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Studies in Tunicate Development. Part I. General Physiology of Development of Simple Ascidians

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II. *Studies in Tunicate Development. Part I.—General Physiology of Development of Simple Ascidians.*

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Contents.

1. *Introduction.*

The development of Ascidians formed the subject of numerous investigations during the latter part of the last century. The interest, however, which was due primarily to the recognition of the chordate characters of the swimming larvæ, and was almost entirely morphological in outlook, then diminished. The present paper is a study from a functional viewpoint and deals with the development typical of most simple ascidians. An account of the abbreviated development to be found within the family Molgulidæ and the accelerated development typical of compound ascidians will be presented in later publications, together with the more general conclusions arising from the study of the class as a whole.

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The results presented here are the outcome of work done at the Marine Biological Laboratories at Plymouth, at St. Andrew's, New Brunswick, and at Woods Hole. Almost all the experimental work was done at Plymouth, that on the variability and floatation of the eggs and on metamorphosis during the summer of 1925 whilst holding a grant from the Committee for Scientific and Industrial Research; that on hatching mechanisms during the springs of 1926–28 whilst occupying the tables of the University of London and University of Leeds. Thanks are due to Dr. E. J. Allen and other members of the Plymouth staff for their interest and assistance. The life histories of the American species were determined during the summer of 1927 at St. Andrew's and Woods Hole whilst holding a travelling fellowship from the International Education Board, to which body, and also to the Biological Board of Canada for the hospitality afforded at St. Andrew's, acknowledgments are made. I wish also to thank Dr. A. G. Huntsman and Prof. E. G. Conklin for their interest and personal assistance in obtaining material, and Prof. D. M. S. Watson for valuable criticism.

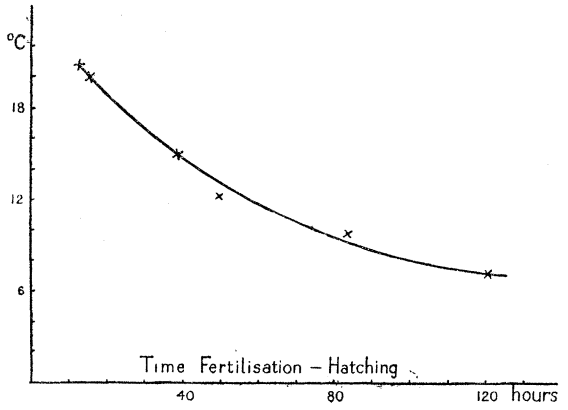
2. *Methods and Material.*

Developmental material was obtained with ease in the case of viviparous species, but in the oviparous forms artificial fertilisations were carried out. Cross fertilisation was invariably made, even in self-fertile species such as the Ascidiidæ. Sufficient sperm was added to the eggs in a fingerbowl of water to cause a faint milkiness, and the combined suspension poured alternately from one fingerbowl to another several times. After one or two hours, according to whether the temperature was high or low and when the eggs had settled to the bottom of the vessel, the water was poured off and replaced. This was repeated and then on three or four occasions during the course of development. In the case of the eggs of *Ascidiella aspersa*, which float to the top of still water, the water was strained away through fine bolting silk.

At Plymouth the following species were obtained fairly readily in quantity:—*Phallusia mammillata*, *Ascidiella aspersa*, *Styelopsis grossularia*, *Polycarpa rustica*; in small numbers only—*Ciona intestinalis*, *Ascidiella scabra*, *Ascidia conchilega*, *Ascidia mentula*, *Ascidia virginea*, *Molgula ampulloides* and *Polycarpa fibrosa*. (*A. mentula*, *A. conchilega* and *A. scabra* are to be obtained much more readily at Roscoff, Brittany). *Boltenia hirsuta*, *Tethyum pyriforme americanum*, *Ascidia prunum*, *Molgula citrina* and *M. retortiformis* were easily obtained in shallow water at St. Andrew's, *Styela partita* at Woods Hole. The principal difficulty in obtaining successful cultures was due to temperature. At Plymouth, Roscoff and Woods Hole, the sea and laboratory temperatures are much the same, and at both places fertilisation and normal development can be obtained for all the species mentioned between 8 and 20° C. at least. At St. Andrew's, however, the temperature of the sea bottom is far below that of the laboratory and successful cultures were only obtained when maintained throughout at temperatures below 11–12° C. This fact probably accounts for the failure encountered at the Marine

Laboratories at Trondhjem and Kristineberg where similar temperature differences obtain. At the latter station the salinity of the water has also to be considered.

The temperature curve given is merely as a rough indication of the time taken from fertilisation to hatching for species with eggs approximately 0·15 mm. diameter;



Temperature Curve.

Temperature curve showing relationship between temperature and the rate of development (approximate only).

Upper four values based on *Phallusia mammillata* (Cuv.), lower two on *Ascidia prunum* (Müll.).

species with smaller or larger eggs (*see* Table III) take a somewhat shorter or longer time respectively.

The question of food for the cultures did not arise for Ascidian larvæ do not feed until considerably after metamorphosis at a time when several protostigmata have been formed. At 15° C. most species do not require food until they are four or five weeks old. Very interesting developmental material for further studies is afforded by *Boltenia hirsuta*, and *Tethyum pyriforme*, both obtainable at St. Andrew's, the former because of the high pigmentation of the lipoidal matter of the eggs and larvæ, the latter for the combination of transparency and large size (compare the tadpoles of Tethyum with that of Styela (figs. 7, A and 10, C).

All the figures illustrating the life history of species, with one exception, are drawn to the same scale and were made from living material with the aid of a camera lucida.

3. *Organisation of the Ascidian Egg.*

(*a*) *Perivitelline Structures.*—The eggs of Ascidians show considerable variation of two types. One is in the quality and quantity of the cell inclusions, the other in the nature of the perivitelline structures.

A fairly typical egg, such as that of *Styela partita* (fig. 10), possesses a chorion enclosing the perivitelline fluid, a few small inner follicle cells ("test cells," "celles de rebut") floating in that fluid, and an investment of vacuolated outer follicle cells adhering to the surface of the chorion. The same structures are present in the egg of *Tethyum*

pyriforme (fig. 6, B), but the ovum is larger, the perivitelline space more extensive, and the inner and outer follicle very much more numerous. In other species, *e.g.*, *Ciona*, *Corella* (fig. 1, D, E), the outer follicle cells have increased in size though not in number.

Within the family Ascidiidæ an additional structure appears in the nature of a fine membrane between the chorion and the ovum.* This usually is very closely applied

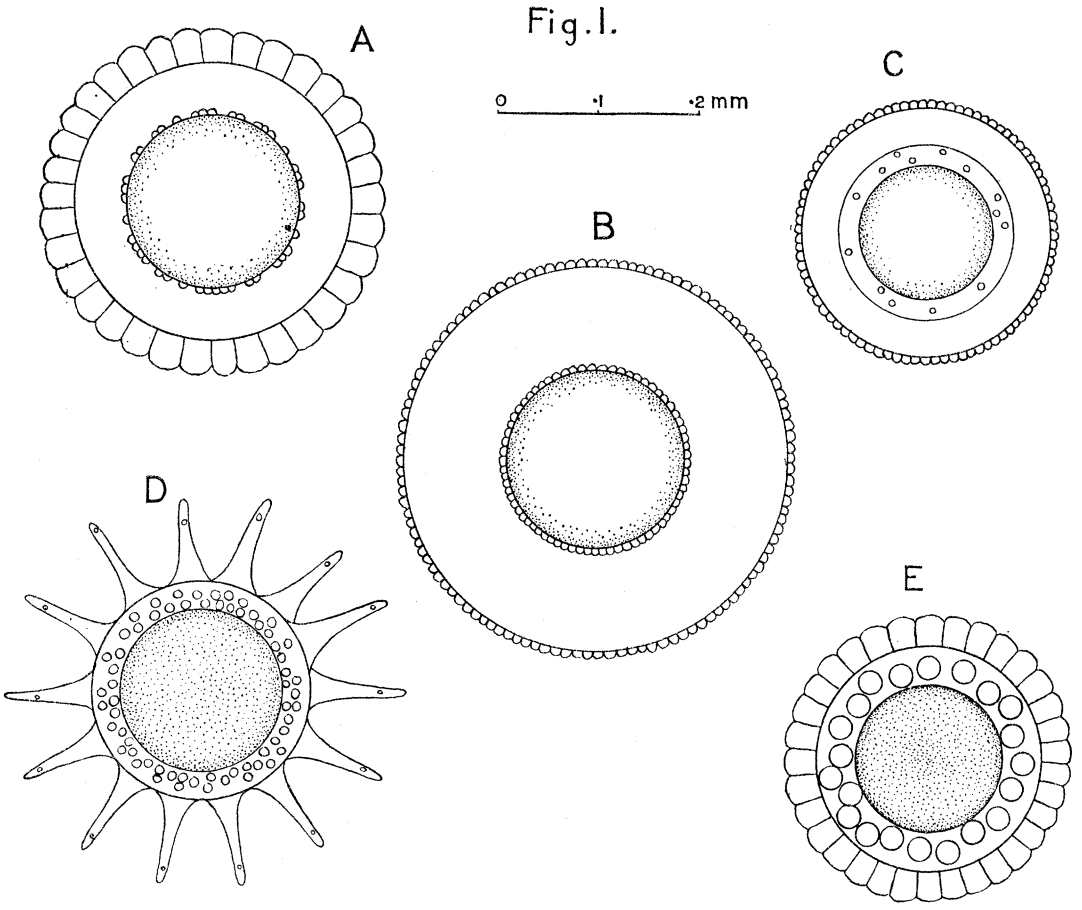


FIG. 1.—Eggs of ascidians after lying in sea-water, showing perivitelline membranes and follicle cells. A., *Ascidiella aspersa* (MÜLL.); B., *Ascidiella scabra* (MÜLL.); C., *Ascidia virginea* (MÜLL.); D., *Ciona intestinalis* (L.); E., *Corella parallelogramma* (MÜLL.).

to the vitelline surface, and the inner follicle cells are pressed by it against the latter to form an investment. In *Ascidia virginea* (fig. 1, C), however, it is well out in the perivitelline space and consequently the inner follicle cells are loose and do not invest. In the other species of the same family this inner membrane only becomes visible during the elevation of the chorion or under certain obscure conditions in the surrounding

* In no case has a fertilisation membrane been seen and no reference is made to such in this paper. All the membranes described are to be found in the unfertilised egg.

water. The ovarian history of these structures has been described well by WERNICKE (1919) and it appears that all follicle cells are rudimentary ova, that the chorion is formed probably as a precipitation membrane by the outer follicle cells, and that the inner follicle cells, or "test cells" as they are often mistakenly called, are enveloped by the growing ovum, supply it with lipoidal (HARVEY, 1927) and possibly other secretions, and at the end of the growth phase are extruded. For details the reader is referred to the original papers.

The perivitelline space and the outer follicle cells are important from the point of view of the floatation of the egg in that they lessen the specific gravity of the egg as a whole. The egg of *Ascidiella aspersa* and *Corella willmeriana** are the only two so far discovered that float to the top of still water, and those of the first will sink very quickly if the follicle cells are washed off. As a matter of fact the tendency to float of the eggs of *C. willmeriana* has resulted in this species being viviparous, for CHILD shows that the adult habitat is such that the siphons are directed downwards, and the buoyant eggs float away from them and so are retained within the atrial cavity until they are hatched. In most other cases, the eggs sink more or less slowly in still water but are caused by very slight agitation to rise from the bottom. This response to agitation is lost if the follicle cells are detached. The floatatory function of these cells is apparently due to their very great vacuolisation (*cf.* WERNICKE, HARVEY and others), and this may be seen in the living egg of those species in which the outer follicle cells are large.

The perivitelline space is formed after the egg is shed from the oviduct and during the first two hours it lies in sea water. It is due to the osmosis of water produced

Fig. 2.

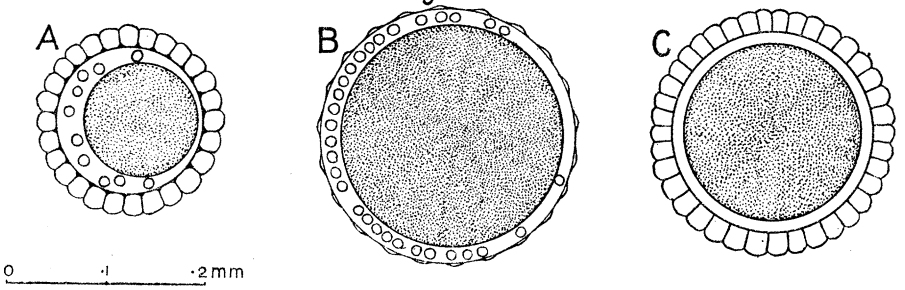


FIG. 2.—Eggs of three species of Molgula showing influence of viviparity and yolk accumulation on the nature of the outer follicle cells. A., *Molgula ampulloides* BENED. (oviparous and little yolk); B., *Molgula complanata* ALD. HANC. (viviparous and much yolk, cells stretched out); C., *Molgula retortiformis* VERRILL (oviparous but much yolk, stretching of cells counteracted by multiplication).

by some colloidal compound, probably protein in nature, between the ovum and the chorion. The colloidal property must be due to a compound of high molecular weight for the chorion is freely permeable to water, salts, urea and to glucose though not to

* The egg of *Corella parallelogramma* sinks in water of salinity 35 per mille (N. J. B.), that of *Corella willmeriana* floats in the water of Puget Sound (CHILD, 1927).

a polysaccharide such as gum arabic. It may be noted that the appearance of the perivitelline space is accelerated if the egg is immersed in hypotonic seawater. The absence of the space while the egg is in the oviduct is probably due to the presence in the oviducal fluid outside the chorion of a similar colloidal compound. The permeability of the membrane is unaffected by the presence or absence of the outer follicle cells.

A very weak solution of gum arabic in seawater* is sufficient to prevent the appearance or cause the disappearance of the perivitelline space, though because of the uncertainty of the exact weight of such molecules or their aggregates the absolute osmotic pressure of the perivitelline substance cannot be determined.

The solutions used and the results obtained are given below :—

3 grm. gum arabic in 10 c.cs. dist. water plus 90 c.cs. seawater inhibits formation of perivitelline sp.

1 grm. gum arabic in 100 c.cs. seawater, perivitelline space not formed	development normal in all cases.
0·8 grm. gum arabic in 100 c.cs. seawater perivitelline sp. $\frac{1}{4}$ formed	
0·6 grm. gum arabic in 100 c.cs. seawater, perivitelline sp. $\frac{3}{4}$ formed	
0·4 grm. gum arabic in 100 c.cs. seawater, perivitelline sp. fully formed	
0·2 grm. gum arabic in 100 c.cs. seawater, perivitelline sp. fully formed	

Equilibrium was attained in approximately the same time in all cases.

A further point of interest is that in all eggs that possess a single membrane, the perivitelline matter is fluid and the ovum usually is somewhat excentric with regard to the chorion. In the Ascidiidæ, however, the matter between the chorion and the inner membrane seems always to be in the gel state, but that between the inner membrane and the ovum, whatever its extent, to be fluid. In this case the ovum may be excentric with regard to the inner membrane, but the sphere formed by the latter is always central with regard to the chorion. These differences are well shown in chorion elevation of the egg of Ascidiella scabra, the perivitelline space of which is very large (fig. 3).

