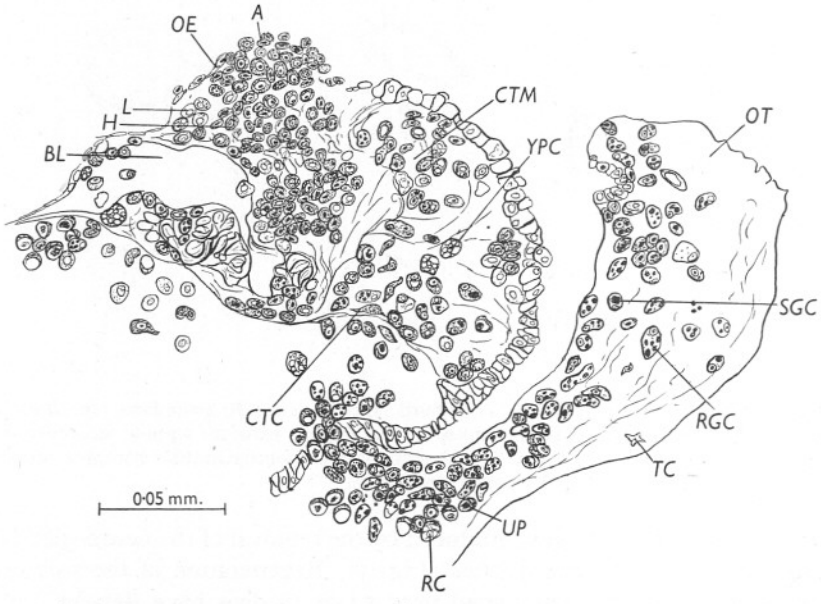

Fig. 5. Six hours after operation—atriopore. *A*, amoebocyte; *BL*, blood lacuna; *CTC*, connective tissue cell; *CTM*, connective tissue matrix; *H*, haemoblast; *L*, lymphocyte; *OE*, original epidermis; *OT*, original tunic; *RC*, reticulate cell; *RGC*, refringent granule cell; *SGC*, cell with single granule; *TC*, tunic cell; *UP*, univacuolar phagocyte; *YPC*, yellow pigment cell.

Janus green B was used in an attempt to stain mitochondria. The reactions observed were interesting, since, with minor exceptions, those regions within the cells which showed an affinity for neutral red also took up janus green B. So widespread was the staining within the cells that it was impossible, in general, to distinguish the mitochondria, which according to Pérès (1943) are never large.

Observations on Preserved Material

Examination of sectioned material shows that during the first hour after siphon removal no visible changes in the affected tissues occur.

During the first 6 hr. after operation two reactions to the injury by the affected tissues can be distinguished. The first may be described as comprising

direct traumatic responses to the operation; the second as comprising processes that represent the earliest stages in the regeneration of the new siphons.

The earliest direct traumatic response is the approximation and apposition of the cut epidermal edges of the mantle which bend inwards and meet (Fig. 6). At first they may overlap, but later they appear to fuse. The epidermal cells in this region alter in shape from characteristic small flattened cells to a cubical, and in places almost columnar, epithelium. In many specimens, where the two epidermal layers do not meet, the gap is plugged by a clump of orange pigment cells, the largest of the blood cells. In other regions the gap is plugged by muscle cells. The differing degrees of intensity of the yellow pigmentation round the cut siphon edges is thus explained. The effect of these processes is to protect the subepidermal tissue from exposure to the water.

The orange pigment cells appear not to have a very long life, since even at this stage a few can be seen degenerating, a process which can occur in two ways. The first is characterized by a change in colour of the pigmentation from yellow or orange to green, and finally by a complete disintegration of the cell and release of the granules into the neighbouring subepidermal regions. Very probably the freed granules are then removed by two types of blood cell, univacuolar phagocytes and amoebocytes. The second method is that described by Pérès (1943). The orange pigment cells again change in colour to green and become reticulate cells (Fig. 5) with an achromatic nucleus. In the hyaline compartments between the refringent bars, one or two small granules can be seen, but in time even these disappear.

Another direct result of the operation is the damage or destruction of cells at the site of the injury. The removal of these cells is performed by amoebocytes and univacuolar phagocytes (Fig. 5). These appear in large numbers from two sources: (i) by emigration from the blood stream, and (ii) by differentiation on the spot, from haemoblasts and lymphocytes. At this stage the number of haemoblasts, although increasing, is not yet large, and, while differentiation of the two types of phagocytic cells is to be observed, the number produced by this means can only be relatively small.

The mobilization of the cells that take part in the regeneration of the siphons begins during this period. These cells are the haemoblasts, the basic blood cells which originate on the epicardium. Examination of sections shows that the initial increase in numbers of the haemoblasts is only, in very small measure, due to the divisions of haemoblasts already present in the injured area (Figs. 5, 6). The number of dividing cells is always small, although it increases slightly during the period of regeneration. Examination of both injured and uninjured animals, vitally stained with neutral red, which facilitates the recognition of haemoblasts in living material, shows that the cavities of the tentacles form reservoirs of haemoblasts. From the bases of the tentacles of injured animals chains of these cells extend towards the cut edges

of the siphons. There is no evidence of a similar migration in uninjured animals. Further examinations of portions of the epicardium from both injured and uninjured animals give pertinent results. While the epicardium of uninjured animals shows a large number of haemoblasts, that of injured animals shows a vastly increased population. So dense is it over considerable areas that the cells are actually touching each other.

