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ON THE ORIGIN OF THE SO-CALLED "TEST-CELLS" IN THE ASCIDIAN OVUM. By J. PLAYFAIR McMURRICH, B. A., *Assistant in the Biological Laboratory, University of Toronto.* With Plate X.

THE following observations have been made in the hope of elucidating to some extent the nature of the so-called "test-cells," so characteristic of the ova of Tunicates. These bodies have been described by various authors as occurring in the eggs of most of the commoner forms, and under normal circumstances probably do not make their appearance until after fertilization. Lacaze-Duthiers⁽¹⁾ states that in *Molgula* a true layer of "test-cells" is wanting, and only the follicle-epithelium surrounds the newly deposited ovum. Under abnormal circumstances, however, they are formed at a much earlier period, and thus in most eggs that have been observed, "test-cells" were present or soon made their appearance.

My observations have been carried on for the most part on ova of *Ascidia amphora*, but I have also confirmed most of them by similar experiments on eggs of *Cynthia ocellata*. I made use only of mature or almost mature eggs, so that I am unable to give as complete an account of some points as could be desired.

The mature eggs of *A. amphora* (Pl. X, Fig. 1) have an average diameter of about .255 mm. and present on optical section two distinctly marked zones, enclosing a semi-transparent granular mass, the yolk. The outer of the two zones is formed by the follicle-epithelium, consisting of a single layer of cells surrounding the whole egg, and presenting on a surface view a polygonal appearance. On examining a single cell which has become separated from the egg, with a rather high magnifying power, its interior is seen to be occupied almost entirely by a number of vacuoles, separated from one another and surrounded by very delicate bands of protoplasm, which, in some of the angles formed by the meeting of the polygonal vacuoles, appear as dark spots. (Pl. X, Fig. 2.) I have not been able to observe the development of these cells, but Semper⁽²⁾ has described it as it occurs in *Molgula nana*, where, in.

the earliest observed stages, they appear as a layer of flat cells on the surface of the egg, which, later on, become prismatic, and in the interior of which 2-4 yellow granules make their appearance. These afterward disappear and large vacuoles take their place, pressing the protoplasm and nucleus to one side. In his figures, the formation of the vacuoles has not advanced as far as in the eggs I studied, but, on comparing his Figure 5, Plate I, with my Figure 2, Plate X, it will at once be recognized that the appearance I have observed is to be accounted for in the same manner, the vacuoles having become exceedingly abundant, and pressed the original contents of the cell to the periphery, small portions only being left in the intervals between the vacuoles.

Fol,⁽³⁾ having succeeded in tracing the origin of these cells still farther back in *Phallusia intestinalis*, states that they are not formed from the ovary, but from the interior of the egg at the boundary between the yolk and the nucleus, and wander thence to the surface, where they form an epithelial layer round the egg. One would fancy at first that the eminent observer had accidentally confused "test-cells" with the follicle-epithelium, but that he has not done so is evident from his also describing the "test-cells" as formed later on. This discovery is of great interest, both from its upsetting all former theories as to the formation of these cells, which have hitherto always been considered as being formed from the ovary, and also from the singular manner in which Kowalewsky's theory in regard to the formation of the "test-cells" from these cells has been turned upside down, these bodies (*i. e.* the "test-cells") being formed independently from the yolk (as will be seen hereafter) from which at an earlier period the follicle-cells had also been derived. If M. Fol's observations are correct, it is evident that the term "follicle-cell" is entirely a misnomer, as is also indeed that of "test-cell," both being to a high degree misleading to one who has not studied the history of the appellations.

Within this layer of so-called "follicle-cells" comes the second zone of the egg, consisting of a transparent, apparently homogeneous structure, which, however, when acted on by acetic acid, becomes markedly granular. This is the egg-shell or "chorion" of some authors.

In the interior of the egg-shell and filling it almost completely in the fresh ovum, is the yolk. On the average it measured

.236 mm. and was of a yellowish gray color, due to the coloration of the constituent granules. In the majority of the eggs of this Ascidian I examined, no nuclei were visible either in the fresh egg, or in those that had been subjected to the reaction of acetic acid and glycerine, or osmic acid and carmine. In some, however, a clear spot was noticeable, usually situated eccentrically (in one instance at the periphery of the egg), and measuring .020-.083 mm. One egg presented a rather peculiar abnormality, which I deem worthy of being recorded. The peculiarity consisted in the presence of two distinct nuclei, both situated eccentrically on the same side of the egg and varying somewhat in size, the larger measuring .06 mm. and the smaller .04 mm. I am certain that I did not observe the male and female pronuclei, as the egg under observation had just been removed from the ovary, so that it could not have been impregnated any length of time, if at all, before my observation of it.