In general it should be noted that in those species which are viviparous and in which there is considerable yolk accumulation (fig. 1, C. 17, A) the outer follicle cells are shrunken and do not cover completely the chorion. This shrunken appearance is not due merely to atrophy through lack of use, but to the positive stretching of a fixed number of cells over a chorion of progressively increasing surface as yolk is accumulated. In viviparous species this stretching is a matter of indifference, but in oviparous species in which the floatating function of the follicle cells is of considerable value, the

* The egg of *Ascidiella aspersa* lying in the oviduct is usually non-spherical and somewhat crenated. This appearance is retained or regained in a solution of gum arabic in sea-water.

tendency to stretch and flatten out is counteracted by an increase in number of the cells. Fig. 2 illustrates this in the case of species of Molgula.

(Of the species mentioned in this paper, those which are viviparous are marked with an asterisk in Table II.)

Fig. 3.

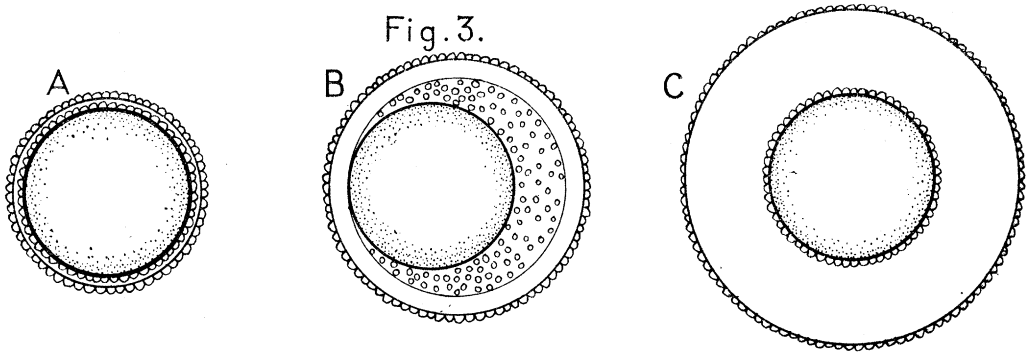


FIG. 3.—Eggs of *Ascidiella scabra* showing formation of perivitelline membranes, space, and transient appearance of inner membrane. A., Egg as found within oviduct; B., Egg in sea-water showing lifting of chorion and temporary outward movement of inner membrane; C., Egg in final condition.

(*b*) *Organ-forming Substances.*—The second type of variability is in the ovum itself and is of two kinds. There is variation in size, *i.e.*, in quantity of inclusions, and there is variation in the nature of the inclusions themselves.

Yolk accumulation seems generally to be associated with viviparity and will be discussed under that head, but an obvious exception may be noted in the case of *Molgula retortiformis* (fig. 2, C). The inclusions of the Ascidian egg have long been of interest, principally as the result of CONKLIN's investigation of their distribution and significance in development. In his studies on *Styela partita* (1905) he showed that different blastomeres in the dividing egg possessed inclusions in varying proportions, that these inclusions were definitely localised under the influence of the spermatozoan, and that as development proceeded the different types became more and more segregated. Primarily each of the first two blastomeres contained three substances, clear protoplasm, grey yolk spherules, and yellow lipoidal inclusions (mitochondria). For details the reader is referred to the original monograph (1905). Briefly, the yellow lipoidal material is accumulated in the form of a crescent in the posterior part of the lower hemisphere of the egg. The first two cleavages are vertical, the third horizontal, the fourth vertical but oblique to the others (fig. 4, A). Thus two tiers of eight cells each are formed, and the inclusions are distributed in the following manner. The upper eight are composed almost entirely of clear protoplasm and gives rise mostly to future ectoderm. Of the lower eight, the middle four contain most of the grey yolk and give rise to endoderm. The anterior two, part of the two yolk-laden cells posterior to them, and part of the anterior two cells of the upper layer give rise to the nervous system and the notochord, and contain yolk in small quantities. The two remaining posterior cells contain most of the yellow lipoidal matter and give rise to the mesenchyme and tail muscles.

Fig. 4.

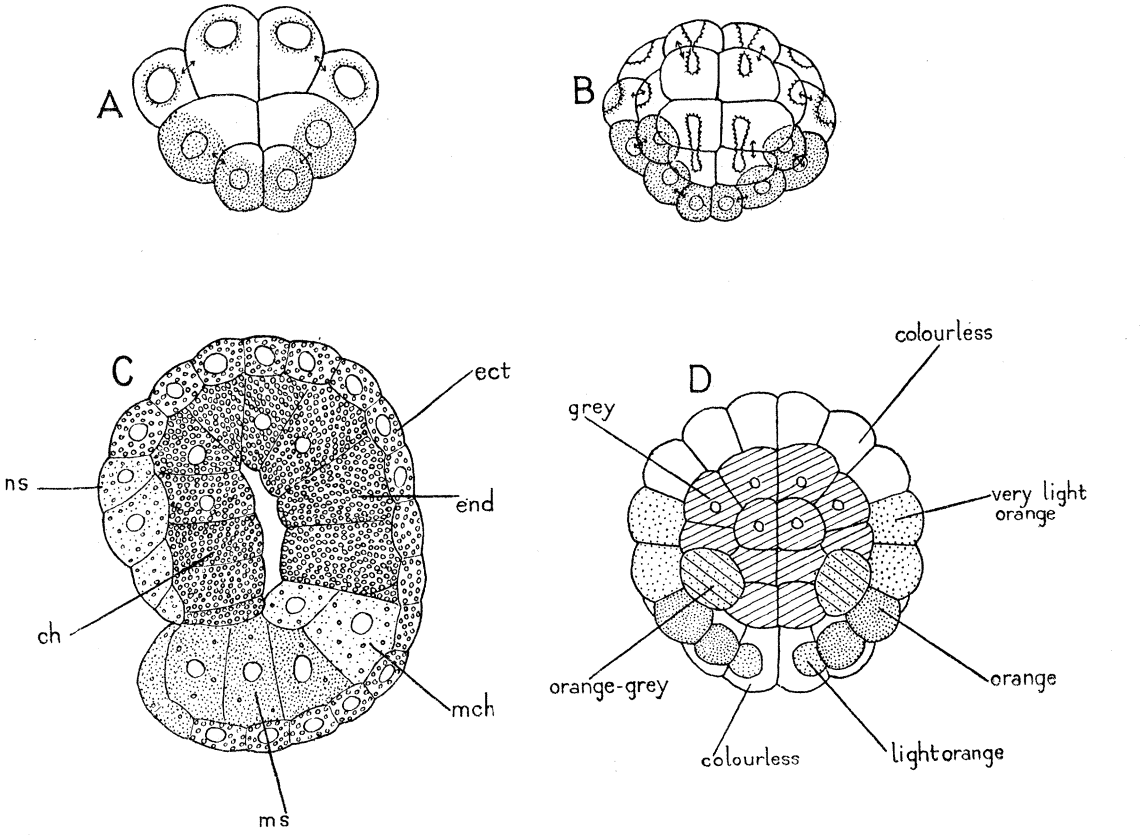


FIG. 4.—Cleavage and organ-forming substances in the eggs of Styela, Ciona and Boltenia. A, B., 16 and 44-cell stage of *Styela partita* (after CONKLIN), C., section of *Ciona intestinalis* at late gastrula stage, Benda stain, showing nature and distribution of inclusions (after DUESBERG); D., 64–74-cell stage of *Boltenia hirsuta* (original). *ch.*, chorda cells; *ect.*, ectoderm; *end.*, endoderm; *mch.*, mesenchyme; *ms.*, mesoderm; *ns.*, neural plate cells. Mitochondrial inclusions as dots in all four figures, yolk spheres as small circles in fig. C, hatched in fig. D, nuclei as large circles.

Gastrulation commences after the 64-cell stage when the following conditions hold. (After CONKLIN.)

Animal hemisphere.	Vegetal hemisphere.
26 ectoderm cells, protoplasmic. 6 neural plate cells, protoplasmic.	10 endoderm cells, yolk-laden. 4 chorda cells, yolk-laden. 4 neural plate cells, protoplasmic. 4 mesenchyme cells, light yellow protoplasm. 2 ant. mesenchyme cells, clear protoplasm. 2 post. mesenchyme cells, clear protoplasm. 6 muscle cells, deep yellow protoplasm.

Thus at the 64-cell stage each type of tissue has already been laid down. CONKLIN subsequently showed (1905–11) that while not visible in the living egg, these substances

existed and had the same fate in the developing eggs of *Ciona intestinalis*, *Phallusia mammillata*, and *Molgula manhattensis* as in *Styela partita*. More recently their course in ovogenesis and development has been followed by DUESBERG (1913–15) and HARVEY (1927). Apart from *Styela partita*, however, no record has been made of any Ascidian eggs in which the lipoidal inclusions are pigmented as well as the yolk spherules, and therefore it is the more interesting to show that such is the case in the eggs and larvæ of *Boltenia hirsuta* (figs. 4, D, and 5).

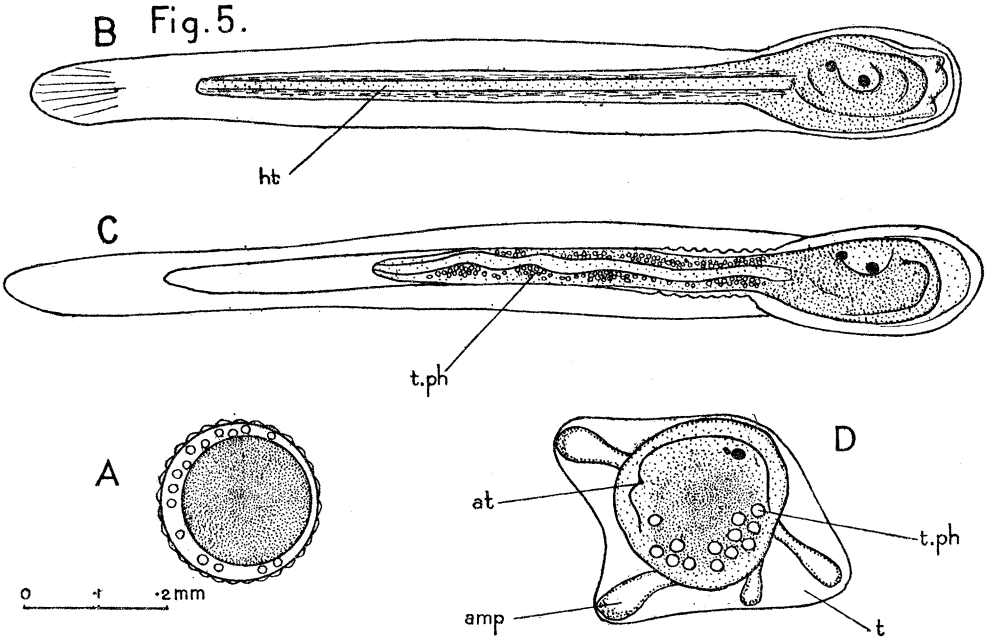


FIG. 5.—Development of *Boltenia hirsuta* (AGASSIZ). A., egg; B., tadpole; C., tadpole commencing metamorphosis; D., metamorphosing individual showing outgrowth of ectodermal ampullæ and first appearance of the single atrial (peribranchial) opening. *at.*, atrial siphon; *amp.*, ampulla; *nt.*, notochord (a continuous vacuole); *t.*, larval test; *t.ph.*, tail phagocytes. The phagocytes (*t.ph.*) figured in C. are a bright orange in colour; a comparison with D. shows the reduction in number and corresponding increase in individual size as the result of fusions.

In this species the colour of the yolk is grey as in Styela, but the lipoids are a vivid orange and show much more clearly in the living embryo than does the yellow substance of Styela. Fig. 4, D, shows the developing egg approximately at the 64-cell stage, and it will be seen that the yolk and the lipoids are distributed in just the same way as they are in the corresponding stage in *Styela partita*, but concentrated in slightly differing proportions in the various mesenchyme and muscle cells. Most of the orange-red colour is found eventually in the tail muscles and its subsequent history will be described under metamorphosis.

Among the different ascidian eggs all conditions of colour and opacity of the yolk spheres are to be found, and it appears that opacity in an egg is more dependent on the high refractive index of the spheres than on their pigmentation, and that such yolk spheres may contain pigment and yet remain transparent.

A summary of the various conditions to be found is given below.*

TABLE I.

	Approx. diam. of ovum. mm.
I.—Yolk spheres alone pigmented.	
(*a*) Eggs clear and colourless.	
Ascidiella aspersa (fig. 1, A)	0·17
Ascidiella scabra (fig. 1, B)	0·17
Diazona violacea	0·16
(*b*) Eggs clear with a faint greenish tinge.	
Phallusia mammillata	0·16
Ascidia mentula	0·15
Eggs clear with a bright red pigment.	
Ascidia conchilega	0·13
Eggs translucent with greenish or reddish pigment.	
Ciona intestinalis (fig. 1, D)	0·16
(*c*) Eggs semi-opaque but without pigment.	
Boltenia echinata	0·17
Ascidia prunum	0·18
Ascidia obliqua	0·15
(*d*) Eggs opaque and pigmented. Yellowish pigment occasionally present.	
Polycarpa fibrosa (fig. 12, F)	0·16
Molgula manhattensis	0·11
Molgula ampulloides (fig. 3, A)	0·11
Molgula arenata	0·11
Molgula occulta	0·11
Molgula bleizi	0·15
Clavelina lepadiformis	0·26
Yellow or reddish pigment.	
Tethyum pyriforme americanum (fig. 6, B)	0·26
Molgula citrina	0·20
Stolonica socialis	0·72
Reddish purple pigment.	
Molgula robusta	0·12
Molgula oculata	0·11
Molgula retortiformis (fig. 3, D)	0·18
†*Polycarpa rustica* (fig. 12, A)	0·18
Styelopsis grossularia (fig. 17, A)	0·48
Boltenia ovifera	0·16
Distomus variolosus	0·59
Amaroucium nordmanni	0·38
2. Mitochondria pigmented.	
Two cases only are known with certainty :—	
Styela partita (fig. 4, A, B ; 11, A)	0·15
Yolk spheres opaque but colourless. Mitochondria yellow.	
Boltenia hirsuta‡ (fig. 4, D ; 5, A)	0·18
Yolk spheres opaque but colourless. Mitochondria occasionally colourless but usually orange.	

* The observations are all original with the exception of *Molgula robusta* (LUCAS, 1927) ; that on *Styela partita* is merely a confirmation of CONKLIN (1905).

† Usually purple, but frequently batches of eggs produced with no trace of the red pigment and consequently a vivid blue.

‡ This species may readily be obtained in shallow water at the Atlantic Biological Station, St. Andrew's, Bay of Fundy. *Boltenia echinata*, of which *B. hirsuta* may possibly be but a variety, judging from specimens obtained at Trondhjem and Kristineberg, apparently does not possess this visible differentiation.