Thus the operation would seem to stimulate the liberation of haemoblasts from the tentacles, and to increase the rate of proliferation of these cells by the epicardium. They then enter by way of the blood stream into the zone of regeneration.

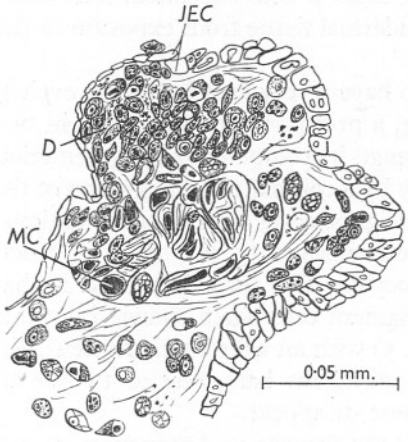

Fig. 6.

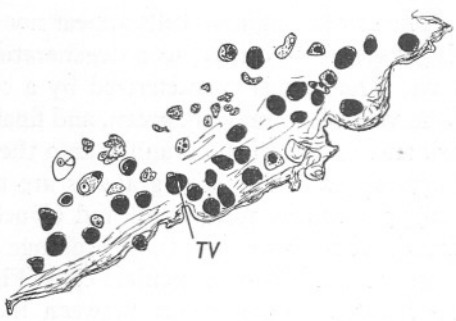

Fig. 7.

Fig. 6. Six hours after operation—mouth. The scale refers to this figure. *D*, debris; *JEC*, epidermal junction; *MC*, muscle cell showing extent of thymonucleic acid in cell.

Fig. 7. Edge of old test showing senescent cells. *TV*, cell with thymonucleic acid vacuole.

The first signs of differentiation from haemoblasts of the three types of cells that participate in the regeneration of the tunic can be distinguished 6 hr. after operation (Fig. 6). In addition, the number of these cells in a fully developed condition in the region has also increased. Some of these have already passed into the tunic adjacent to the old mantle (Fig. 6).

Finally, certain cells are eliminated from the animal by a process analogous to desquamation. Senescent cells, having migrated into the tunic, move towards the free edge and undergo degenerative changes *en route*. No matter what their origin, they all acquire a relatively enormous vacuole, and at the same time cytoplasm and nucleus decrease in volume. Ultimately only a crescentic area of cytoplasm containing a very small achromatic nucleus is to be distinguished (Fig. 7). The vacuole stains deeply with toluidine blue, the methyl green of Unna's Pappenheim, and is positive to Feulgen. These

reactions suggest that as an accompaniment to the deterioration of the cells there is a nuclear breakdown which results in a liberation of thymonucleic acid into the vacuole. In other words, there appears to be a modified form of chromatolysis (cf. Wigglesworth, 1942). At the free edge the cells seem to drift together (Fig. 7) and as they pass out of the tunic become surrounded by a thin bag formed by the test. This finally breaks away from the animal altogether.

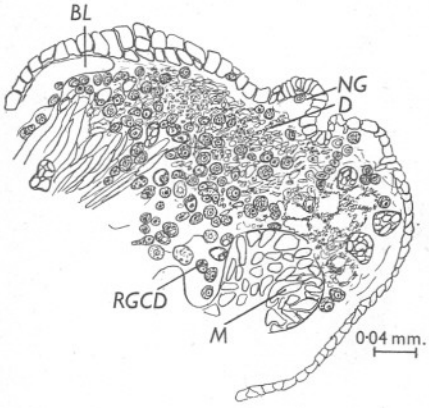

Fig. 8.

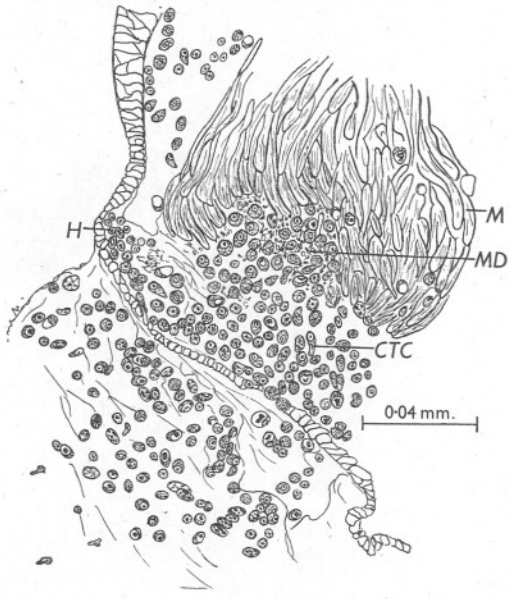

Fig. 9.

Fig. 8. Twelve hours after operation—mouth. *BL*, blood lacuna; *D*, debris; *M*, muscle; *NG*, new growth; *RGCD*, differentiating refringent granule cell.

Fig. 9. Sixteen hours after operation—mouth. *CTC*, connective tissue cell; *H*, haemoblast; *M*, muscle; *MD*, muscle debris.

