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MECHANISMS OF DETERMINATION IN THE DEVELOPMENT OF GASTROPODS

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I. Introduction

The eggs of the 'Spiralia' (i.e., those groups of animals that exhibit a spiral cleavage), according to the classical view belong to the 'mosaic eggs', characterized by a precocious and irreversible determination of the cells. One might expect the processes bringing about such an early determination to differ essentially from those governing the development in other groups, where determination occurs at a much later stage, and the developing embryo retains for a long time the capacity of regulation.

However, investigations of the last 25 years have shown that the differences in modes of development between the eggs of Spiralia, on the one hand, and 'regulation eggs', like those of sea urchins and vertebrates, on the other, are much less fundamental than was originally assumed. At present there seems to be no doubt that the main trends of development are the same in all groups of the animal kingdom. The existing differences can be seen as variations of the same general theme, in which now one, then another of the component processes plays a more prominent or more easily recognizable part. Therefore the analysis of development in each group contributes its share to the construction of a general picture of development. For instance, experimental embryology of vertebrates has especially elucidated the importance of induction; research on sea urchins has shown the role of gradients in development; the study of insect development made possible the analysis of gene

actions during development. In the same way, in the study of the development of Spiralia it is the differential distribution of cytoplasmic substances (ooplasmic segregation) that is most striking. This does not mean however that this process does not also occur in the other groups, nor that the other mechanisms mentioned above are lacking in spirally-cleaving eggs.

In this paper a survey of the different mechanisms of determination in the development of the gastropods will be given (for a more extensive treatment cf. Raven, 1958b).

II. Ooplasmic Segregation

When the fully grown oocyte in the gonad of gastropods is ready to ovulate, the various components and inclusions of its cytoplasm are generally more or less evenly distributed throughout the egg (Fig. 1A).

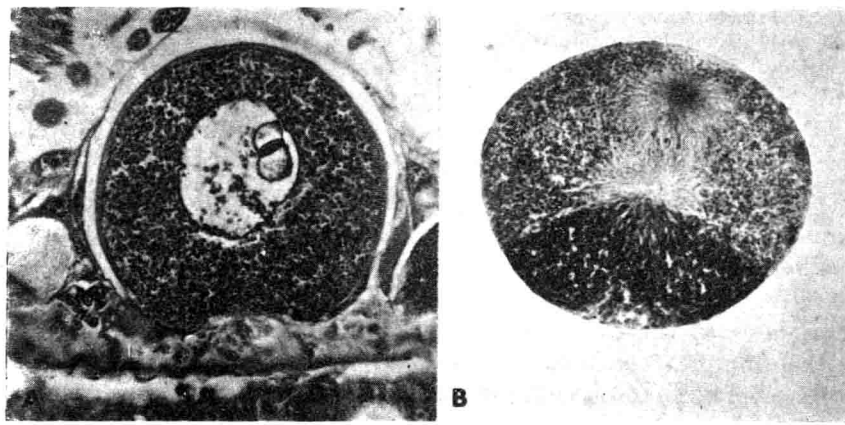

FIG. 1. Ooplasmic segregation in *Limnaea stagnalis*. A. Fully grown oocyte in the gonad. Uniform staining and distribution of cytoplasmic components. B. Fertilized egg immediately after oviposition. First maturation spindle. Vegetal pole plasm below. Subcortical 'patches' in equatorial region at right. Staining: azan.

Distinct localizations of certain substances or particles are not observed at this stage. Immediately after ovulation and fertilization, however, in most cases a movement of various egg substances occurs, through which they are accumulated or concentrated at certain places in the egg cell. These processes, which may be summarized under the term ooplasmic segregation, bring about a situation, in which different parts of the egg differ more or less in their cytoplasmic composition. The ooplasmic segregation continues during cleavage. The segregated egg substances are distributed unequally among the cleavage cells, which in this way

are endowed from the outset with a different chemical composition of their cytoplasm.

In the eggs of *Limnaea stagnalis* a special plasm accumulates at the vegetal pole during their passage through the female genital duct (Raven, 1945). This vegetal pole plasm is rich in protein yolk granules (Fig. 1B). In the recently laid egg it occupies a well-defined sector at the vegetal pole, but soon it extends beneath the surface towards the animal side. For some time the animal pole, where the two polar bodies are extruded, is free from it, but then it spreads over this region also, forming a continuous layer of nearly uniform thickness, the subcortical plasm, around the egg.

A second cytoplasmic differentiation, occurring in various pulmonates (*Limnaea*, *Myxas*, *Planorbis*, *Physa*, *Succinea*), is the animal pole plasm. Immediately after oviposition it has not yet been formed, but it appears during or shortly after the maturation divisions by the accumulation of a special plasm beneath the egg cortex surrounding the animal pole. This occurs at various moments in different species, e.g., prior to the extrusion of the first polar body in *Succinea putris* (Jura, 1960); between first and second maturation division in *Limnaea palustris*, *L. ovata* and *Myxas glutinosa*; some time after the extrusion of the second polar body in *Limnaea stagnalis* (Raven, 1945). The animal pole plasm is very rich in mitochondria. The latter are first concentrated around the maturation spindles and asters. They are transported by this means towards the animal pole, where they accumulate in dense layers immediately beneath the cortex surrounding the animal pole.

At first and second cleavage, the cytoplasmic substances in *Limnaea* are distributed about equally among the blastomeres. Prior to the third cleavage, however, the subcortical plasm and animal pole plasm unite at the animal pole into a common mass of dense cytoplasm. Most of this plasm passes into the micromeres, which consequently consist for the greater part of pole plasm substance, whereas the macromeres consist mainly of vacuolated cytoplasm. This differential distribution of cytoplasmic substances is repeated at the following cleavages, so that the relative amount of pole plasm substance in the cells of the blastula decreases from the animal towards the vegetal pole (Raven, 1946). It lies in the superficial region in all cells. In older blastulae vacuoles appear in this region, filled with egg capsule fluid, which is taken up by pinocytosis (Elbers and Bluemink, 1960). A similar differential distribution of pole plasm substance has also been found in *Succinea* (Jura, 1960).