CONKLIN, DUESBERG, MEVES and HARVEY have all demonstrated that the yellow crescent contains most of the mitochondria, and DUESBERG suggests that it is the concentration of these bodies in the cells derived from the crescent that causes them to become muscle and mesenchyme cells in later development. This correlation is borne out by the centrifugation experiments of CONKLIN (1905) on Styela eggs, in which he succeeded in displacing the yellow material in one case entirely into one of the first two blastomeres, and in another into the postero-ventral cells of the 8-cell stage instead of the postero-dorsal. In both cases it is said that muscle cells developed only where this substance was localised. It should be borne in mind, however, that as the identification of muscle cells in early embryos is by their staining properties, those due to the presence of mitochondria are invalid since they will be found wherever the mitochondria have been displaced, and, therefore, either other staining properties must be used or else some morphological character such as the presence of myofibrillæ.

4. *Development and Function of the Notochord.*

After gastrulation the blastopore is closed by the posterior growth of its anterior lip in the manner typical of chordate development. During this process the chorda cells are inrolled so as to lie in the roof of the gastrocoel, and further posterior growth of the dorsal lip carries the entire mass of chorda cells into the hinder half of the embryo. The dorsal lip fuses with the ventral lip, there being no concrescence of lateral lips, and the fused region thus formed grows posteriorly to form the tail anlage, with its dorsal layer of neural cells, deeper chorda cells, lateral mesoderm, and external lateral and ventral ectoderm. There is also a strand of solid endoderm below the chorda cells. The larval nervous system, as is well known, is formed by invagination from the neural plate, commencing posteriorly and advancing forwards.

The chorda cells at this stage form a solid mass of cells which later, as CONKLIN describes, by shoving and interdigitation decreases in width and increases in length until finally they become arranged in a single column.

This process is seen to advantage in the development of *Tethyum pyriforme* (fig. 6, C, D, E).

The one characteristic property of notochordal cells of all animals is their turgidity and great or less degree of vacuolisation, implying a progressive imbibition of water in development. It seems probable then that the correlated increase in volume of individual cells, assuming they be contained within a relatively non-elastic cylinder, *i.e.*, of mesoderm and ectoderm, is sufficient to account for the sliding movements and interdigitation described above, if it so happens that one end of the cylinder is extensible. That this is the case is fairly clear, for, assuming the ectodermal sheath of the tail anlage to be reasonably firm, the chorda cells will tend to slide more and more into the long axis of the embryo. As large yolk-laden endodermal cells block the anterior end, the thrust will come mainly at the weakest part of the whole ectoderm, *i.e.*, the tip of the tail.

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Fig. 6.

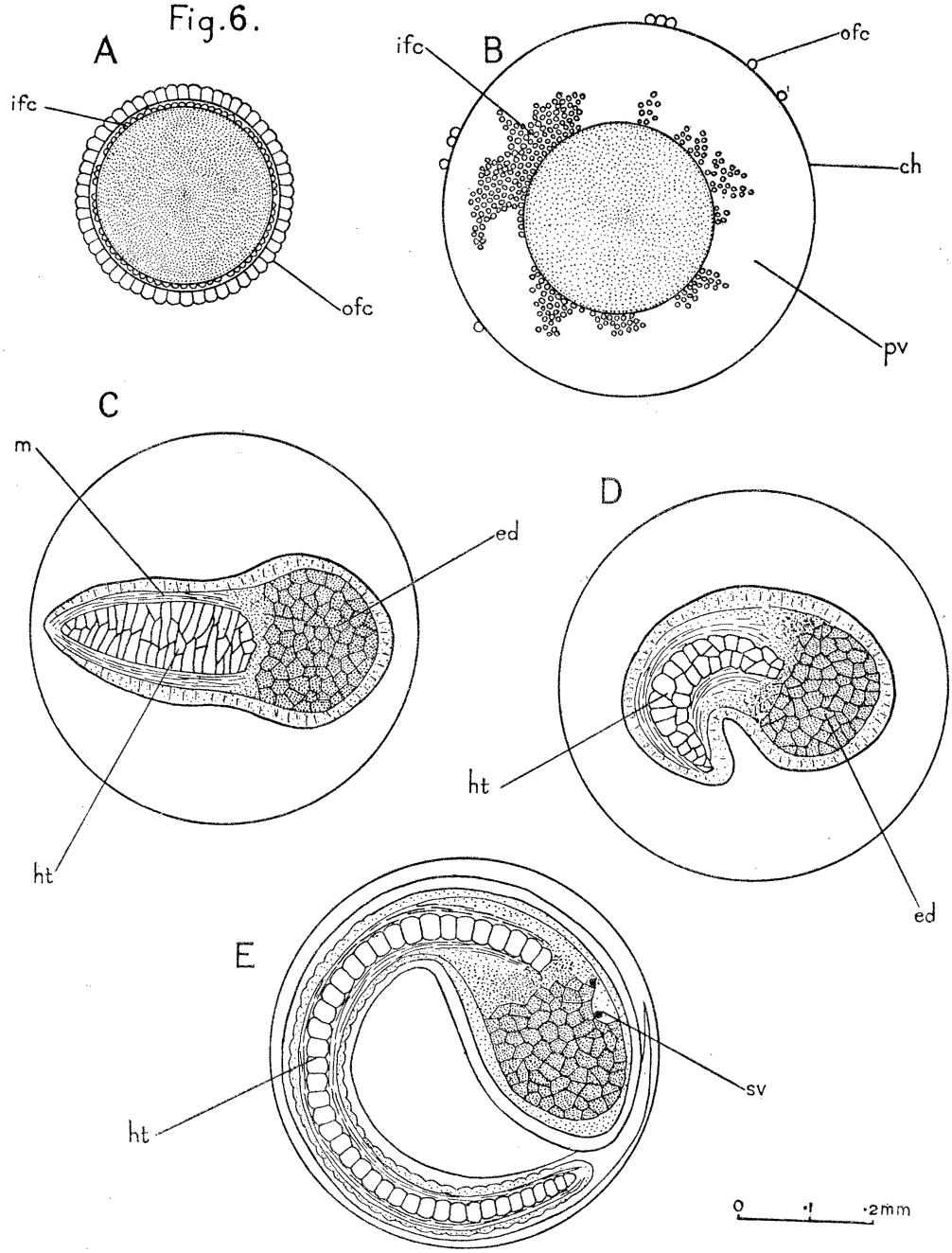

FIG. 6.—Development of *Tethyum pyriforme americanum* HUNTSMAN (*Halocynthia pyriformis* RATHKE).* A., egg as shed from oviduct; B., same after lying in sea-water, note appearance of extensive perivitelline space, the scattering of the inner follicle cells in it, the somewhat excentric position of the ovum, and the tendency to fall away of the outer follicle cells; C., D., dorsal and lateral view of embryo with short tail, note that notochord cells form a column 4 cells thick; E., later embryo showing first appearance of sensory pigment and arrangement of notochord cells into a single column. *ch.*, chorion; *ed.*, endodermal cells; *i.f.c.*, inner follicle cells; *m.*, muscle cells; *nt.*, notochord cells, *o.f.c.*, outer follicle cells; *pv.*, perivitelline space; *sv.*, sensory vesicle.

* During the summer of 1927 about 50 per cent. of the larger individuals caught at St. Andrew's contained large numbers of embryos, usually all at the same stage of development within any one animal. During the summer of 1928 no individuals were found to contain embryos. This suggests that viviparity is by no means a fixed character of the species. It is notable that the elongate ovaries of this species open far away from the atrial siphon, and that the egg shows no sign of being influenced by viviparity.

Further, this thrust may be expected to produce the same stimulatory effect as do the growing tips of the skeletal arms in echinoderm larvæ. This is supported by regeneration experiments on the frog by Morgan and Davis (1903), where it was found that the tail was re-formed only when the notochordal cells began growing into the papilla, and if part of the notochord was removed no outgrowth occurred until the notochord had reached the cut surface. Conklin suggested (1905–6) that the advancing tip of the notochord possesses an organising influence, and later that it may be identical with the "organiser" of Spemann in the amphibia. This is supported somewhat by Spemann's conclusion that the "organiser" is situated below the ectoderm, *i.e.*, in the meso-endoderm of the dorsal lip, and by the fact that the development of both the ascidia and amphibia follows the same plan, even to the extent that the eggs of both possess a mitochondrial crescent, which later forms tail mesoderm. Probably, however, the notochord possesses a mechanical influence alone, in stretching the muscle cells and stimulating the cells of the ectoderm to divide, for the amphibian "organiser" has a much more subtle influence, which is definitely non-mechanical (experiments by Spemann, Brachet and Geinitz).

Conklin states that the interdigitation of notochord cells is not dependent on their being crowded together from right and left sides, since it occurs normally when cells of one side only are present, *i.e.*, in lateral half-embryos. However, since it occurs rarely, if at all, in anterior half-embryos, in which the ectoderm and mesoderm of the tail are missing, it must depend upon a certain amount of lateral compression. While there is no regeneration of the missing parts (Conklin, 1906), in the case of lateral half-embryos the cells of the ectoderm tend to slide over the open side. Thus several factors facilitate interdigitation. The number of notochord cells in any given transverse plane has been halved, and the pressure will be primarily in a dorsal and ventral direction, while the ectodermal cells over the open side will tend to counter-balance what pressure there may be towards that side.

The development of the nervous system and the various organs in the trunk is well known and only those structures that exhibit variation will be described at present.

In connection with the larval nervous system a cerebral vesicle is developed, which typically contains two sense organs, an otolith, and a simple eye. In some families, however, the otolith alone is present, while in the Styelidæ all conditions are to be found. Table II shows the occurrence of the different types.

The other structure the development of which varies among the different families is the atrial siphon. The better known type is that of the Phlebobranchiata* where two independent invaginations of the dorsal ectoderm occur, one on either side of the nervous system, to form the peribranchial sacs. In the more typical of these forms the atrial invagination, which is median and dorsal and which involves the apertures

* It is agreed with Van Name (1921) that the order names Phlebobranchiata, Aplousobranchiata, and Stolidobranchiata of Lahille should be used rather than Seeliger's Diktyobranchia, Krikobranchia, and Ptychobranchia.

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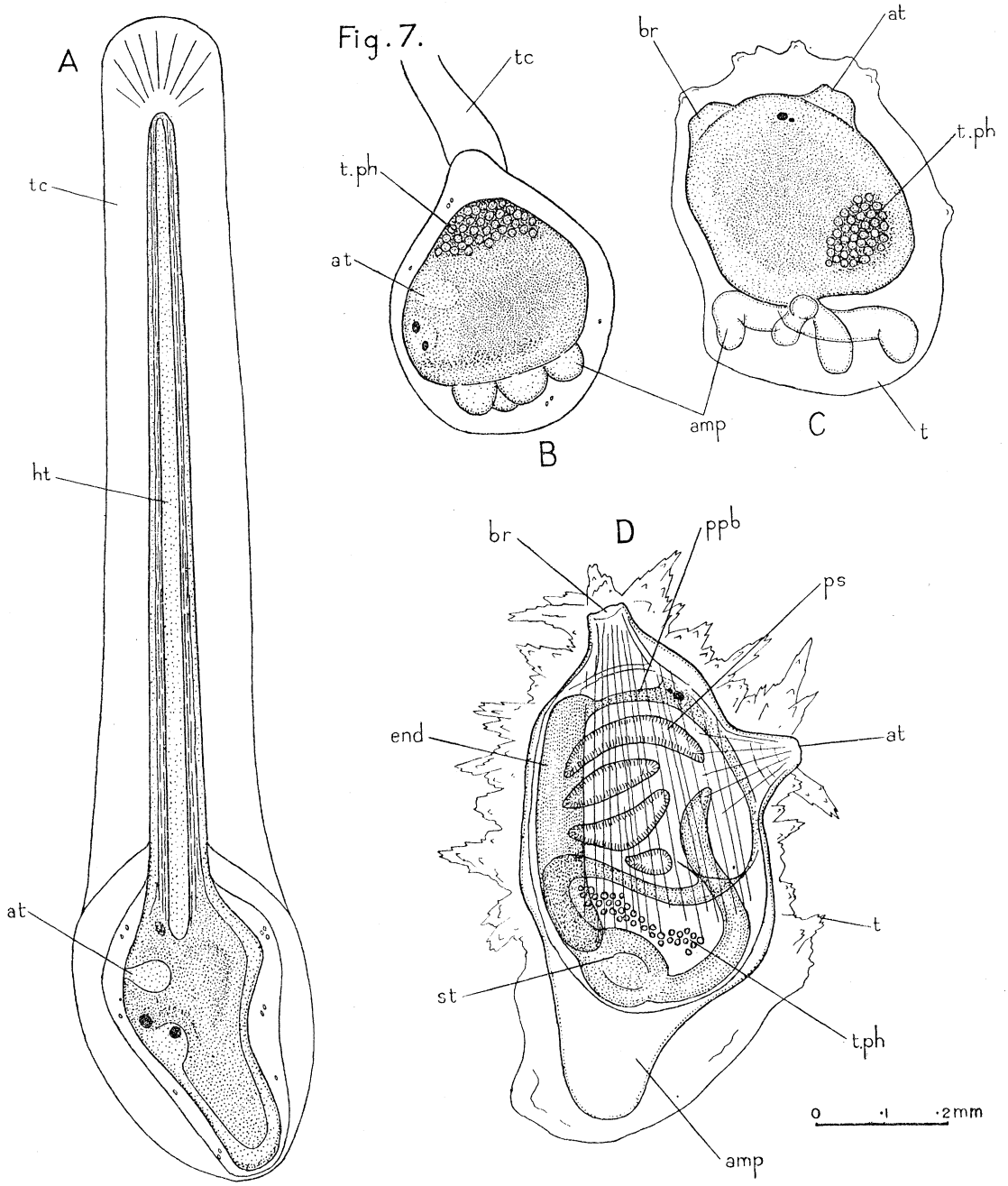

FIG. 7.—Development of *Tethyum pyriforme* (continued). A., tadpole, showing fusion of notochord cells to form a single continuous cylinder, and single dorsal atrial (peribranchial) invagination; B., metamorphosing individual, showing storage of phagocytosed tail and anterior outgrowth of 4 ampullæ; C., older metamorphosing individual, showing extended outgrowth of ampullæ, appearance of branchial and atrial siphons, and rotation of body; D., fully metamorphosed individual; note fusion of ampullæ to form a single short extension, differentiation of alimentary canal and protostigmata, inclusion of sensory pigment near permanent ganglion, and precocious crenation of larval test, responsible in the adult for its characteristic velvety texture. *amp.*, ampulla; *at.*, atrial opening; *br.*, branchial siphon; *end.*, endostyle; *nt.*, notochord; *p.p.b.*, peri-pharyngeal band; *ps.*, protostigma; *st.*, stomach; *t.*, test; *t.c.*, tail cuticle; *t.ph.*, tail phagocytes.