Twelve hours after the operation (Fig. 8) the two epidermal layers have completely fused all round the reduced 'siphons'. In the subepidermal connective tissue there is a slight decline in the number of orange pigment cells, univacuolar phagocytes, amoebocytes and reticulate cells and an increase in the free pigment granules and in the numbers of small and medium-sized cells with refringent granules. The processes described as occurring 6 hr. after the operation continue.

By 16 hr. (Fig. 9) the numbers of those cells concerned with the regeneration of the missing siphons has markedly increased. The subepidermal lacunae are packed with haemoblasts and with cell-types differentiating from them. Univacuolar phagocytes, amoebocytes and yellow pigment cells remain, but they are now few.

In certain regions (Fig. 9) there appears to have been a mass migration of cells with one or several refringent granules into the tunic. In one specimen in which, as a result of the operation, the old test was torn from the epidermis posterior to the actual cut, new test growth was well advanced. The epidermal cells had already laid down the ground substance of the tunic, and cells with one or many granules were in the process of adding to the ground substance exactly as described by Pérès (1948*a*).

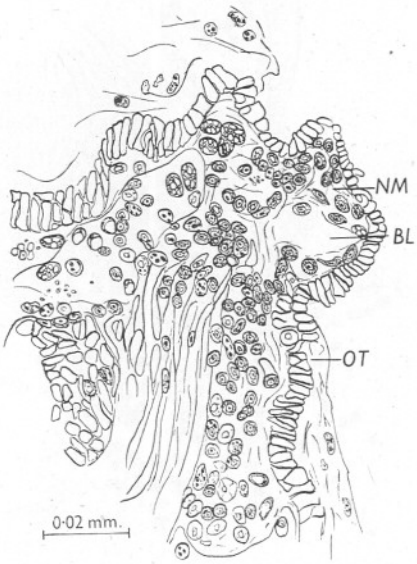

Fig. 10.

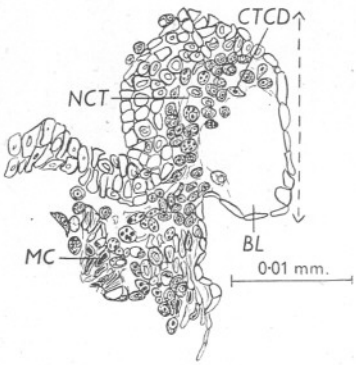

Fig. 11.

Fig. 10. Twenty-four hours—mouth. *BL*, blood lacuna; *NM*, new connective tissue matrix; *OT*, original tunic.

Fig. 11. Thirty-six hours—mouth. *BL*, blood lacuna; *CTCD*, differentiating connective tissue cell; *MC*, muscle cell; *NCT*, new connective tissue matrix; - - - , new growth.

In 24 hr. (Fig. 10) the haemoblast population is at its maximum density and the first evidence of actual regeneration of the mantle is observed. Between 16 and 24 hr. after the operation, the epidermal cells have begun to divide and a slight outpushing of the epidermis all round the cut edges of the siphons is apparent (Fig. 10). The bulge becomes filled with blood plasma carrying haemoblasts. Elsewhere in the region differentiation of haemoblasts and lymphocytes continues, in particular into cells with many granules or with a single granule, some of which migrate into the tunic, while others remain in the subepidermal region to perform other functions.

By 36 hr. (Fig. 11) the epidermal outpushing has increased in length and, in the subepidermal space, all stages in the differentiation of the haemoblasts into connective tissue cells can be distinguished (Fig. 11). The many cells

which have fully differentiated have begun to secrete the connective tissue matrix (Fig. 11) characteristic of the subepidermal regions of the normal mantle.

It is to be remarked that very rarely in the regenerating regions do the different types of blood cell differentiate from lymphocytes. Differentiation almost invariably appears to involve haemoblasts alone. Pérès has stated (1943) that the various blood elements, in young animals differentiate from

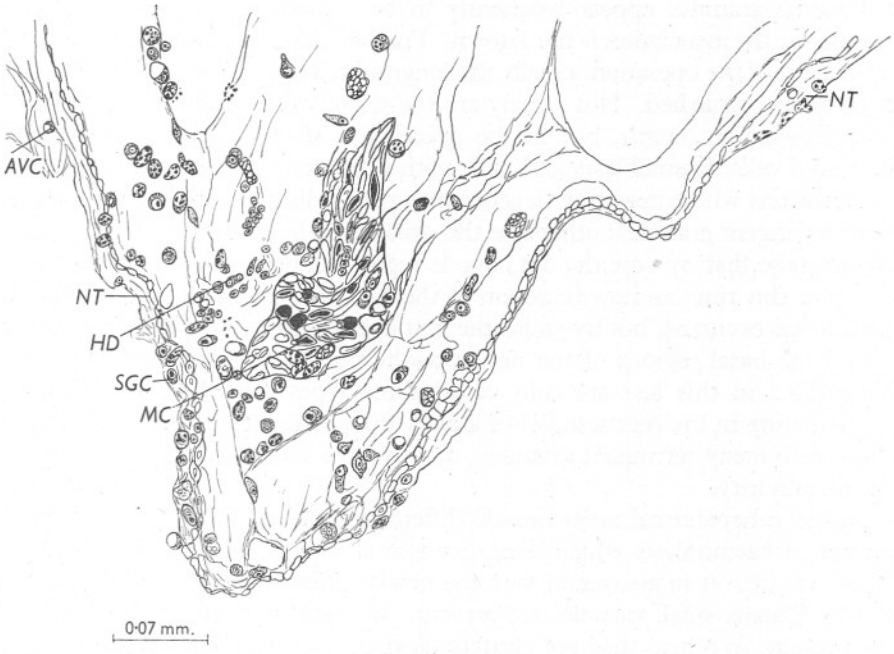

Fig. 12. Fifty-six hours—mouth. *AVC*, acidophil vacuole cell; *HD*, dividing haemoblast; *MC*, muscle cell; *NT*, new tunic; *SGC*, cell with single granule.