These were all the points to be observed in a perfectly fresh ovum, but in one that had been removed from the ovary for a short time, or which had been subjected to the action of various reagents, there was to be seen surrounding the yolk a layer of bodies, which have received the name of "test-cells" from the idea that they were the cells of the developing ovum, from which, eventually, the characteristic test of the adult Ascidian was formed. Kowalewsky⁽⁴⁾ in his paper on the development of *Ascidia cinerea* states his belief that such is the fate of these cells, which, he also maintains, have their origin from the epithelial cells of the egg-follicle. Later on, however, in his paper on the development of *Pyrosoma*,⁽⁵⁾ he withdraws the statement that the mantle is formed from the "test-cells," but still adheres to the opinion that these are merely follicle-epithelial cells, which have migrated inwards towards the yolk. Before the appearance of his first paper, however, Kupffer,⁽⁶⁾ after investigating the subject, came to the conclusion that the "test-cells" formed at the surface of the egg itself, which theory had been independently adopted by Metschnikoff.⁽⁷⁾ Before the publication of Kowalewsky's second paper, Hertwig⁽⁸⁾ shewed that the "test-cells" take no part in the formation of the mantle, this being formed as a secretion of a homogeneous substance from the epidermis, into which, later on, cells migrate from the epidermis. Hertwig's observations were made on *Phallusia mamillata* and *P. virginea* (?), and have been confirmed by Semper⁽²⁾ by

observations on *Clavelina vitrea* and *Cynthia depressa*. In the same year that Semper published his observations, a paper by Ussow⁽⁹⁾ appeared, in which the old theories first advanced by Kowalewsky are revived and most emphatically insisted upon. He says: "The outer mantle of the Tunicates is developed, not as a secretion product of the epidermal cells of the inner mantle, (Hertwig, Arsenjew,) but by the increase in number and growth of the so-called 'test-cells' (Kupffer, Kowalewski)," and again: "The result of my observations on the formation of the so-called 'test-cells' is in complete accord with that of A. Kowalewski. The yellow bodies are in fact nothing but cells of the Graafian follicle"

Semper shews that in the several species in which he examined the ova, the "test-cells" were formed in the interior of the egg, and that by the action of reagents, or even by exposure to seawater, these bodies might be produced in immature eggs. He holds that they are devoid of a nucleus and of a cell-wall, and discarding the term "test-cells," substitutes instead that of "test-drops."

My own observations having been confined to mature or almost mature eggs, I cannot confirm Professor Semper's statement as to the production of these peculiar bodies in immature eggs by means of reagents, but these have the effect of producing them in most cases almost immediately in mature eggs, even the exposure to seawater for a short time being sufficient for the purpose. Produced in this manner these bodies are small and roundish in shape, and in their interior numerous clear highly-refractive granules are to be seen. I could detect no nucleus either in the fresh or in the stained "drop," and a limiting membrane was also apparently wanting.

As regards their mode of origin I am in accord with the observations of Kupffer,⁽⁶⁾ Metschnikoff,⁽⁷⁾ etc. When an egg has been removed from the ovary for a few minutes, there appear in the interior of the yolk, numerous clear spots situated nearer the periphery than the centre. In no case did they make their appearance at the centre of the yolk, and though in Figure 3, (Pl. X,) some appear to be very close to it, these in reality are peripheral and appear indistinctly when an optical section of the egg is made and accordingly have been represented. I accordingly conclude that their origin is peripheral as stated by Metschnikoff.⁽⁷⁾ They

gradually migrate outwards, until they form a layer at the periphery of the yolk (Pl. X, Fig. 4), and then pass outside of it altogether. The yolk at the same time contracts and leaves a space between its circumference and the egg-membrane, in which the "test-cells" lie, forming at first a layer round the yolk (Pl. X, Fig. 5), but as the contraction of the yolk proceeds, and the space becomes larger, they move away from the surface of the egg and scatter themselves irregularly. (Pl. X, Fig. 6.)