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of the two sacs, is not formed until late in the post larval development. The time at which it appears may vary greatly and this will be discussed later, but in certain families, notably the Molgulidæ, Pyuridæ, Styelidæ, and Botryllidæ, *i.e.*, in the Stolidobranchiata, and nowhere else, the invagination is from the first median and dorsal though it bifurcates over the nervous system to form the peribranchial sacs. Fig. 8 illustrates this difference in development, while Table II shows the occurrence of the two types. The question of variation in development of branchial and peribranchial structures will be dealt with in a later publication dealing with the development of compound ascidians.

Before hatching, a further change may occur in the notochord, and that is the breakdown of the wall separating adjacent cells, so that a long turgid cylinder is formed. This fusion in many forms, notably those of the Ascidiidæ, may be delayed

Fig. 8

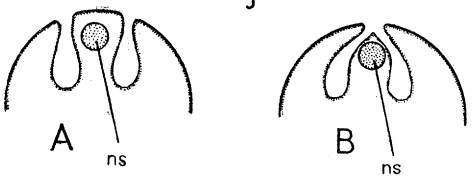

FIG. 8.—Diagram to show the two types of peribranchial invagination. *ns.*, larval nerve cord.

till after hatching and occur during the free swimming period. In most it occurs before hatching, while in a few, *e.g.*, *Styelopsis grossularia* (fig. 17) it does not occur at all. The change is well shown in *Tethyum pyriforme* (figs. 6, 7).

GRAVE has recently shown that the muscle cells in each strand lying throughout the length of the tail are contiguous and act as a functional unit, *i.e.*, as one muscle fibre which is attached to the basal and distal end of the notochord.

A study of the tail movements in a living tadpole, *e.g.*, of a species of the Ascidiidæ, shows that the notochord bends into almost a perfect arc, first to one side, then straightening, and then to the other; that is, it behaves like a single steel rod. GRAVE has demonstrated in the tadpoles of Botryllus and Amaroucium that the muscle strands in the tail have a slight spiral arrangement and distort the arc of contraction so as to give a spiral movement to the locomotion. The various tropisms and activities are well described by him (1920–26) and by MAST (1921).

5. *Post-embryonic Development.*

Before discussing in detail the larval and post-larval phases of development, it is convenient to give a brief anticipation of them. Figs. 7, 14 and 15, A., illustrate the essential points. There is the phenomenon of hatching itself, a free-swimming period of greater or less duration, the metamorphic changes and post-metamorphic growth and differentiation.

Table II.

(Observer's name placed after specific name where no personal observations made. An asterisk after the specific name denotes viviparity.)

Species.	Type of larva.	Larval sense organs.	Peribranchial invaginations.
Stolidobranchiata.			
Bostrichobranchus pilularis	Anural	None	Fused dorsally.
*Molgula citrina**	Urodele	Otocyst	,, ,,
,, *complanata**	,,	,,	,, ,,
,, *canadensis**	,,	,,	,, ,,
,, *manhattensis*	,,	,,	,, ,,
,, *ampulloides*	,,	,,	,, ,,
,, *oculata*	,,	,,	,, ,,
,, *occulta*	Anural	None	,, ,,
,, *bleizi**	,,	,,	,, ,,
,, *robusta* (Lucas)	,,	,,	,, ,,
,, *retortiformis*	,,	,,	,, ,,
*Boltenia hirsuta**	Urodele	Otocyst and eye	,, ,,
*Tethyum pyriforme americanum**	,,	,, ,,	,, ,,
Styela partita	,,	Otocyst and minute eye	,, ,,
*Styelopsis grossularia**	,,	Single sense organ	,, ,,
*Distomus variolosus**	,,	,, ,,	,, ,,
*Stolonica socialis**	,,	,, ,,	,, ,,
*Polycarpa rustica**	,,	Otocyst	,, ,,
,, *fibrosa (comata)**	,,	,,	,, ,,
*Botryllus schlosseri**	,,	Single sense organ	,, ,,
*Botrylloides leachii**	,,	,, ,,	,, ,,
Phlebobranchiata.			
Corella parallelogramma	,,	Otocyst and eye	Separate.
,, *willmeriana* (Child)*	,,	,, ,,	?
Ascidia mentula	,,	,, ,,	Separate.
,, *virginea*	,,	,, ,,	,,
,, *conchilega*	,,	,, ,,	,,
,, *prunum*	,,	,, ,,	,,
,, *obliqua*	,,	,, ,,	,,
Ascidiella aspersa	,,	,, ,,	,,
,, *scabra*	,,	,, ,,	,,
Phallusia mammillata	,,	,, ,,	,,
*Perophora listeri**	,,	,, ,,	Separate at first, fused at hatching.
,, *viridis*	,,	,, ,,	,, ,,
Aplousobranchiata.			
*Clavelina lepadiformis**	,,	,, ,,	,, ,,
*Distaplia clavata**	,,	,, ,,	,, ,,
*Cystodites draschii** (Herdman)	,,	Single sense organ	Fused (early history unknown).
Sycozoa (*Colella*) species* (Herdman and Caullery)	,,	,, ,,	,, ,,
*Aplidium pallidum**	,,	Otocyst and eye	Separate at first, fused at hatching.
*Amaroucium nordmanni**	,,	,, ,,	,, ,,
*Diplosoma listerianum**	,,	,, ,,	,, ,,

Metamorphosis may be considered to consist of the following phenomena, the phagocytosis of the tail and larval nervous system, the outgrowth of ectodermal tubes or ampullæ from the body-wall to form respiratory and fixatory organs, and a differential growth of the body-wall between the anterior attached region and the mouth, causing a rotation of the mouth and atrial sacs to the posterior, now distal, part of the body.

The phagocytes containing the tail substance migrate into the trunk about the time the ectodermal tubes first appear, as may be seen in fig. 7, B. The extent of rotation may be seen by comparing the relative position of the sensory pigment in figs. 7, B, C and D. CHILD has demonstrated in the case of *Corella willmeriana* that the anterior lip region, the growth of which is responsible for the rotation, is a rapid reducer of $KMnO_4$.

6. *Hatching Mechanisms.*

There are fundamentally two methods by which an ascidian embryo may hatch, by digestion of the egg membranes or by their rupture. The evidence may be anticipated, and the conclusion stated here, that the first method is the primitive one.

Hatching enzymes have been recorded for various of the lower vertebrates, by GRAHAM KERR for Lepidosiren in 1900, by BLES for Xenopus in 1906, and more recently for numerous Teleosts by both WINTREBERT (1912–26) and REMOTTI. A short review of their occurrence is given by BOURDIN (1926). In some cases the enzyme apparently is completely responsible for the hatching process, in others, such as the Perch, body movements are necessary as well. The glands producing the enzymes, in the Teleosts at least, seem to be mono-cellular epidermal cells that disappear shortly after hatching has occurred. Among Invertebrates, from personal observations, hatching enzymes, almost certainly proteolytic, have been noted in Loligo, many Opisthobranchs, and certain other forms, but in these it is yet uncertain whether the enzyme is derived from the embryo or is supplied by the parent when spawning.

(*a*) *Hatching Enzymes.*—Among Ascidians the existence of a hatching enzyme was first suspected while following the development of the tadpole of *Ascidiella aspersa*. In the eggs of this species the outer follicle cells, which are relatively large, are not only mutually very cohesive, but exert a back pressure on the chorion, so that the digestion of the latter by an enzyme would allow the follicle cells gradually to sink inwards on to the enclosed embryo while yet remaining a complete investment. Fig. 9 shows that this is the case, and the embryo often has to complete the process of hatching by wriggling through these cells. A confirmatory observation for the existence of an enzyme was that in somewhat concentrated cultures, not only did normal embryos hatch, but also very abnormal ones and even unsegmented eggs.

The enzyme was then isolated and its existence proved as follows:—Cultures were made of *Phallusia mammillata* and after the embryos had commenced to form tails, the water in which they were developing was reduced to a minimum. Good respiratory conditions were ensured by spreading the concentrated culture over the bottom of a

Petri dish. After hatching, the culture was poured into a narrow tube, the larvæ allowed to settle to the bottom and the supernatant water poured off. Unfertilised eggs of the same species were then put into the decanted fluid and it was found that after a certain number of hours (5 to 50 according to the temperature and concentration of the enzyme) the membranes disappeared and the follicle cells fell away, leaving naked unsegmented ova. The process was followed under the microscope and it was seen that the follicle cells and membranes disappeared in an order opposite to that in which they do normally during development.

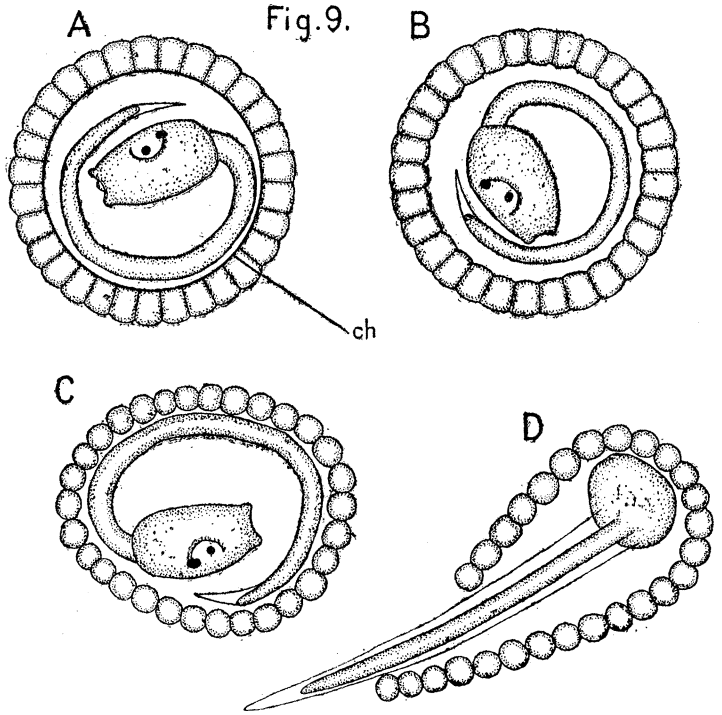

Fig. 9.—The hatching of *Ascidiella aspersa* through digestion of the egg membrane. A., fully formed tadpole before hatching; B., digestion proceeding, chorion broken down; C., follicle cells collapsing on to tadpole after disappearance of the supporting chorion; D., tadpole wriggling through follicle cells. *ch.*, chorion.

Similar experiments were carried out with *Ascidia mentula*, *Ascidia prunum*, *Ascidia conchilega*, and *Ascidiella aspersa*, and the existence of an enzyme shown in each case. It was further shown that the enzyme produced by Phallusia can digest the egg membranes of *Ascidiella aspersa* and *Ascidia conchilega*, that of Ascidiella can digest the egg membranes of Phallusia though less readily than those of its own eggs, while in the case of *Ascidia conchilega* the enzyme produced can digest the egg membranes of its own eggs much less readily than it can those of Phallusia.

Therefore within the Ascidiidæ at least the enzyme is not specific. Outside this family, however, optical evidence (of the nature indicated in fig. 9) alone has been relied upon for the presence of a hatching enzyme, principally because of the difficulty

in obtaining the large quantities of eggs necessary for such experiments. It is hoped, however, that this will be remedied in the future by the use of more delicate tests.

Three typical experiments :—

Ascidiella aspersa enzyme.

13.4.27.

Temp. 15° C. Unfertilised eggs of *Ascidiella aspersa* and *Phallusia mammillata*.

8.0 a.m. put into enzyme containing water.

12 noon 25 per cent. Ascidiella eggs hatched, 25 per cent. hatching. No Phallusia eggs affected.

5.0 p.m. 100 per cent. Ascidiella eggs hatched. No Phallusia eggs hatched

9.0 p.m. 20 per cent. Phallusia eggs hatched.

Phallusia mammillata enzyme.

11.4.27.

Temp. 15° C. Unfertilised eggs of *Phallusia mammillata* and *Ascidia conchilega*.

9.0 a.m. put into enzyme water.

7.0 p.m. All Phallusia and Ascidia eggs hatched.

Ascidia conchilega enzyme.

11.4.27.

Temp. 15° C. Unfertilised eggs of *Ascidia conchilega* and *Phallusia mammillata*.

9.0 a.m. Put into enzyme water.

8.0 p.m. 50 per cent. Phallusia eggs hatched. No Ascidia eggs hatched.

9.0 a.m. 100 per cent. Phallusia eggs hatched. 20 per cent. Ascidia eggs hatched.

By stopping development at various stages with a 0·0005 per cent. solution of KCN in sea-water it was determined in the case of *Phallusia mammillata* that the enzyme is formed, presumably by ectodermal cells, during the early stages of tail formation, since when development was stopped at stages onwards from where sensory pigment was just visible and the tail but half its full length, hatching occurred.

That the enzyme is a protease is demonstrated by the following experiments.*

(1) 5 c.cs. calcified milk buffered to *p*H 8·0 plus 1 c.c. enzyme water of *Phallusi mammillata* incubated at 30° C., two tubes with active enzyme, control tube with boiled enzyme.

After 20 hours, milk coagulated in tubes containing active enzyme, unchanged in tube containing boiled enzyme.

After 4 days, control tube still in original state.

(2) A similar result was obtained with enzyme water of *Ascidiella aspersa*.

(3) Tests for amylase and lipase with 2 per cent. starch solution and an emulsion of olive oil stained with Nile blue sulphate produced negative results.

* Toluol was used for sterilisation in all such experiments.

(4) Action of 1 per cent. Liquor Trypsin Co. (Allen & Hanbury) in sea-water of *p*H 7·9 and temperature 12° C.

(*a*) On unfertilised eggs of *Phallusia mammillata*.

After 15 hours, 5 per cent. with membranes digested.

After 24 hours, 30 per cent. with membranes digested.

(*b*) On unfertilised eggs of *Ascidiella aspersa*.

After 15 hours, 5 per cent. with membranes digested.

After 20 hours, 75 per cent. with membranes digested.

After 24 hours, 90 per cent. with membranes digested.

The effect of temperature and the hydrogen-ion concentration was determined by a modification of the method of DERNBY, used by BODANSKY and ROSE, and more recently by YONGE.

Five test-tubes were used, containing each 5 c.cs. 10 per cent. gelatin solution buffered to *p*H 8·3, plus 1 c.c. enzyme water of *Phallusia mammillata*; while two tubes, differing only in so far as the enzyme water had been boiled, formed the controls. The five tubes containing enzyme were incubated for four days at temperatures 23, 30, 37, 48 and 64° C. respectively, while the control tubes were incubated for the same time at temperatures 30 and 64° C.

The results obtained on cooling to various temperatures are given below :—

Temperature of incubation.	Cooled to 12° C.	Cooled to 6° C.	Cooled to 2° C.
64° C.	Control gelatin set	Set	Set.
	Enzyme gelatin fluid	Set	Set.
48° C.	Enzyme gelatin fluid	Semi-fluid	Set.
37° C.	Enzyme gelatin fluid	Fluid	Fluid.
30° C.	Control gelatin set	Set	Set.
	Enzyme gelatin fluid	Fluid	Semi-fluid.
23° C.	Enzyme gelatin fluid	Fluid	Barely set.

Therefore the optimum temperature seems to be in the region of 37° C., while the enzyme is not completely destroyed at 64° C.