haemoblasts, in older animals from lymphocytes. It would therefore appear that in old specimens of *Ciona* regeneration is associated with the type of cell differentiation characteristic of young animals.

In one specimen killed 36 hr. after operation, the inhalant siphon had been cut sufficiently far posteriorly to involve the anterior pharynx. Here regeneration of new gill bars was in process. It was of interest to note that the endoderm and ectoderm, separated by connective tissue, which lined the anterior end of the pharynx, were identical in appearance. The ectoderm and endoderm had joined round the cut edge, and could not be distinguished from each other. Laterally and internally, slight bulges were visible at intervals, the forerunner of new gill bars and papillae.

By 48 hr. the ectodermal outpushings at the apices of the regenerating siphons have become even longer. In the posterior region of the regenerated zone a clumping of haemoblasts in the recently secreted connective tissue matrix has occurred. Differentiation of these cells along three lines has already been initiated. One group has begun slightly to elongate to form new muscle cells, another to differentiate into cells with refringent granules, and a third, much smaller than the others, consists of haemoblasts in the process of differentiation into cells with a single refringent granule. Cells with several refringent granules appear frequently to be associated with muscles. The reason for the association is not known. Further changes in muscles damaged as a result of the operation, chiefly the longitudinal muscles of the mantle, can now be distinguished. Not merely are the slightly damaged cells now intact and growing in length, but in the spaces left after the removal of badly damaged cells, haemoblasts and cells with refringent granules are found.

In the test where new growth is occurring the cells present are mostly those with refringent granules; otherwise the situation is little different from that at 24 hr., save that by now the cut edge is regular in outline.

Up to this time no new secretion of the tunic by the newly differentiating mantle has occurred, but by 56 hr. the first appearance of a very delicate tunic round the basal regions of the new growths can be distinguished (Fig. 12). Embedded in this test are cells of all three types described by Pérès, as participating in the regeneration of a new tunic. In general, the majority are those with many refringent granules; those with a small acidophil vacuole are in the minority.

In the subepidermal areas muscle differentiation continues and additional groups of haemoblasts which later give rise to muscle cells can be observed (Figs. 12–13). Also associated with the newly differentiated muscles are cells in which many small vacuoles are present. In some only one or two granules are present, in others they are entirely lacking. These are probably stages in the degeneration of the refringent granule cells.

In certain regions of the newly regenerating siphons there appears to have been no appreciable growth in length between 48 and 56 hr.; in others growth has continued. The third day sees the appearance of lobes characteristic of the siphons; and the differential growth just mentioned is undoubtedly associated with the development of these lobes. This type of growth continues through the third day.

By 60 hr. the picture of the regenerative processes is very little different from that observed at 56 hr. In the new tunic, which by now has here and there fused with the old, the number of cells with single acidophil vacuoles has increased, and some of the cells with many refringent granules have entered into the second phase of activity (Pérès, 1948*a*). The original granules are much paler and, in some cells, difficult to distinguish, while densely staining spheroidal masses, probably proteins, are appearing.

From 76 hr. until the end of the fourth day (Figs. 14–16) the regeneration of the siphons continues by repetition of the processes already described. For the first time near the outer edge of the tunic secreted by the newly regenerated mantle, a few cells with large vacuoles whose contents stain with toluidine blue, methyl green, and are positive to Feulgen, are seen.