I should imagine that there is in a manner a separation of the egg into two portions; an outer, consisting of protoplasm with comparatively few yolk granules, and an inner, containing most of the yolk granules and a small amount of protoplasm. The outer zone is of no further use in the process of development, and gradually splits up into these "test-drops," their formation commencing at the inner part of the zone and proceeding outwards, until we have numerous "test-drops" and nothing left of the egg but a dense mass of food-granules, closely packed together in the remaining protoplasm, from which the embryo is formed. Metschnikoff⁽⁷⁾ describes this separation of the egg into two portions. He says: "In the greenish protoplasm of a young egg of *Ascidia intestinalis*, fine yolk-granules collect round the nucleus; the number of these becomes continually greater, whereby only the peripheral portion of the protoplasm retains its greenish coloration. This layer now separates itself distinctly from the central granular portion and splits up into a great number of round bodies which are the first 'Tunic-cells.'" From this description one would imagine that the author implied that the "Tunic-cells" were formed at the extreme periphery of the egg, which, however, is not the case, for they make their appearance in its interior.

On treating a fresh ovum with a dilute solution of acetic acid (1 or 2 drops of commercial acid to a watch-glassful of water) for about half an hour, its appearance becomes considerably changed. (Pl. X, Fig. 6.) The interglobular protoplasm of the "follicle-cells" becomes much more distinct, and, in consequence, the globules themselves become more plainly marked off. The transparent, apparently homogeneous egg-membrane becomes, as mentioned above, distinctly granular. The yolk contracts very much, measuring on the average about .116 mm., half its original size. This contraction leaves a clear space between the yolk and the egg-membrane, which, however, is larger in one-half of its cir-

cumference than in the other, owing to the eccentric position assumed by the contracted yolk. In this clear space are numerous "test-cells," not forming a layer round the yolk, as they usually do in an egg that has been subjected for a short time only to the action of acetic acid or sea-water, but scattered irregularly around the yolk. The "test-cells" measure .008 mm. and present the appearance described above. In eggs that have been left in acetic acid for a much longer period (6-20 hours) no further changes occur, showing that the acid has exerted its full influence on them.

After exposure to sea-water for six hours, very much the same appearance is presented as with dilute acetic acid. The "follicle-cells," however, shew a tendency to separate from the egg-membrane, which, on its part, does not present a granular appearance. (Pl. X, Fig. 7.)

Upon running some strong picro-carmin under a cover-glass, below which were some ova in sea-water, very important changes occurred. At first no "test-cells" were to be seen, but, as the picro-carmin gradually reached the egg, and the picric acid exerted its action upon it, it gradually assumed a yellow hue, while, at the same time, there appeared at its periphery many small spherical bodies of a round or oval shape, the same size as the "test-cells," and containing in their interior several highly refractive granules, which, in fact, render them apparent. No "test-cells" appear outside the yolk, which retains its original size. The egg-membrane assumes a pink hue, and, after some time, becomes distinctly granular. The "follicle-cells" do not stain for some time and show a tendency to separate from the egg-membrane. (Pl. X, Fig. 8.) The reaction produced by very dilute picro-carmin is also rather important. After being subjected to this reagent for about half an hour, the eggs presented an appearance intermediate between that produced by the continued action of dilute acetic acid and that following the employment of strong picro-carmin. (Pl. X, Fig. 9.) The yolk contracts to a slight degree, and "test-cells" make their appearance, filling up the small space between the partly contracted yolk and the egg-membrane.

I also employed osmic acid in the following manner. The eggs were placed in a watch-glass containing sea-water, to which 1 or 2 drops of $\frac{1}{4}$ per cent. osmic acid had previously been added, and

allowed to remain there for from five to ten minutes, when they were removed and stained with Beale's carmine. In most cases no change occurred, the yolk remaining of its original size, and no "test-cells" or clear spots made their appearance in the yolk, with the exception of one instance, in which I did perceive a number of clear spots in the periphery of the yolk.

By these results two questions are suggested: 1st. What are these "test-cells?" 2d. How are the various phenomena caused by the various reagents to be explained? I shall give the second question priority. The explanation that seems to me to be the simplest, and that which bears the stamp of probability most distinctly, is, that these phenomena are caused by the varying effects of the different reagents in producing a contraction of the protoplasm of the yolk. Thus, osmic acid, which "fixes" the protoplasm immediately, allows of little or no contraction, and hence no "test-cells" appear; with picric acid (which evidently is the constituent of the picro-carmine that is active in producing the phenomenon) a slight contraction takes place before the protoplasm becomes "fixed," whereby the "test-cells" are formed, but the contraction is not sufficient to cause them to pass outside the yolk; and, in the last place, with acetic acid and sea-water there is no fixing of the protoplasm, and the contraction goes on to such an extent that the "test-cells" are driven completely outside the yolk. Strong evidence in support of this theory is afforded by the variation in the action of picric acid, according to the strength in which it is used. For, as we have seen, in a dilute solution so much contraction of the yolk is produced, that the "test-cells" do partly pass out.