To determine the upper *p*H limit for enzyme action the following experiments were made :—

A series of Sorensen's glycocoll—NaOH buffers from *p*H 9·0 to *p*H 12·6 were made into 10 per cent. gelatin solutions, and to 5 c.cs. of each 1 c.c. enzyme water of *Phallusia mammillata* was added. Control tubes contained boiled enzyme, and all were incubated at 32° C. for four days.

By cooling to various temperatures, as before, it was found that there was seemingly full activity up to a *p*H value of 10·0, but that above *p*H 10·5 all enzyme activity ceased. Therefore the upper limit is between *p*H 10·0 and *p*H 10·5. The lower limit was determined in a different manner.

Embryos of *Phallusia mammillata* were allowed to develop in normal sea-water of *p*H 8·1 to within an hour or so of their full term, and were then divided among vessels of sea-water of *p*H values varying from 5·5 to 9·0. Hatching was found to be inhibited below *p*H 6·8, and to occur in 95 per cent. at *p*H 7·2.

The inhibition below *p*H 6·8 is complete and has been demonstrated in addition for *Ascidiella aspersa*, *Ascidia mentula* and *Ciona intestinalis*, but transference to water of *p*H 8·0 allows hatching to proceed.

The enzyme mechanism as a whole, however, seems to be very sensitive, for while in species such as *Phallusia mammillata* and *Ascidiella aspersa* very abnormal embryos may hatch, in others, *e.g.*, *Styela partita*, and to a less marked degree in *Ciona intestinalis*, slight abnormality in development is to be correlated with the failure of the digestion method of hatching. This does not preclude later hatching by means of rupture, and, in fact, it may be stated generally that the more sensitive the enzyme process, the more efficient is the later rupture method.

This method is typically associated with metamorphosis. Usually about the time the tail is phagocytosed, the larval test begins to swell and a varying number of ectodermal outgrowths appear. Rupture of the egg membrane may then be brought about in two ways, by the swelling of the larval test alone, or by the pushing out of the test locally by the ectodermal tubes or ampullæ. In some species, hatching by digestion is the only possible way, while in others hatching by rupture alone can occur, but species exist among which one may find every degree of efficiency of the two methods and the various types are summarised below.

TABLE III.

(*a*) Digestion the normal method of hatching.

1. Swelling of test and outgrowth of ampullæ never sufficiently vigorous to rupture if digestion fails.
   - *Phallusia mammillata.*
   - *Ascidia mentula.*
   - *Ascidia conchilega.*
   - *Molgula oculata.*
2. Outgrowths sufficiently vigorous to cause rupture in 5–10 per cent. cases.
   - *Ascidiella aspersa.*
   - *Ascidiella scabra.*
   - *Boltenia hirsuta.*
3. Outgrowths sufficiently vigorous to cause rupture in 40–50 per cent. cases.
   - *Ciona intestinalis* (fig. 15).
   - *Molgula ampulloides.*
   - *Molgula manhattensis.*
   - *Polycarpa fibrosa.*
   - (? *Corella willmeriana*).*
4. Outgrowths sufficiently vigorous to cause rupture in 90–100 per cent. cases.
   - *Styela partita* (fig. 10, G, H).
   - *Tethyum pyriforme americanum.*

(*b*) Digestion so easily disturbed and hatching by rupture so efficient and common in nature that difficult to say which is normal.
   - *Polycarpa rustica* (fig. 11, C, D).
   - *Polycarpa pomaria*

(*c*) Hatching by rupture alone.
   All species of the Molgulidæ in which there is yolk accumulation or anural development, all compound Ascidians so far examined, and most cases where there is marked accumulation of yolk.

* CHILD, 1927.

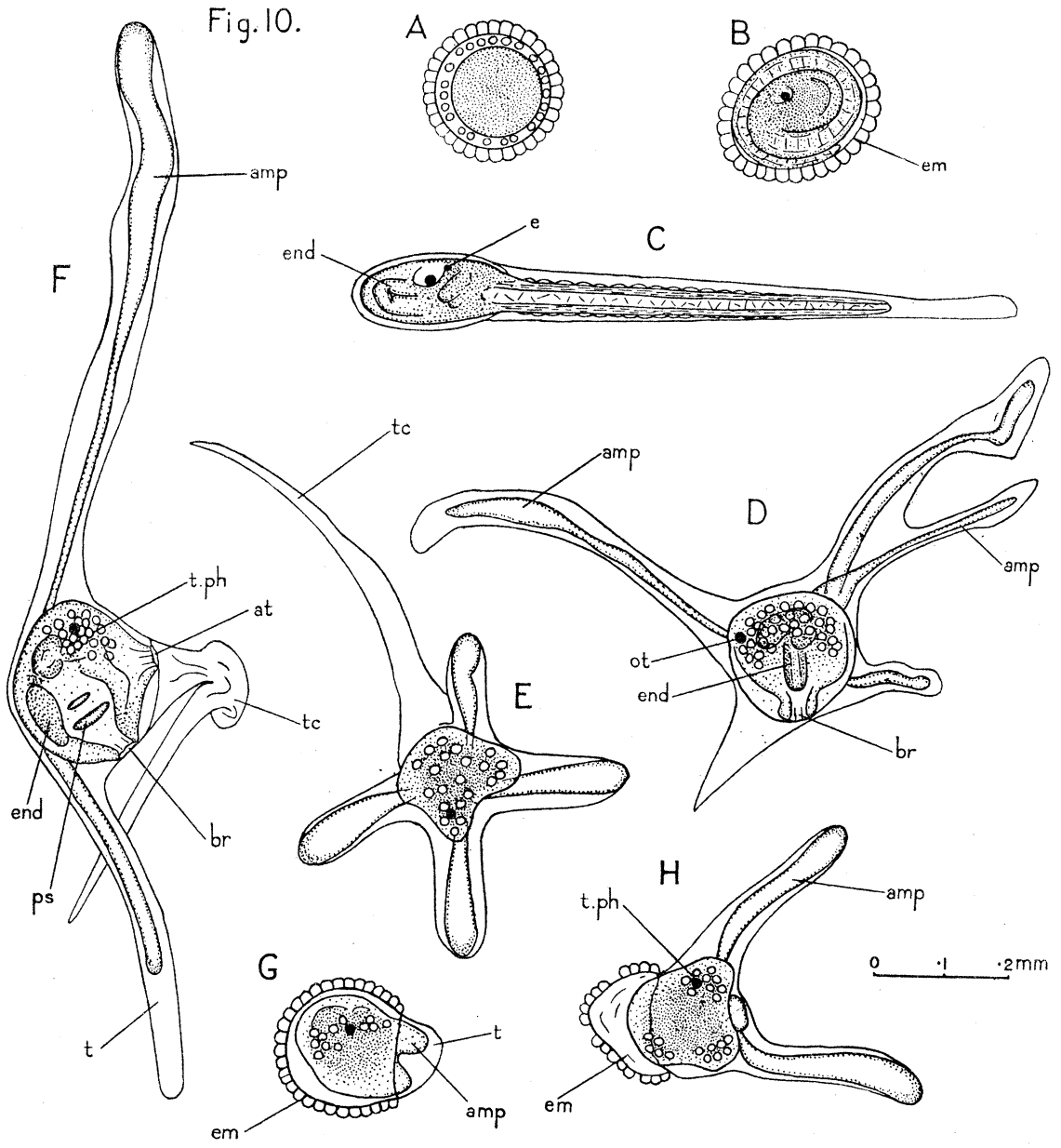

Fig. 10.—Development of *Styela partita* (Stps.). A., egg; B., tadpole on point of hatching; C., hatched tadpole; note notochord cells do not fuse; D., E., metamorphosing individuals, showing tail phagocytes, appearance of respiratory ampullæ and branchial siphon; F., older metamorphosing individual, with first 2 protostigmata, greater extension of ampullæ, and appearance of single atrial opening; G., H., two individuals that failed to hatch as tadpoles through the digestion method and hatching later by means of the swelling test and ampullæ; B., C., 20 hours from fertilisation; E., D., G., H., 46 hours; F., 68 hours (temperature 19° C.). *amp.*, ampulla; *at.*, atrial opening; *br.*, branchial siphon; *e.*, vestigial eye; *end.*, endostyle; *ot.*, otocyst; *ps.*, protostigma; *t.*, test; *t.c.*, tail cuticle; *t.ph.*, tail phagocytes.

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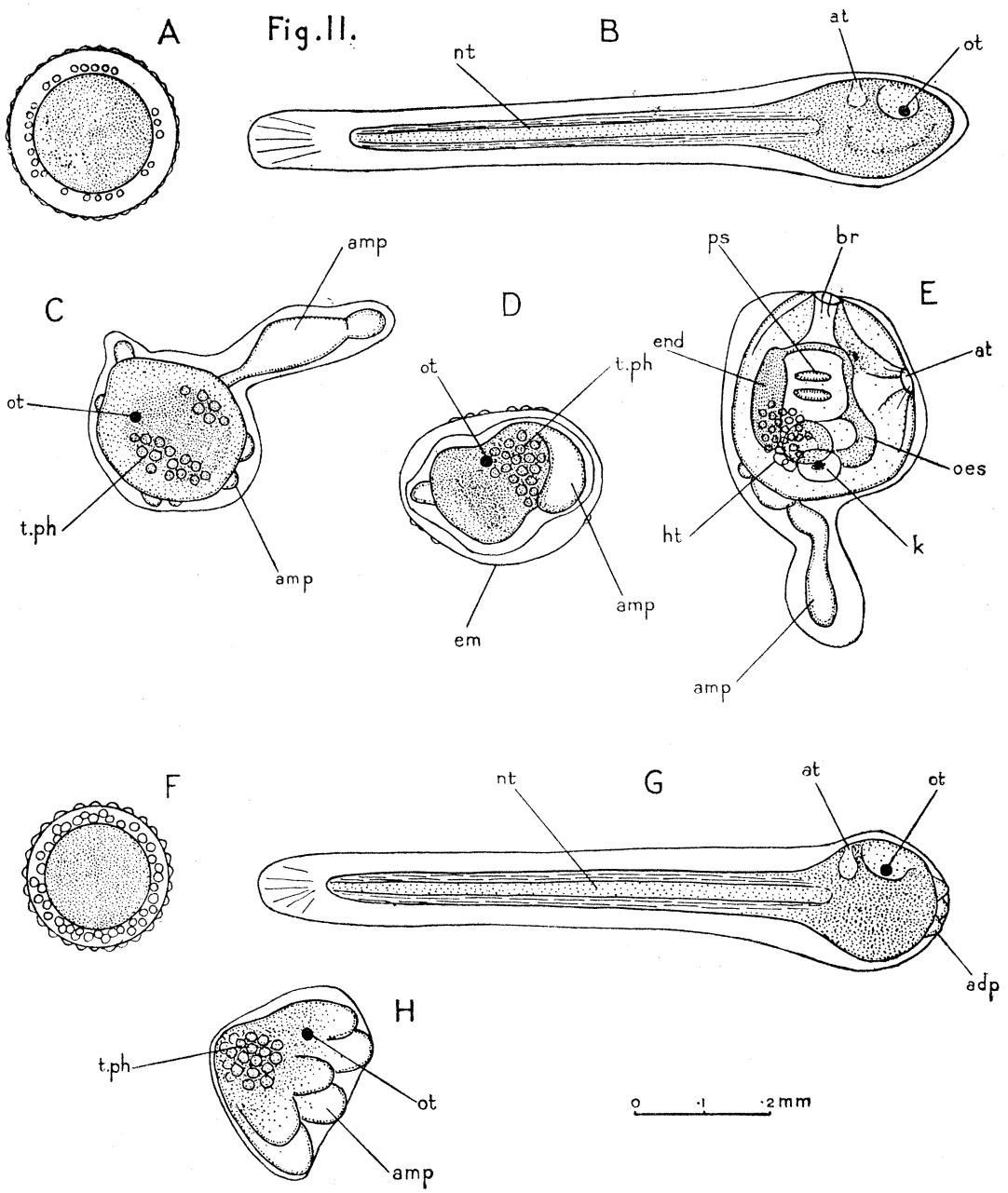

FIG. 11.—Development of *Polycarpa* (*Pandocia*) *rustica* LAC. DUTH. & DEL., and *Polycarpa fibrosa* (*comata*) STPS. A., egg of *P. rustica*; B., tadpole of same, showing fusion of notochord cells, single peribranchial invagination, and presence only of otocyst; C., metamorphosing individual, showing tail phagocytes, outgrowth of ampullæ, one of which being primary and with waves of constriction; D., similar individual about to hatch through rupture, having failed to hatch through digestion as tadpole; E., fully metamorphosed individual, showing differentiation of alimentary canal, etc., and appearance of the primary renal vesicle. F., egg of *P. fibrosa*; G., tadpole of same; H., metamorphosing individual. *ad. p.*, adhesive papillæ; *amp.*, ampullæ; *at.*, atrial invagination; *br.*, branchial siphon; *end.*, endostyle; *ht.*, heart; *k.*, renal vesicle; *nt.*, notochord; *œs.*, œsophagus; *ot.*, otocyst; *ps.*, protostigma; *t.ph.*, tail phagocytes.

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7. *Metamorphosis.**

After a shorter or longer free-swimming period the tadpole sinks to the bottom and commences to metamorphose. As the factors controlling the length of the free-swimming period and the onset of metamorphosis are the same, the two phases will not be treated separately. Here again, while temperature and molecular concentration have no marked effect, the whole process can be controlled through the hydrogen-ion concentration of the medium. The effect of the oxygen tension may be considerable but it has not yet been investigated.†

Experiments fall into two classes—those in which the larvæ were reared throughout at a given hydrogen-ion concentration, and those in which they were reared at one concentration and transferred suddenly to water having a different concentration. Anticipating the conclusions, it may be stated that any increase in hydrogen-ion concentration tends to induce metamorphosis, while low concentrations, *e.g.*, *p*H 9·0, inhibit metamorphosis to a greater or lesser extent. The various *p*H values were obtained by adding HCl or NaOH to sea-water and aerating violently for about twelve hours, in order to bring the CO_2 tension into equilibrium with that of the atmosphere. The CO_2 tension was thus the same in all experiments. Concentrated acid and alkali were used, in order to avoid undue dilution of the sea-water. Increasing the CO_2 tension while keeping the *p*H constant seems to have the same effect as decreasing the *p*H. The experiments are summarised below.

Experiments on *Phallusia mammillata*.

1. Larvæ developing throughout at *p*H 9·0 have a very prolonged free-swimming period, and very slow metamorphosis. Individuals may be fixed eventually with functional protostigmata and heart, but with tails wagging at their side or but incompletely absorbed. Phagocytosis may commence in the tail at any point, rarely at the tip or normal region however.

2. Larvæ hatching at *p*H 9·0 and then transferred to *p*H 6·0–6·3 complete tail absorption and metamorphosis more quickly than the control in sea-water of *p*H 8·1, but at the same rate as the control when the latter is transferred on hatching to *p*H 6·0.

3. Larvæ developing at *p*H 6·8 fail to hatch, when absorption of the tail may be as fast as, or slower than, the control at *p*H 8·1, but not faster.

* In all the following experiments on metamorphosis, variability, etc., in the case of Phallusia several thousand larvæ were used, while in the case of Asciella, Ascidia, and Ciona several hundred.