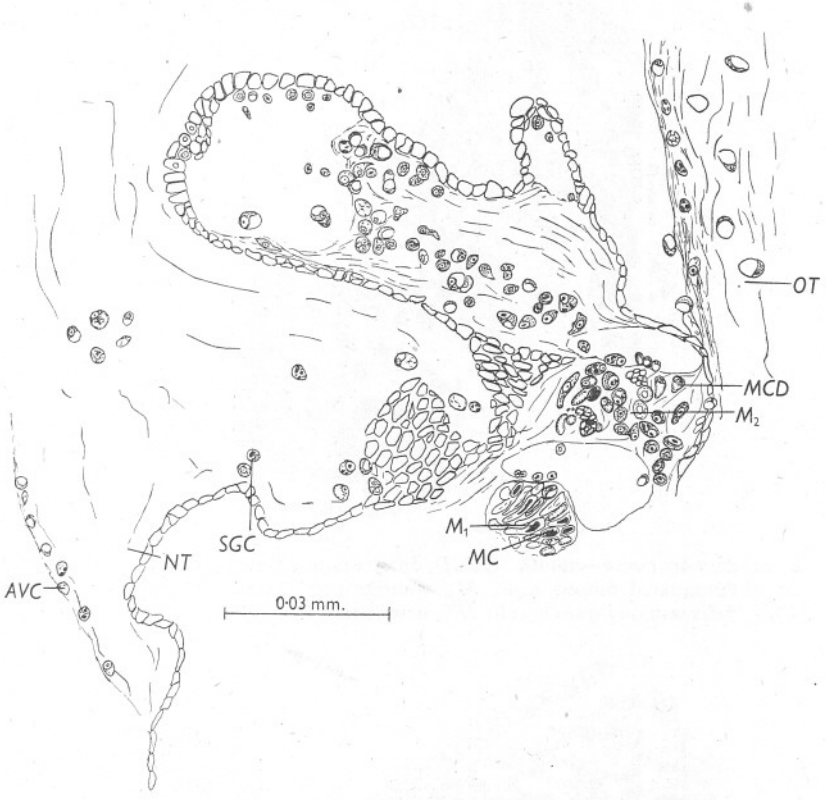

Fig. 13. Sixty hours—atriopore. *AVC*, acidophil vacuole cell; M_1, almost completely differentiated muscle cells; M_2, younger cells differentiating into muscle cells; *MC*, muscle cell; *MCD*, differentiating muscle cell; *NT*, new tunic; *OT*, original tunic; *SGC*, single granule cell.

The peculiar nature of the muscle cells should now be mentioned. Sections through individual fibres of fully developed muscles show that thymonucleic acid is evenly distributed throughout the cell, positive results having been given by both the Feulgen test and Unna's pappenheim. During the development of a fibre from a haemoblast the thymonucleic acid in the cytoplasm steadily increases in amount. With its appearance the small nucleus present during the earlier stages of growth ceases to be microscopically recognizable. The fully differentiated muscle cell is therefore apparently non-nucleated.

The description of the regeneration processes just given is characteristic of the majority of animals examined. There are minor variations in the time of tunic secretion by the newly formed mantle. It may be slightly earlier than has been stated.

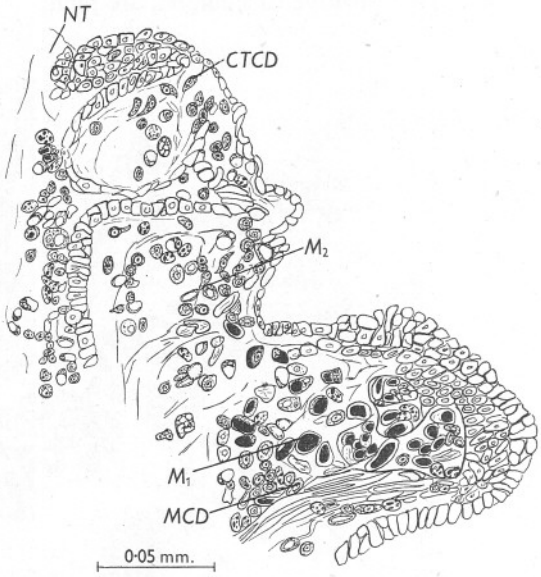

Fig. 14. Seventy-six hours—mouth. *CTCD*, differentiating connective tissue cell; M_1, almost fully differentiated muscle cells; M_2, younger cells differentiating into muscle cells; *MCD*, differentiating muscle cell; *NT*, new tunic.

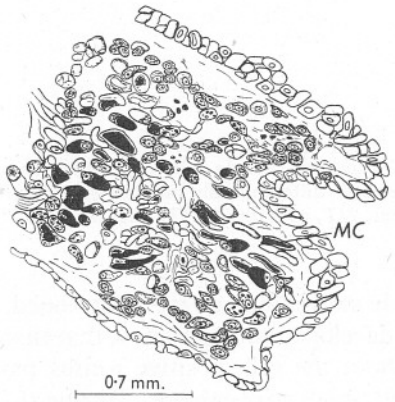

Fig. 15.

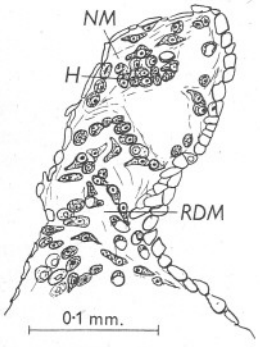

Fig. 16.

Fig. 15. Eighty hours—mouth—further growth. *MC*, muscle cell.

Fig. 16. Eighty hours—atriopore—continued growth. *H*, haemoblast; *NM*, new connective tissue matrix; *RDM*, recently differentiated matrix.

Discussion

The regenerative processes of specimens of *Ciona intestinalis* from which the siphons have been removed involve the haemoblasts, ectoderm, and a small region of the pharyngeal endoderm. The operation stimulates division and migration of the haemoblasts to such an extent that in a few hours a large number will have appeared at the site of the injury. Once there, they may differentiate directly into connective tissue cells, muscle cells and into various types of blood cell, or, much less frequently, differentiation of the blood cells may be preceded by a division of the haemoblast into two lymphocytes. The ectoderm and endoderm of certain regions of the pharynx are microscopically indistinguishable, and in some animals from which the most anterior region of the pharynx has been removed it is impossible to tell whether ectoderm or endoderm is proliferating to give rise to the new endoderm. The participation of haemoblasts in the formation of the new epithelia is in doubt. Certainly in some sections haemoblasts are seen wedged among the new ectoderm and endoderm cells. They may, however, be merely in transit through the epithelium.