Accordingly, then, the "test-cells" are formed by a contraction of the protoplasm of the egg, and thus we can readily understand their formation in a developing egg, where the contraction produced by the process of cleavage would be quite sufficient to cause their extrusion from the yolk.

We are now in a position to discuss the question as to the nature of these "test-cells." Semper⁽²⁾ regards them as merely polar globules, comparing them, in respect to their number, with those of the Mollusca. This theory is, however, untenable, for by the researches of Hertwig on the formation of the polar globules in the eggs of *Hæmopsis*, *Nephelis*,⁽¹⁰⁾ *Asteracanthion*, *Mytilus*,⁽¹¹⁾ and other forms, we know that the polar globules are formed by a true

cell-division, and are themselves true cells, containing a nucleus, whereas no such process has been observed during the formation of the "test-cells," and I for my part am sure that it does not obtain, and, as Semper himself insists, the "test-cells" are not true cells, but merely "drops." Fol,⁽³⁾ too, states that in *Phallusia intestinalis* polar globules (two in number) are formed after the disappearance of the original nucleus and after the formation of "test-cells." Accordingly then, there is no morphological homology between the polar globules and the "test-cells." In the eggs of certain forms, however, such as, in the Amphibia, *Rana*,⁽¹⁰⁾ and in the Pisces, the Trout,⁽¹²⁾ after the disappearance of the germinal vesicle, peculiar bodies are extruded from the yolk without any spindle-formation or cell-division, for which Hertwig proposes the name of excreted bodies (Excretkörper) in contradistinction to the polar globules formed by cell-division. These structures have been supposed by the various authors to be the remains of the germinal vesicle, and thus, as far as their mode of formation is concerned, probably do not allow of comparison with the "test-cells," but since they resemble these latter in being bodies whose presence in the egg is not necessary to its further development, and since the cause of their appearance is evidently the same, viz: the contraction of the yolk induced by a stimulus, I think there can be no objection to classifying the "test-cells" with them as Excretkörper.

Wyville Thomson,⁽¹³⁾ however, has described bodies as occurring in *Antedon rosaceus* which bear a closer homology to "test-cells" than even these structures. He says: "Consequently on the contraction of the yolk, a number of minute spherical pale yellow oil-globules are apparently pressed out into the space within the Vitelline membrane." These bodies differ from "test-cells" only in the fact that they are oil-globules, whereas "test-cells" are distinctly protoplasmic in their nature, and contain in their interior several oil-globules usually. This distinction, however, is of comparatively little moment, and both in their mode of formation and general appearance these Excretkörper—for so they also may be denominated—are evidently closely related to "test-cells" and perhaps identical with them.

I consider these "test-cells" to be simply masses of albuminous material containing two or three granules of the food-yolk, and presume that they are in reality only portions of the protoplasm

of the egg, which have been forced out by the contraction. If an egg, in which the "test-cells" have passed outside the yolk, be subjected to pressure sufficient to rupture the yolk-membrane, allowing the yolk to come into contact with the "test-cells," and at the same time leaving the egg-shell intact, the "test-cells" commingle completely with the yolk and cannot be distinguished again. The granules to be observed in a "test-cell" have a perfect resemblance, both in shape and appearance, to those remaining in the yolk as food, so that it may be presumed that they are in reality the same, and were originally situated in the yolk, in that portion of the protoplasm which formed the "test-cell," and were extruded with it.

The reason why portions of the yolk, originally of use to the embryo, have become useless and are extruded, must remain undecided until the life-histories of more of the lower types of Ascidians have been fully worked out, but in all probability the explanation is to be sought for in a change in the life of an ancestral form, whereby the development became more rapid and less food-yolk was required, while, at the same time, little or no diminution in the amount of yolk in the egg was produced.

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EXPLANATION OF FIGURES.

Figure 2 is drawn with Hartnack obj. 9, oc. 2; all the rest are drawn with Hartnack obj. 7, oc. 2.

FIGURE 1.—Fresh egg of *Ascidia amphora*.

FIGURE 2.—Follicle-cell.

FIGURE 3.—Egg after short exposure to sea-water.

FIGURE 4.—Egg after longer exposure to sea-water.

FIGURE 5.—Egg after still longer exposure to sea-water.

FIGURE 6.—Egg after exposure for half an hour to the action of dilute acetic acid.

FIGURE 7.—Egg after exposure to sea-water for six hours.

FIGURE 8.—Egg after the action of strong picro-carmin.

FIGURE 9.—Egg after the action of very dilute picro-carmin.

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