† An experiment by P. Weiss (1928, p. 72) with *Ciona intestinalis* indicates that the effect of decreasing the oxygen tension is similar to that of decreasing the hydrogen-ion concentration, both changes tending to inhibit metamorphosis :—40½ hours after fertilisation the 2 control cultures in normal sea-water showed 59–66 per cent. larvæ at middle metamorphosis ; 2 cultures in sea-water poor in oxygen showed but 7–10 per cent. at middle metamorphosis.

4. Larvæ developing at *p*H 6·8 on being transferred to water of *p*H 9·0 hatch and complete general metamorphosis more quickly than the control developing throughout at *p*H 9·0, but tail absorption is just as incomplete.

5. Larvæ hatched at *p*H 8·1 invariably have curved tails, the enforced curvature produced by confinement within the egg membrane not being overcome by the turgidity of the notochord.

6. Larvæ hatched at *p*H 9·0 invariably have straight tails.

Experiments on *Ascidiella aspersa*.

As in Phallusia, the free-swimming stage is prolonged at *p*H 9·0 while metamorphosis of the trunk is fairly rapid and complete, the tail being hardly affected. A change from a higher to a lower *p*H value tends to induce metamorphosis.

Experiments on *Ciona intestinalis*.

The hydrogen-ion concentration has the same influence on the duration of the free-swimming period and on metamorphosis as in Phallusia and Ascidiella, but to a less marked degree.

When metamorphosis does occur at an abnormally high *p*H it usually is very slow and it becomes apparent that phagocytosis of the tail is not a necessary companion to the further development of the trunk organs. Thus frequently tadpoles have been seen swimming about, with the larval ampullæ fairly well formed, the trunk loosening out and protostigmata forming and, most striking of all, with a beating heart (see fig. 12). This last structure normally does not function until metamorphosis is complete. Further when development has taken place in water of *p*H 9·0 or above, after fixation has occurred and most of the adult organs are formed and functional, it is not uncommon to find the tail persisting and twitching at the side of the body.

In cases where the *p*H is low enough to prevent hatching, while metamorphosis is normal, its onset is usually retarded, and this seems to be correlated with the relative inactivity of the enclosed larvæ.

Thus from the evidence just given it seems possible that the onset of phagocytosis of the tail is caused primarily by an increasing acidity of the tissues concerned, and that normally this is brought about by the activity of the tadpole. Intercellular acidity in the tail could be the result of lactic acid accumulation, or of carbon dioxide, though the latter probably would diffuse away too quickly to have much effect.*

The experimental results may then be interpreted as follows :—That the transference of tadpoles from water of a high *p*H to water of a low *p*H produces a change in the

* Caswell Grave (1927) found that in the case of *Symplegma viride* there exists apparently a direct quantitative relation between the duration of the free-swimming period of the larva and the light to which the larva is exposed. This does not invalidate the above theory, since the light could control through the larval sense-organs the degree of larval activity, and therefore of inter-cellular acidity in the tail tissues.

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Table IV.

Influence of the hydrogen-ion concentration on the free-swimming life and metamorphosis of ascidian tadpoles.

Phallusia mammillata. (Time from fertilisation) at 15° C.	*p*H 8·1.	*p*H 9·2.	*p*H 6·8.
40 hours	All hatched.	All hatched.	None hatched.

	Divided among		*Divided among*		
	*p*H 8·1	*p*H 6·1.	*p*H 6·1.	*p*H 9·2.	
50 hours	All swimming ...	Tail absorption started	Tail absorption started	All swimming ...	None hatched.

	Divided among	
	*p*H 9·2.	*p*H 6·8.
80 hours	Tail absorption started	Tail absorption complete				
110 hours	Tail absorption complete					
160 hours	Metamorphosis complete		Tail absorption complete	—	Mostly hatched ...	None hatched.
250 hours	—	—	—	Tail absorption started		
340 hours	—	—	—	—	Tail absorption started	None hatched.
415 hours	—	—	—	Tail absorption half completed. Trunk metamorphosis well advanced.		

[The above table represents a typical experiment out of the fifteen made in this connection.]

intercellular fluid analogous to that produced as the result of muscular activity of the tadpole, and that it is this change which induces phagocytosis and probably metamorphosis generally. Tadpoles developing throughout in water of low *p*H would be

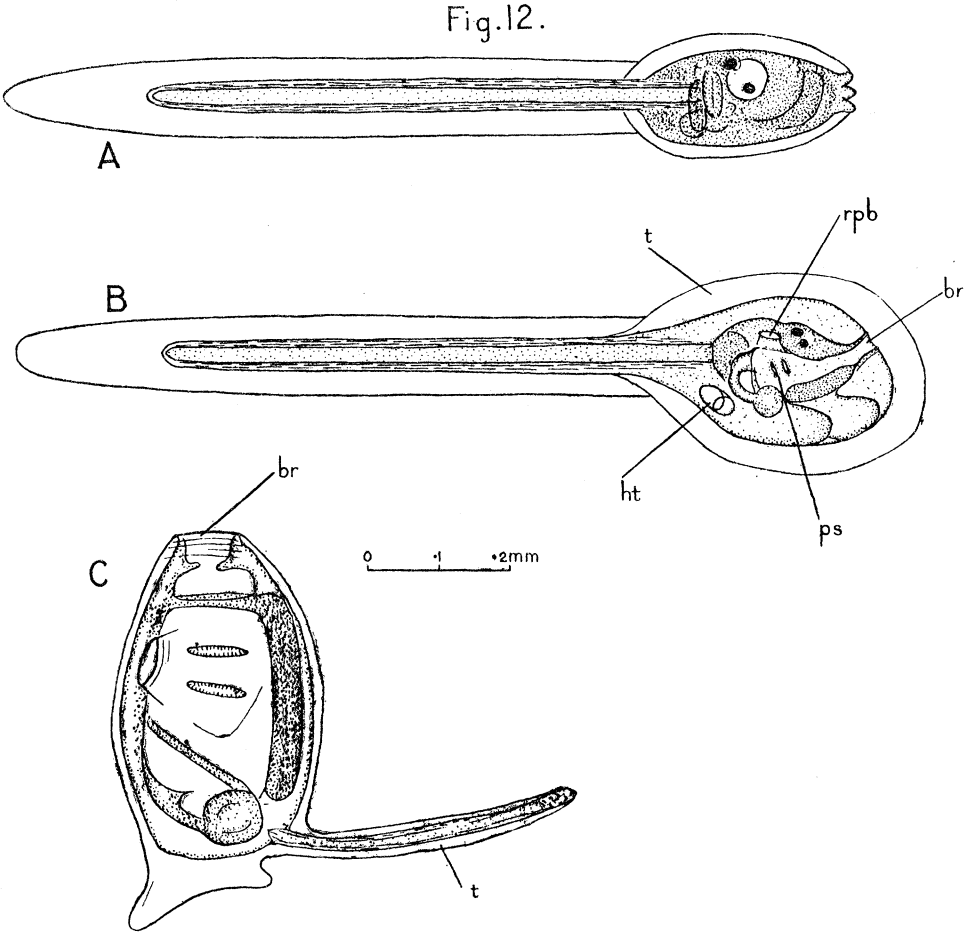

FIG. 12.—Tadpoles of *Ascidiella aspersa*. A., recently hatched; B., tadpole 10 days old, with functional heart, outgrowth of ampullæ and generally advanced differentiation of body, characteristic of tadpoles developing in hyper-alkaline sea-water or from physiologically old eggs; C., metamorphosed and fixed individual with tail still intact and vibrating. *br.*, mouth or branchial opening; *ht.*, heart; *pb.*, right peribranchial or atrial opening; *ps.*, protostigma; *t.*, test in B., tail in C.

in some degree acclimatised, and the absolute acidity would be less effective than an increase in acidity. Tadpoles transferred from water of a low *p*H to water of a high *p*H would find the production of an intercellular acidity by muscle contraction extremely difficult, while the fact that tadpoles which have developed throughout in water of a high *p*H have the same retardation of phagocytosis implies that there is a critical *p*H above which phagocytosis is more or less inhibited.

It should be noted that in typical tadpoles that have existed as such for a few hours only, phagocytosis of the tail commences at the tip and progresses towards the base until all the tissue has been stored within the trunk. This progression may be due to

the fact that in such tadpoles the cells of the tail increase in age from the base to the tip, and according to CHILD'S theory of susceptibility one would expect the cells in the tip to be affected first.* This is supported by the phenomena seen in those tadpoles that have existed as such for many days (those in water of high pH), where the difference in age between the base and tip cells of the tail becomes relatively small. In this case phagocytosis, when it does occur, may commence at any point, and it more often occurs at the base or middle of the tail than at the tip, when only the tissue between the trunk and the most proximal region of phagocytosis becomes stored within the trunk.

In viviparous ascidians, such as *Styelopsis grossularia*, eggs are fertilised a few at a time, but the tadpoles are retained until twenty or thirty have been formed. They lie inactive in the atrial cavity, some for relatively long periods, but on being shed they become very active and metamorphosis may commence within the hour. So here again it seems that it is the sudden activity, and therefore an increase in intercellular acidity, that is effective. It is not uncommon, however, in such species to find a certain proportion of tadpoles metamorphosed within the atrial cavity, the proportion varying greatly from one species to another.

Styelopsis grossularia *Clavelina lepadiformis*	1–2 per cent. metamorphose within the atrial cavity.
Molgula citrina (American) ..	2–3 per cent. metamorphose within the atrial cavity (within egg membrane).
Molgula citrina (European) ..	80–85 per cent. metamorphose within the atrial cavity (within egg membrane).
Tethyum pyriforme americanum *Polycarpa pomaria*	Occasionally whole batches of embryos metamorphose within the atrial cavity.

The effect of increasing the hydrogen-ion concentration on cells is twofold, as is shown by the following experiments. Eggs in the 2-cell stage were put into sea-water of pH values from 4 to 6, and it was found that development came to a standstill anywhere from the 4-cell stage to the elongated gastrula, the sooner the greater the concentration of the hydrogen-ion, and that not only was cell division gradually inhibited but that the surface tension was altered so that originally contiguous cells became spherical. Secondly, tadpoles that had been swimming for nine or ten hours in sea-water of pH 8·1 were transferred to water of pH 5·5 and within half an hour two changes had occurred. The tail was phagocytosed and more than half absorbed, while every cell in the body had become spherical, thus bringing all further activity to a standstill.

These phenomena are especially interesting in the light of certain experiments on phagocytosis by W. O. FENN (1922). He determined in the case of leucocytes that as the hydrogen-ion concentration of the medium increased, so did the phagocytosis

* CHILD (1927) shows that the power to reduce potassium permanganate is greatest at the growing tail tip throughout its development, but that the gradient thus demonstrated *tends* to disappear just before the onset of tail absorption.

of quartz particles by the leucocytes, while the adhesiveness of the leucocytes decreased; and that the concentration for maximum phagocytosis and minimum adhesiveness was *p*H 7·0. These results support the suggestion that the primary factor inducing phagocytosis in the ascidian tadpole is an increase in intercellular acidity.

In normal metamorphosis, however, the change once initiated is not confined to the tail. In *Styelopsis grossularia* the whole organism is affected immediately the tip of the tail begins to break down. There is no circulation nor body cavity at this stage, and the change is so rapid that it is doubtful whether diffusion of substances can account for it, and the inducing factor may conceivably be electrical or neuroid. As soon as phagocytosis commences several changes occur. The larval test, which has been inconspicuous and free from cells, now swells and becomes invaded by numerous cells, according to JULIN (1887) of double origin, ectodermal and mesodermal, which are the future test cells. At the same time the deep red colour of the entire larva changes to a yellow orange, while the ellipsoid trunk is caused by the contraction of numerous strand-like muscles to become somewhat medusoid in shape (see fig. 17).

In 1892 WILLEY discovered that cutting off the tails of tadpoles of *Styelopsis* hastened the preliminary metamorphic changes in the trunk region. The significance, however, is obscure, for the change may have been induced either by the removal of the possibly inhibiting presence of the tail, or more likely by the formation of a current of injury.

Other experiments on the metamorphosis of ascidian tadpoles have been made recently by WEISS (1928). He investigated the effect on the tadpoles of *Ciona intestinalis* of pituitrin and thyroid extract. The effect of immersion on an average of four hours in sea-water containing one of the above substances on the time taken to reach that stage of metamorphosis where the tail is completely absorbed was noted. It was found that whereas pituitrin had no influence whatever, in the case of thyroid extract, averaging the results of seven experiments, the control culture showed 45 per cent. of its tadpoles had reached middle metamorphosis when the thyroid containing culture showed 81 per cent. at that stage. The average number of tadpoles used in each culture was 450.

These results are interesting in that they show the tissues of the ascidian tadpole to be susceptible to the principal agent effective in producing metamorphosis in amphibia. There is no suggestion made, however, that the equivalent of a thyroid hormone is produced by the endostyle of the ascidian, for metamorphosis normally commences before there is any circulation and frequently body cavity, and while diffusion might be rapid enough the endostyle can hardly be said to be formed in many ascidian tadpoles at this stage of development. Further, SPAUL (1928) found that an extract of the endostyle of *Ciona intestinalis* had no effect on the metamorphosis of amphibian tadpoles.

The exact origin of the phagocytes of the tail has not been determined, though JULIN (1887) suggested that they arise from the endodermal strand of cells lying below the notochord.

Boltenia hirsuta is interesting in this respect, in that the muscles of the tail are a reddish orange and their fate can be followed optically. The first obvious change is that they break down into small spheres (fig. 5) which are possibly the phagocytes themselves or the muscle tissue already phagocytosed by other cells. As the process proceeds the small spheres gradually coalesce, at the same time involving the remaining cells of the tail, until a dozen or so large reddish phagocytes are formed, which migrate into the trunk (fig. 5, D). During this process the refractive index of the phagocytes increases, and even in those species in which they are not pigmented they become very obvious.

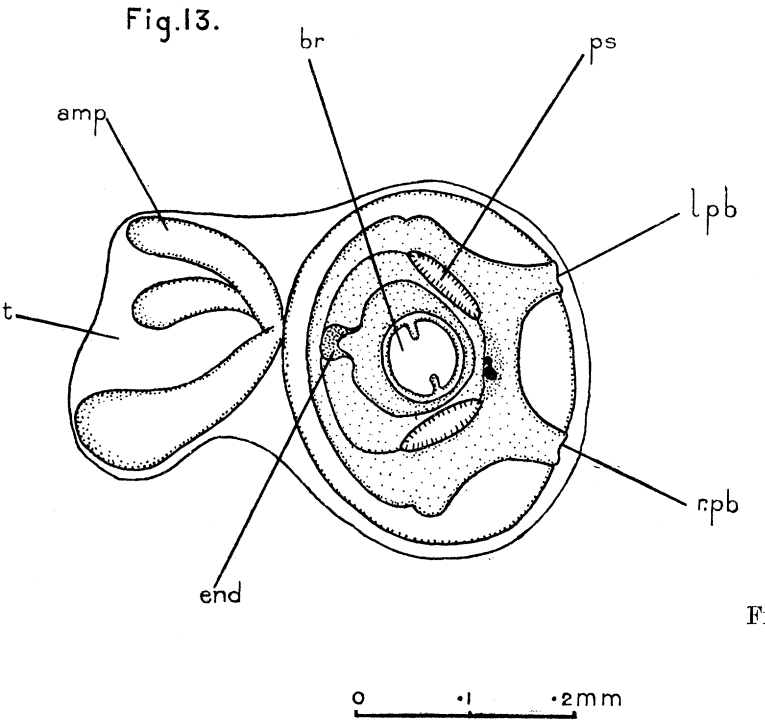

FIG. 13.—Metamorphosed individual of *Ascidiella aspersa*. Dorsal view showing branchial siphon and wide separation of peribranchial apertures. The cluster of ampullæ seen laterally merge later into a short single process (see fig. 14 amp.). (For key to lettering see fig. 14.)