There have been many accounts of asexual reproduction and regeneration among ascidians, but almost all of them suffer from inadequate accounts of the histological processes involved. Nevertheless, an attempt will be made to review the processes, to relate the phenomena of siphon regeneration in *Ciona* to those of ascidian regeneration in general, and to formulate certain principles which may go some way towards explaining the outstanding powers of asexual reproduction and regeneration in ascidians.

Asexual reproduction and regeneration in the phlebobranchs and aplousobranchs (in Harant's classification, 1933) either involve internal organogenesis from the epicardium, a pharyngeal outgrowth, or from a mesenchymatous septum, or from the blood cells, as in *Ciona*. As will be seen later both organogenesis from the epicardium and from the septum can occur in one and the same animal.

Before these methods can be discussed, more detailed descriptions of the processes involved must be given.

Asexual reproduction in *Diazona violacea* involves the formation of buds by transverse constrictions of the post-abdomen (Berrill, 1948*b*). Each bud contains ectoderm, muscles, two sections of the alimentary canal and part of the epicardium. From the epicardium are proliferated cells from which the pharynx, peribranchial sacs, cerebral ganglion and the anterior part of the post-pharyngeal alimentary canal of the new individual develop. The pericardium also has its origin in this structure. The two sections of the parent alimentary canal unite posteriorly and the loop so formed increases in length. This account lacks any detailed histology.

However, Brien's (1933) work on *Archiascidia neapolitana* (Julin) may

clarify the picture. When the thorax has been naturally or artificially lost, the ventral wall of the epicardium thickens and proliferates cells, described by Brien as embryonic in appearance, and it is from these cells that the two pharyngeal lobules develop that later coalesce to give the definitive pharynx. Significantly Brien describes the cells proliferated by the epicardium as 'embryonic', and very probably these are haemoblasts. In 1933 Pérès had not described the various types of blood cell to be found in ascidians and this term was therefore unknown to Brien. As noted above, Berrill (1948*b*) describes the epicardium in *Diazona* as dividing to give rise to cells from which the branchial sac, atrium, etc., develop. These cells may well be similar to those described as embryonic in *Archiascidia* and thus also be haemoblasts.

Asexual reproduction in *Clavelina phlegrea* (Salfi) is in its major aspects (Brien, 1930) an almost exact replica of that of *Archiascidia neapolitana*, but in another species, *Clavelina lepadiformis*, and in the Perophoridae, an apparently different process is involved. Here each of the bud chambers that develop upon a stolon contains a 'blastogenic mass', consisting in *C. lepadiformis* of a mass of blood cells, which Brien (1930) calls lymphoblasts, and of mesenchyme cells. These cells are the products of the disintegration of a mesenchymatous septum to which the lymphoblasts had become attached. In this mass an 'endoblastic vesicle' appears, on the future dorsal side of which there develops a neurogenital 'massif'. This vesicle is the forerunner of the pharynx peribranchial cavities, epicardium and the remainder of the alimentary canal. The neural and genital cells dissociate from each other. Further development consists of the differentiation of organs whose 'anlagen' have now appeared.

It therefore seems that, according to the current view, two methods of asexual reproduction and regeneration can occur in the one genus *Clavelina*. Further, it has been found experimentally that the two processes can occur in the same species *C. lepadiformis*. Asexual reproduction is normally at the expense of a mesenchymatous septum. If, however, as a result of transverse cuts, a bud chamber is formed in the oesophageal region, the new organs develop from the epicardium (Brien, 1933; Berrill & Cohen, 1936). The epicardium, a pharyngeal outgrowth, is generally said to be an endodermal organ. Thus, clearly, according to current ideas, two very different methods of asexual reproduction can occur, not merely in the same genus, but even in the same species. This conclusion seems to be very much open to doubt.

As already stated, the epicardium is generally regarded as an endodermal structure. Berrill (1951) has, however, suggested that it may possibly be of mesodermal origin. There are now good reasons for the acceptance of this idea. Enterocoely is common among protochordates and echinoderms, possible ancestors or close relations to the ancestors of the urochordates. To suggest, therefore, an enterocoelic origin for the epicardial tubes seems not unreasonable. This would make the cavity of an epicardial tube a portion of the coelom. In its continued growth, in *Ciona* the epicardium comes to invest the abdominal

organs; the potential cavity in this double-layered structure would thus form a perivisceral coelom, whose absence in ascidians has seemed anomalous. Further, the epicardium has been stated by Pérès (1947*c*) to be the centre of haemoblast proliferation, particularly in larval life. He also notes that in the Polyclinidae it undergoes haemoblastogenesis in the adult condition. In cephalochordates and in most vertebrates, according to Brachet (1944), blood cells are of mesodermal origin.

If the mesodermal nature of the epicardium be conceded, then epicardial and septal budding are but different manifestations of a single process. This hypothesis, however, does not account for the phenomena of siphon regeneration in *Ciona* and an attempt will now be made to show that this apparent defect can be overcome.

Pérès (1947*c*) pointed out the epicardial origin of haemoblasts, and Brien (1933) noted the proliferation of embryonic cells from the epicardium during asexual reproduction in *Archiascidia neapolitana*. He also described the mesenchymatous septum in *Clavelina lepadiformis* as consisting, very largely, of lymphoblasts (haemoblasts). It might also be suggested that the already differentiated mesenchymatous cells would be unlikely to play an important role in organogenesis. Thus, in both species the same cells, the haemoblasts, are almost certainly extensively involved in asexual reproduction, and in both they have the same origin, the epicardium. Berrill's (1948*b*) account of asexual reproduction in *Diazona violacea* offers no serious objection to this view. The epicardial origin of cells involved in organogenesis is noted. It is true that he states that blood cells play no part in the process, but this statement refers to the nutritive trophocytes.

It therefore seems reasonable to suggest that, in the phlebobranchs and aplousobranchs, asexual reproduction occurs at the expense of haemoblasts, cells proliferated in an undifferentiated condition, from the epicardium. The position of *Ciona*, a phlebobranch, in so far as regenerative processes are concerned, is now clear. In this animal, the cells responsible for the regeneration of all tissues other than the epithelia, of the siphons, are haemoblasts originating in the epicardium.

As a point of interest, according to Pérès (1947*b*), the cerebral ganglion of *Ciona* differentiates from neoblasts. These cells appear to be very like haemoblasts. The cerebral ganglia of *Diazona* and *Clavelina*, if the views outlined above be accepted, also differentiate at the expense of haemoblasts.

Finally, a third method of asexual reproduction in ascidians is seen in the pallial budding of the stolidobranchs. Here mesenchyme and ectoderm alone are involved in organogenesis; the mesenchyme is that tissue lying between the two layers of ectoderm of the outer atrial wall. The vesicle that gives rise to the future pharynx, nerve complex and atrial cavity is lined, at its first appearance, by ectoderm. A neural tube and two peribranchial cavities evaginate and become isolated from the parent vesicle, after which process

differentiation to form the definitive organs takes place. The future genital cells are carried from the parent gonads to the bud by the blood stream (*Botryllus*), or differentiate from mesenchyme cells, *in situ* (*Heterocarpa glomerata*). It must be emphasized that detailed histological investigations of the processes involved have never been attempted, and further work along these lines may considerably modify our present information.

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Summary

Regeneration of the siphons of normal specimens of *Ciona intestinalis* has been found to be completed, in every particular other than that of length, in 4 days.

Removal of the neuroglandular complex retards the regeneration rate.

With the exception of the epidermis and of the basic tunic, the new structures differentiate from haemoblasts.

A tentative synthesis has been made of the information available upon asexual reproduction and regeneration in the Ascidiaceae.

REFERENCES

Azema, M., 1937. Recherches sur le sang et l'excrétion chez les Ascidies. *Ann. Inst. Océanogr. Paris*, T., 17, pp. 1–150.

Bacq, Z. M. & Florkin, M., 1935. Mise en évidence dans le complexe 'ganglion nerveux-glande neurale d'une Ascidie (*Ciona intestinalis*) des principles pharmacologiquement analogues à ceux du lobe postérieur de l'hypophyse des Vertébrés. *Arch. internat. physiol*, T. 40, pp. 422–8.

Baker, J. R., 1945. The structure and chemical constitution of the Golgi element. *Quart. Journ. Micr. Sci.*, Vol. 85, pp 1–71.

—— 1949. Further remarks on the Golgi element. *Quart. Journ. Micr. Sci.*, Vol. 90, pp. 293–307.

Barth, L. G., 1940. The process of regeneration in hydroids. *Biol. Rev.*, Vol. 15, pp. 405–20.

Berrill, N. J., 1929. Studies in tunicate development. Part I. *Phil. Trans. Roy. Soc.* B, Vol. 218, pp. 37–78.

—— 1932. Mosaic development of the ascidian egg. *Biol. Bull. Woods Hole*, Vol. 8, pp. 381–6.