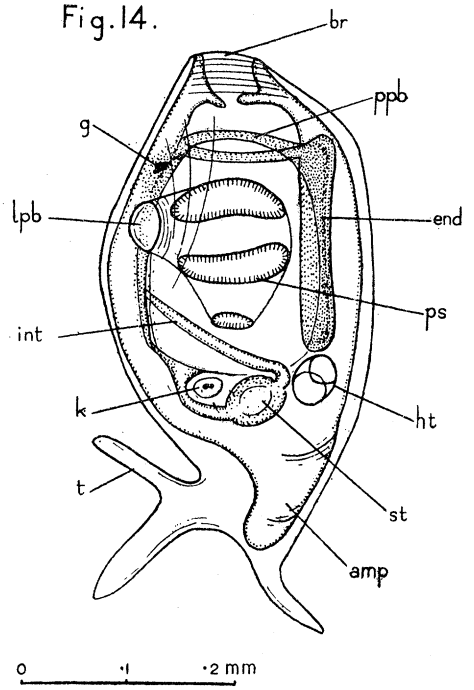

FIG. 14.—Metamorphosed individual of *Ascidiella aspersa*. Lateral view (tail phagocytes absorbed by stomach and mid-gut). *amp.*, ampulla; *br.*, branchial siphon; *end.*, endostyle; *g.*, ganglion (including sensory pigment); *ht.*, heart; *int.*, intestine; *k.*, renal vesicle; *l.pb.*, left peribranchial or atrial opening; *pph.*, pharyngeal band; *ps.*, protostigma; *st.*, stomach; *t.*, test.

In such members of the Styelidæ as *Styelopsis grossularia*, *Styela partita* and *Polycarpa rustica*, the phagocytes are similar to those of *Boltenia hirsuta*, *i.e.*, large, few in number and more or less scattered in the metamorphosing trunk, but in the majority of species, especially of the Molgulidæ and Ascidiidæ, the phagocytes are smaller, more numerous and form a more or less compact mass, eventually lying close to the stomach, by which they are finally absorbed.

The differential growth of the anterior lip of the mouth is well known and is figured well by WILLEY for *Ciona intestinalis* (1893). The result of this growth is that the mouth and atrium are rotated from their position near the region of fixation to one where they are less likely to become engulfed by mud or sand, etc. The rotation is extensive, as can be seen from fig. 7, B and D, and as a result the mouth or branchial siphon becomes dorsal, the gut and tail phagocytes ventral, and the atrial or peribranchial apertures lateral. That this process may be more or less inhibited without interfering with any other aspect of development can be seen from 15 D. This is a

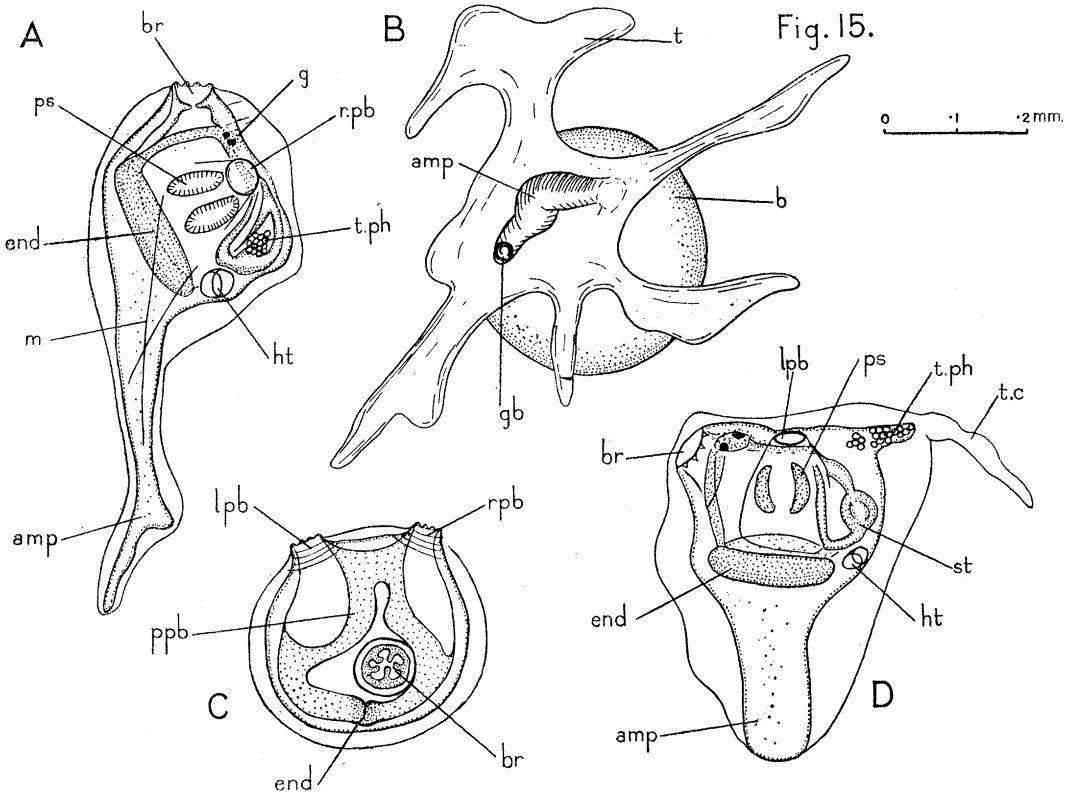

FIG. 15.—Metamorphosed individuals of *Ciona intestinalis* (L.). A., lateral view as of Ascidiella; note single and moderately long ampulla; B., top view of individual floating at surface film of water; note gas bubble in tip of ampulla causing buoyancy, and spreading of the test along the surface film; C., dorsal view of normally fixed individual showing, as in Ascidiella, the wide separation of the left and right peribranchial openings; D., individual developed and metamorphosed in sea-water of *p*H 6·3 showing slightly retarded development, almost complete absence of rotation, and incomplete migration of tail phagocytes. *amp.*, ampulla; *b.*, body of individual; *br.*, branchial siphon; *end.*, endostyle; *g.*, ganglion; *gb.*, gas bubble; *ht.*, heart; *l.pb.*, *r.pb.*, left and right peribranchial openings; *m.*, muscle fibre; *p.ph.*, peripharyngeal band; *ps.*, protostigma; *t.*, test; *t.c.*, tail cuticle; *t.ph.*, tail phagocytes.

tadpole of *Ciona intestinalis* that developed throughout in sea-water of *p*H 6·3 and it should be noted that while the siphons, endostyle and tail phagocytes are approximately in the same relative positions they occupy in a newly attached tadpole, the

general expansion of the trunk, the formation of protostigmata, and the development of a functional heart has progressed normally.

Phagocytosis normally involves the larval nervous system, the adult nervous system being a new formation, and while the sense organs are destroyed in most forms at this time, in the Molgulidæ and in *Tethyum pyriforme* the otolith persists and is included near the permanent ganglion (see fig. 7).

8. *Respiratory and Fixatory Function of Ectodermal Ampullæ.*

The remaining phenomenon associated with metamorphosis is the outgrowth of ectodermal tubes or ampullæ. These structures are invariably present and may vary in number among different species from three to twenty-five. They were first noted in *Molgula ampulloides* by P. J. van Beneden in 1846, later Herdman (1885), Bancroft (1899), and Pizon (1900) suggested their respiratory function in the fully formed colony of Botryllus, while Willey (1900) assigned a similar function to them in the larvæ of *Molgula manhattensis*. The alternative function, that of fixation, was put forward by Herdman (1893) for them in the larvæ of *Polycarpa glomerata* (?*Distomus variolosus*) and by Willey (1893) for *Styelopsis* as well. These ampullæ are outgrowths from the anterior or lateral regions of the body-wall, and in many cases one is formed considerably in advance of the rest, and has been called the mental process or chin. This primary outgrowth is always anterior, may be relatively short and wide, as in *Ciona intestinalis* (fig. 15) or very long and attenuated, as in *Molgula ampulloides*, *Molgula manhattensis*, and *Styela partita* (figs. 18 and 11, F.). Somewhat later accessory outgrowths occur from the lateral parts of the body, and in the majority of cases, as the ampullæ grow out, their bases grow together, so that a cluster of ampullæ is formed on either side of the body. This is well seen in the various species of Molgula (fig. 18). In the Ascidiidæ a single cluster of three or four ampullæ is formed, while in others a ring may be formed round the anterior part of the larva, twenty-four in Styelopsis, eight in Botryllus (see figs. 13 and 17).

Table V shows the various conditions. With the exception of *Corella willmeriana* (Child, 1927) and *Molgula robusta* (Lucas, 1927) the observations are original.

In reality the ampullæ possess the double function of fixation and respiration, though the latter seems to be the more important.

Most swimming tadpoles possess three anterior adhesive papillæ, which stick to any solid structure they meet. The adhesiveness, however, is associated with the general surface of the tadpole and one not infrequently finds the latter attached by the tip of the tail, though the anterior end is the more usual region. In any case the fixation brought about by this adhesive property is but temporary, and permanent fixation is made by the extensions of the larval test, initiated by the outgrowing ampullæ. Thus the test pushed out by the ampullæ adheres firmly to the substratum and remains as the organ of attachment after the ampullæ have dwindled and disappeared. In fact,

TABLE V.

Molgula ampulloides ...	One very long primary ampulla, finally 2 ampullæ on one side, 3 on the other.
,, *manhattensis*	
,, *occulta*	5 short ampullæ, 2 on one side, 3 on the other.
,, *oculata*	7 short ampullæ, 3 on each side, 1 anterior.
,, *bleizi*	7–9 short ampullæ, 3 on one side, 4 on the other.
,, *retortiformis* ...	Final condition a cluster of 6–8 ampullæ on each side.
,, *citrina*	Final condition a cluster of 4–5 ampullæ on each side.
,, *robusta*	5 ampullæ, 2 on one side, 3 on the other.
,, *arenata*	3–4 ampullæ.
Bostrichobranchus pilularis ...	One very long primary ampulla, occasionally secondary ones.
Tethyum pyriforme	4 anterior short ampullæ, later fuse into one.
Styela partita	2–4 very long ampullæ.
Polycarpa rustica	One long primary ampulla, several secondary.
Styelopsis grossularia ...	24 very short ampullæ arranged in a ring round the anterior end of the larva.
Botryllus schlosseri ...	8 short anterior ampullæ.
Botrylloides leachii ...	
Ciona intestinalis	One anterior ampulla, which may become subdivided.
Corella willmeriana	3 short antero-ventral ampullæ.
Ascidia, Ascidiella, Phallusia species	3–4 ampullæ arising from antero-ventral surface of tadpole and finally forming a single cluster.
Diplosoma gelatinosum ...	3 anterior short ampullæ.
Amaroucium pellucidum	Ampullæ anterior, but break up into small and very numerous vesicles. (This has also been observed by GRAVE, 1921.)
,, *nordmanni* and other species	

N.B.—These characters are independent of environmental changes in temperature, salinity, light, depth, periodical exposure, etc. (See BERRILL, 1928, p. 173.)

the ampullæ themselves are fixatory only in so far as they push the test out into processes which can adhere to surrounding objects and possibly enter crevices. *Ciona intestinalis* affords a good example of this (fig. 15, B.), for frequently a bubble of gas is secreted at the tip of the ampulla, which causes the metamorphosing larva to float towards the surface of the water (hence the common habitat of Ciona under ships and buoys), and the organism becomes attached to the surface film by the test near the distal end of the ampulla. Observations on such forms show at once that the test, even at this stage of development, can extend along surfaces, while the fact that test processes of adult *Phallusia mammillata* and *Ascidiella aspersa* have been found extending many inches into crevices in which the animal itself could have existed at no stage of its development, and the fact that re-attachment can occur after separation shows that it persists as an active structure throughout life.

Many observers have noted that waves of contraction occur in the ampullæ, especially where these are long, as in *Molgula manhattensis*. Such waves occur in almost all ampullæ that are reasonably elongated and the maximum rate of wave propagation seems to be much the same in them all. The contraction commences at the base of the ampulla and passes slowly as a wave of constriction towards the distal end, causing marked movement of the enclosed fluid and corpuscles in and out of the developing body cavity. The maximum rate of propagation seems to be about 30 per hour, while

the time taken to traverse the ampulla is between three and four minutes. The change in form and the rate of movement can be seen in fig. 16.

The ectodermal ampullæ invariably have enlarged cells at their distal end, of a glandular appearance, and it is possible that they are mainly responsible for the formation of the internal fluid. The respiratory and circulatory function of these structures is

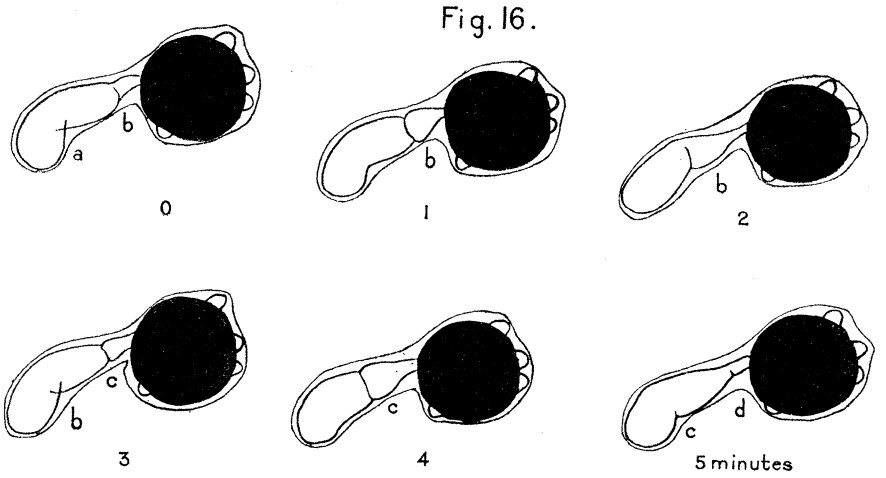

FIG. 16.—Metamorphosing individual of *Polycarpa rustica* drawn at intervals of one minute to show nature and rate of wave of constriction in the primary ectodermal ampulla. *a*, *b*, *c*, *d*, four successive waves.

indicated in several ways. The outgrowths occur at the end of the differentiation phase of development, and at the time growth proper commences and a body cavity is being formed, *i.e.*, when there is probably an increase in activity and before the protostigmata and heart have developed to produce an oxygenated circulatory fluid; while they shrink and disappear just about the time the latter structures become functional.

In some forms there seems to be actual degeneration, *e.g.*, *Styela partita*, but in most species where the outgrowths are less elongated their disappearance is due rather to their fusion and blending with the main surface of the organism. This is shown in fig. 7, C. and D., and fig. 14. CHILD (1927) shows the same phenomenon in his figures 9 and 10 for *Corella willmeriana*. His statement that the three anterior adhesive papillæ give rise to the ectodermal ampullæ (three in this case) may be no more than the result of a coincidence, in that the number of both is the same and the later outgrowths may stretch and obliterate the papillæ. In the great majority of species they are very obviously independent structures, and in the tadpoles of most compound ascidians they may be seen to co-exist.

The later development of the metamorphosed larvæ can be seen in figs. 7 D., 13, 14 and 15, A., and B., and will be discussed in detail in a later paper in connection with the acceleration of development typical of the social ascidians.

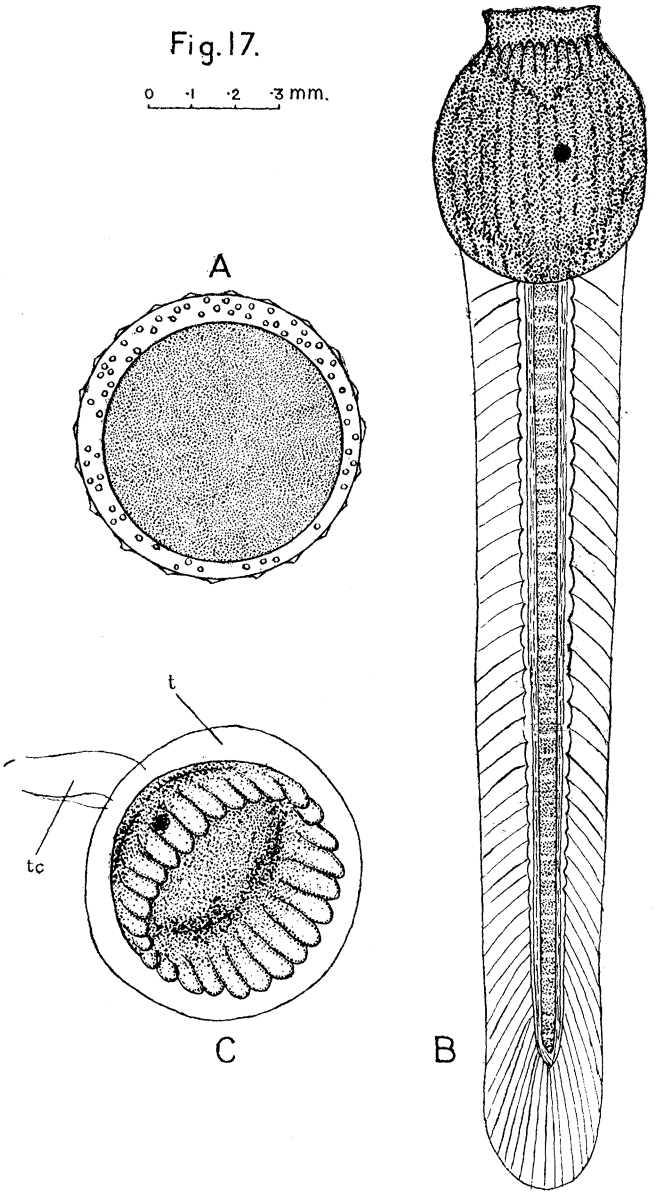

FIG. 17.—Development of *Styelopsis* (*Dendrodoa*) *grossularia* BENED. A., egg, showing stretching of outer follicle cells; B., tadpole with single sense organ and characteristic fin-ray appearance of the tail cuticle; C., anterior view of metamorphosing individual showing medusoid shape, and appearance of the marginal ampullæ. *t.*, test; *t.c.*, tail cuticle.

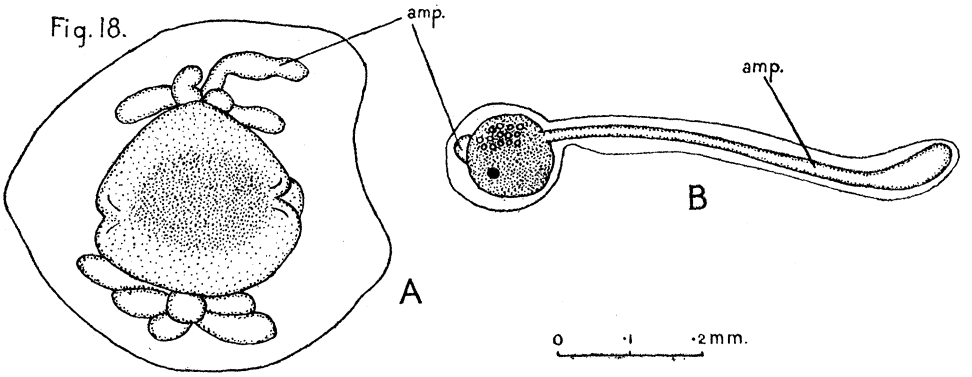

FIG. 18.—Ectodermal ampullæ of *Molgula manhattensis* and *Molgula retortiformis*. A., *M. retortiformis*. B., *M. manhattensis*. *amp.*, ampulla.

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9. *The Influence of Molecular Concentration.*

The total concentration of the salts in the water in which ascidian embryos develop has little influence of a differential nature, the principal effect of hyper- or hypo-tonic solutions being retardation and (above or below certain limits) inhibition. Various concentrations of sea-water were made, hypotonic solutions by diluting normal sea-water with glass distilled water, hypertonic solutions by diluting artificial sea-water isotonic with 1·2 molar NaCl with distilled water. The salts used for the artificial medium were those of Kahlbaum and Co. The *p*H of every solution was 8·1, and in every case the eggs were immersed at the 2-cell stage. Fig. 19 indicates the general nature of the results obtained.

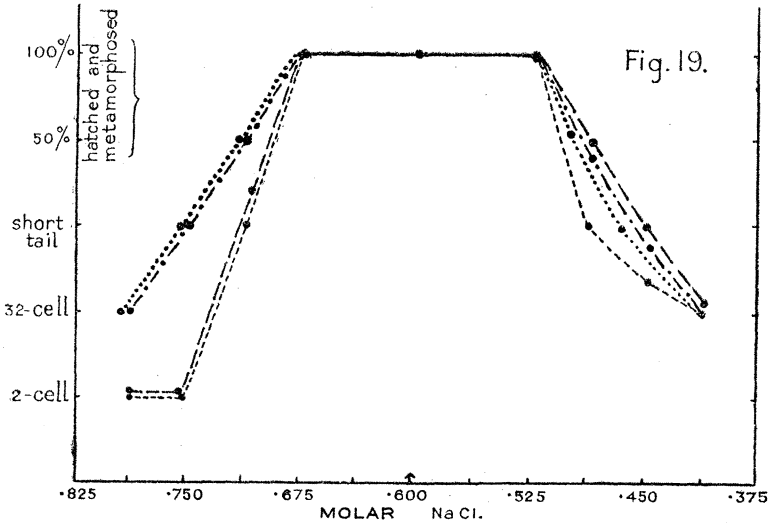

Fig. 19.—Diagrammatic representation of the influence of salt concentration on the development of ascidians (Phallusia).

The retardation occurs to much the same extent, whether it be due to hypertonism or hypotonism, and it is seen that normal development can occur through a wide range of concentrations (from 0·525 to 0·675 mol. conc.). Retarded development occurs between concentrations 0·675 and 0·7125 mol. and 0·525 and 0·4825 mol.; greater or lower concentrations cause arrest of development at any stage, the earlier the more extreme. Hypotonic solutions, as distinct from hypertonic, produce a certain extent of opacity in the egg or embryo, an opacity first appearing about 0·525 mol. and complete at 0·4125 mol. This may be due to the dilution of the salts and consequent precipitation of proteins within the cells (*cf.* Gray, 1920, on trout eggs); but it is especially interesting to note that though normal eggs and embryos are perfectly translucent, fairly typical development can occur in hypotonic solutions that produce a most marked degree of opacity.

The approximate range for normal development in terms of salinity for such forms as *Phallusia mammillata* and *Ascidiella aspersa* is 30 per mille to 40 per mille.

10. *Variability of Eggs and Larvæ.*

When the development of a large number of eggs is followed, the variability within a batch from one individual, or between batches from different individuals, becomes very striking. The variability may be seen in the susceptibility of the unfertilised eggs themselves, in the rate of early cleavage and, to a lesser extent, of chorion lifting, and in the nature of the tadpole. This last may approach the low *p*H type with a short free-swimming period and quick metamorphosis, or the high *p*H type with both phases prolonged.

In general, there is a correlation between healthy eggs, rapid division and the production of tadpoles that metamorphose quickly, on the one hand, and more susceptible eggs, slow division and the production of tadpoles with prolonged larval and post-larval stages on the other. With the second type is often found a fairly high percentage of very abnormal cleavage and development.

Some experiments were made with the object of ascertaining the origin of such variability and the conclusion was reached that it depends primarily upon two factors, the length of time an egg remains in the oviduct and the physiological condition of the parent during that time.

The members of the Ascidiidæ all possess more or less lengthy oviducts and therefore eggs may be extracted from different regions, that have lain in the duct for a shorter or longer time. The species mostly used was *Phallusia mammillata*, for the reason that the oviduct may be four or five inches in length and packed throughout with large quantities of mature viable eggs.

In these forms the germinal vesicle of the egg breaks down as soon as the egg is shed from the ovary into the oviduct, and nuclear changes continue to the metaphase of the first maturation division. In this condition the egg remains, however long it is retained, and the maturation divisions are completed only after fertilisation.

If then the oviducal fluid be not inert but can affect the egg in any way, its influence, *e.g.*, in Phallusia where the eggs may take several weeks and possibly months to pass through the oviduct, may be very marked. That this is the case is obvious, for in species of the Ascidiidæ it is the rule rather than the exception to find a certain number of eggs cytolised within the oviduct. In *Ascidiella aspersa*, a species that dies in large numbers towards the end of summer, the percentage of eggs cytolised within the oviduct may be as high as 75 or 80. This has also been noted by Chabry (1887), though the species he used may have been *Ascidiella scabra* and not *A. aspersa* (*cf.* Berrill, 1928, p. 171).

Assuming then that the whole egg content of an oviduct be shed at once, and that the great majority of the eggs be "normal" and have remained within the oviduct for an optimum time, then a relatively small percentage of eggs from the ovarian and distal ends of the oviduct will have remained there for too short and too long a time

respectively. For the sake of convenience these three types will be called young, normal and old.*

Their relative percentages within any one oviduct will be influenced by several factors. An increase in the frequency with which batches of eggs are expelled will tend to increase the relative percentage of young eggs, while undue retention will tend to produce more and more of the old. Unhealthy conditions in the parent, involving a greater toxicity of the oviducal fluid, will increase the relative percentage of old eggs, while the same conditions holding during the growth stages of the ova will produce a similar result by increasing the susceptibility of the eggs to that toxicity. The physiologically young and old eggs thus tend to produce swimming tadpoles approaching the types produced by high and low hydrogen-ion concentration respectively, and among the larvæ resulting from such old eggs many retain their tail through and after metamorphosis (fig. 12, C.). This type occurs very commonly in certain species of Molgula, and will be described in full in the account of that group.

11. *Summary.*

1. The life-histories and general physiology of development have been investigated in the following species—*Tethyum pyriforme americanum*, *Boltenia hirsuta*, *Styela partita*, *Styelopsis grossularia*, *Phallusia mammillata*, *Ascidiella aspersa* and *scabra*, *Ascidia conchilega*, *A. mentula*, *A. prunum and Ciona intestinalis*.

2. In the eggs of all species there are the following perivitelline structures, an outer layer of follicle cells resting on the chorion or egg membrane proper, and inner follicle cells lying in the perivitelline fluid. In species of the Ascidiidæ there is in addition a fine membrane between the inner cells and the chorion, and the space thus delimited by the two membranes contains matter in the gel state.

3. The perivitelline space as a whole is formed in the unfertilised egg when it is first shed into the sea-water, as a result of the osmotic pressure exerted by colloidal substances lying between the ovum and the chorion. This pressure is counter-balanced while the eggs lie within the oviduct by the presence of substances with a similar osmotic pressure in the oviducal fluid. The perivitelline compound involved is probably protein in nature, and has, in the cases of Ascidiella and Phallusia, a total osmotic pressure equivalent to that produced by a solution of 0·8 per cent. gum arabic in sea-water.

4. In the eggs and embryos of *Boltenia hirsuta* the protoplasm proper is transparent, the yolk spheres are grey, while the lipoidal inclusions are of a bright orange colour, giving thus a picture of "organ-forming substances" even more spectacular than is the case in *Styela partita*.

* In *Ciona intestinalis* eggs found in the oviduct of individuals at the commencement of the breeding season possess greenish pigment, at the latter part of the breeding season, red pigment, while during an intermediate period individuals may be found in which the eggs at the distal part of the oviduct are red, at the ovarian part, green; *i.e.*, physiologically young eggs are green, physiologically old are red.

5. The interdigitation of notochord cells to form a single column, and their subsequent fusion to form a continuous cylindrical rod closed at either end, is seen exceptionally clearly in the development of *Tethyum pyriforme*. This species affords very good material for the study of development from almost any point of view.

6. Variations in the structure of tadpoles of simple ascidians are connected primarily with the presence or absence of one or both sense organs and with the nature of the peribranchial invaginations.

7. In all species of the Ascidiidæ investigated, and probably in most simple ascidians in which there is little yolk accumulation, there is a proteolytic enzyme developed for the purpose of hatching.

8. This enzyme is produced by the embryo during the early stages of tail formation, has an optimum temperature in the region of 37° C., and is active only in media of *p*H limits 7 to 10.

9. An alternative method of hatching which is efficient at a later stage of development is that of rupture by means of ectodermal ampullæ which push out the larval test. In general the more sensitive the earlier method, the more efficient is the later.

10. The onset and rapidity of metamorphosis of Phallusia, Ascidiella and Ciona tadpoles may be controlled by varying the hydrogen-ion concentration of the water. Hyper-alkaline sea-water tends to inhibit metamorphosis altogether, while changes in the direction of increased acidity tend to induce metamorphosis. It is possible under natural conditions that the change is initiated by an increase in intercellular acidity produced in the tail as the result of muscular activity.

11. The ectodermal ampullæ responsible for the later method of hatching have but a transitory existence. Their primary function seems to be that of a temporary respiratory organ, functional only until the heart is developed; a secondary though important function is that of fixation.

12. Alteration in the molecular concentration of the sea-water in which ascidian embryos develop has but little effect other than retardation. Normal development may occur over a salinity range 30–40 per mille. Such development occurs in hypotonic solutions that produce marked opacity of the eggs and embryos.

13. The variability of eggs and larvæ of ascidians is great, and seems to be due primarily to the length of time an egg has lain within the oviduct and to the toxicity of the oviducal fluid during that time.

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