BERRILL, N. J. 1935*a*. Studies in tunicate development. Part III. *Phil. Trans. Roy. Soc.*, B, Vol. 225, pp. 255–326, 327–79.
—— 1935*b*. Cell division and differentiation in asexual and sexual development. *Journ. Morph. Philadelphia*, Vol. 57, pp. 353–427.
—— 1936. Studies in tunicate development. Part V. *Phil. Trans. Roy. Soc.*, B, Vol. 226, pp. 43–70.
—— 1947. The development and growth of *Ciona*. *Journ. Mar. Biol. Assoc.*, Vol. 26, pp. 616–25.
—— 1948*a*. Structure, tadpole and bud formation in the ascidian *Archidistoma*. *Journ. Mar. Biol. Assoc.*, Vol. 27, pp. 380–8.
—— 1948*b*. The development, morphology and budding of the ascidian *Diazona*. *Journ. Mar. Biol. Assoc.*, Vol. 27, pp. 389–99.
—— 1950. *The Tunicata*. Ray Society Publication, London.
—— 1951. Regeneration and budding in tunicates. *Biol. Rev.*, Vol. 26, pp. 456–73.
BERRILL, N. J. & COHEN, A., 1936. Regeneration in *Clavelina lepadiformis*. *Journ. Exp. Biol.*, Vol. 13, pp. 352–62.
BOURNE, G., 1950. *Cytology and Cell Physiology*. Oxford.
BRACHET, J., 1944. *Embryologie chimique*. Paris.
BRIEN, P., 1927. Contribution à l'étude de la blastogenèse des Tuniciers. Bourgeonnement chez *Apilidium zostericola* (Giard). *Arch. biol.*, T. 35, pp. 155–205.
—— 1930. Contribution à l'étude de la régénération naturelle et experimentale chez les Clavelinidae. *Ann. Soc. zool. Belg.*, T. 61, pp. 19–112.
—— 1933. Régénération thoracique chez *Archiascidia neapolitana* (Julin). *Bull. Biol. France-Belge*, T. 67, pp. 100–24.
BRIEN, P. & BRIEN-GAVAGE, E., 1927. Contribution à l'étude de la blastogenèse des Tuniciers. III. Bourgeonnement de *Clavelina lepadiformis* Müller. *Rec. Inst. Zool. Torley-Rousseau Bruxelles*, T. 1, Fasc. 1, pp. 31–81.
—— —— 1928. Contribution à l'étude de la blastogenèse des Tuniciers. IV. Recherches sur le bourgeonnement de *Perophora listeri* (Weigm). *Rec. Inst. Zool. Torley-Rousseau Bruxelles*, T. 1, pp. 123–52.
TEN CATE, 1928. Contribution à la question de la fonction du système nerveux de *Ciona intestinalis*. *Arch. Neerland Sci.*, Bd. 13, pp. 391–401.
CHILD, C. M., 1941. *Patterns and Problems of Development*. Chicago.
COHEN, A. & BERRILL, N. J., 1936. The development of isolated blastomeres of the ascidian egg. *Journ. Exp. Zool.*, Vol. 74, pp. 91–117.
DAS, S. M., 1936. Structure and function of the ascidian test. *Journ. Morph.*, Vol. 59, pp. 539–601.
DRACH, P., 1948. *Traité de Zool.*, Vol. 11, pp. 738–46. Masson et Cie, Paris.
FOX, H. M., 1924. Note on Kammerer's experiments with *Ciona*, concerning the inheritance of an acquired character. *Journ. Genetics*, Vol. 14, pp. 89–91.
GARSTANG, W., 1928. The morphology of the Tunicata and its bearings on the phylogeny of the Chordata. *Quart. Journ. Micr. Sci.*, Vol. 72, pp. 51–187.
HARANT, H. & VERNIÈRES, P., 1933. *Tuniciers*. Faune de France, Paris.
HIRSCH, G. C., 1939. *Form- und Stoffwechsel der Golgi-Körper*. Borntraeger, Berlin.
HIRSCHLER, J., 1914. Über die Restitutions and Involutions—vorgänge bei operieten. Exemplaren von *Ciona intestinalis*. *Archiv.-mikr-Anat. Bonn.*, Bd. 85, pp. 205–27.
HUXLEY, J. S., 1926. Studies in dedifferentiation. VI. Reduction phenomena in *Clavelina lepadiformis*. *Pubb. Staz. zool. Napoli*, Vol. 7, pp. 1–34.
KAMMERER, P., 1923. Experiments on *Ciona* and *Alytes*. *Nature*, Vol. 112, pp. 826–7.
SELYS-LONGCHAMPS, M. DE, 1916. Sur le burgeonnement des Polystyelinés. *Stolonica* et *Heterocarpa*. *Bull. Sci. France-Belgique*, T. 50, pp. 170–276.

PÉRÈS, J. M., 1943. Recherches sur le sang et les organes nerveux des Tuniciers. *Ann. Inst. Océanogr. Paris*, T. 21, pp. 229–359.

—— 1947*a*. Recherches sur le sang et la tunique commune des Ascidies composées. *Ann. Inst. Océanogr. Paris*, T. 23, pp. 345–473.

—— 1947*b*. Remarques sur le complexe neuroglandulaire de *Ciona intestinalis* et les propriétés de ses extraits. *Bull. Lab. Marit. Dinard*, T. 29, pp. 29–34.

—— 1947*c*. Interdépendance du sang, du mesenchyme et de la tunique chez les Ascidies. *Bull. Mus. Hist. nat. Marseille*, T. 7 (2–3), pp. 83–90.

—— 1948*a*. Recherches sur la genèse et la régénération de la tunique chez *Ciona intestinalis* L. *Bull. Inst. Oceanogr. Monaco*, No. 936, pp. 1–12.

—— 1948*b*. Recherches sur la genèse et la régénération de la tunique chez *Clavelina lepadiformis* Müller. *Arch. Anat. Micr. Morph. exp.*, 3, pp. 230–60.

ROSE, S. M., 1939. Embryonic induction in the Ascidia. *Biol. Bull. Woods Hole*, Vol. 77, pp. 216–32.

SCHULTZE, L. S., 1899. Die Regeneration des Ganglions von *Ciona intestinalis*. *Jena Zeitschr. Naturw.*, Bd. 33, pp. 263–344.

SPEMANN, H., 1938. *Embryonic Development and Induction*. Oxford.

WEBB, D. A., 1939. Observations on the blood of certain ascidians with special reference to the biochemistry of vanadium. *Journ. Exp. Biol.*, Vol. 16, pp. 499–523.

WIGGLESWORTH, V. B., 1942. The significance of chromatic droplets in the growth of insects. *Quart. Journ. Micr. Sci.*, Vol. 83, pp. 141–52.