Sometimes a vegetal pole plasm may temporarily be constricted off from the rest of the egg, forming a so-called polar lobe. Such polar lobes have been described in the eggs of various Prosobranchiata, e.g., *Bithynia*, *Crepidula*, *Nassa*, *Ilyanassa*, *Ocinebra*, *Urosalpinx*, *Fulgur*.

They may be formed at the time of the maturation divisions, and again at early cleavage divisions. In the first case they protrude at the moment of the maturation divisions, but are afterwards incorporated again into the egg. A polar lobe constricted off from the vegetal pole at first cleavage, afterwards fuses with one of the cleavage cells, which thereby becomes CD; it then surpasses its sister cell AB in size by the volume of the polar lobe. At the second cleavage, a similar polar lobe forms at CD, which subsequently fuses with its daughter cell D. In this way the substance of the polar lobe is assigned as a whole to one of the first four blastomeres.

The corpuscular inclusions of the egg cytoplasm may be subjected to considerable displacements during ooplasmic segregation. In many cases the protein yolk granules, which were initially distributed more or less evenly throughout the egg, are concentrated in the vegetal part of the egg during or shortly after maturation, leaving an extensive yolk-free area at the animal pole. This is most pronounced in the eggs of Opisthobranchiata (e.g., *Cymbulia*: Fol, 1875; *Aplysia*: Ries and Gersch, 1936; *Navanax*: Worley and Worley, 1943), but it occurs to a lesser extent also in some Prosobranchiata (e.g., *Neritina*: Blochmann, 1882; *Columbella*: Spek, 1934). This vegetal concentration of the yolk may continue during cleavage, bringing about a still sharper separation of egg substances. In general it leads to a distribution, in which the cells of the first quartet contain hardly any protein yolk, those of the second quartet somewhat more, and so on, while most of the yolk passes into the endomeres.

A very unequal distribution of the yolk among the cells may already have taken place at the first cleavages in some Opisthobranchiata, e.g., in *Cymbulia* and *Cavolinia*, where nearly all the yolk is found at the 4-cell stage in A, B and C, whereas D consists almost exclusively of hyaline protoplasm (Fol, 1875).

In the pulmonates *Limnaea* (Raven, 1945), *Physa* (Mancuso, 1953), *Succinea* (Jura, 1960) and *Arion* (Lams, 1910) fat droplets are rather uniformly distributed at first, but are shifted towards the vegetal part of the egg during the maturation divisions. The same occurs in *Bithynia* (Attardo, 1955a). Consequently the micromeres get only few fat droplets, while most of the fat passes into the endomeres.

On the contrary, in the opisthobranchiates *Aplysia* (Ries and Gersch, 1936) and *Navanax* (Worley and Worley, 1943) the concentration of the protein yolk in the vegetal two-thirds of the egg is accompanied by an accumulation of the fatty yolk in the animal part, where it occupies a layer between the protein yolk and the clear hyaloplasm area at the animal pole. During cleavage it passes mainly into the micromeres.

In *Ilyanassa* the lipid droplets are likewise accumulated in the animal

part of the egg. At the 4-cell stage they arrange themselves in each quadrant into a narrow zone at the boundary between the clear animal cytoplasm and the protein yolk. Very few droplets pass into the cells of the first micromere quartet, somewhat more into the second, while the cells of the third quartet get numerous fat droplets. In the D-quadrant, nearly all the rest of the fatty yolk passes into 4d; in the other quadrants a more uniform distribution among the daughter cells takes place (Clement and Lehmann, 1956).

The Golgi bodies ('Speichergranula') in *Aplysia* (Fig. 2) are at first distributed rather evenly beneath the surface, but during maturation they concentrate in a narrow ring at the boundary between the protein yolk and fatty yolk zones. At cleavage they pass into particular cells: the micromeres 2c and 2d and the macromeres A and B, but especially (Fig. 2, I) the cells 3c, 3d, C and D (Ries and Gersch, 1936) (Fig. 2). In *Navanax* a similar concentration of the Golgi bodies in a narrow supra-equatorial ring takes place, but this occurs at the 4-cell stage; both the first micromeres and macromeres get part of these corpuscles (Worley and Worley, 1943).

In the pulmonates *Limnaea* (Raven, 1945), *Myxas*, *Planorbis*, *Succinea* (Jura, 1960) and *Arion* (Lams, 1910) the mitochondria have a strong tendency to concentrate around the maturation spindles; they lie, often in rows, between the astral rays. This leads to their accumulation in the neighbourhood of the animal pole, where they come to lie in the animal pole plasm and then become heaped up immediately beneath the cortex. They are then distributed with the animal pole plasm.

In *Aplysia* (Ries and Gersch, 1936; Attardo, 1957) and *Ilyanassa* (Clement and Lehmann, 1956) the mitochondria also accumulate around the animal pole. In *Aplysia* they pass mainly into the first micromeres 1a–1d and the cells 2a, 2b, 3a and 3b. At the 24-cell stage the trochoblasts $1c^2$ and $1d^2$ are very rich in mitochondria. In *Ilyanassa* the cells of the first three quartets of micromeres all get a rich supply of mitochondria; the rest passes mainly into the fourth micromeres 4a–4d, whereas the cells 4A–4D only get few mitochondria.

Particular granules of an unknown nature have been observed by Blochmann (1882) in the uncleaved egg of *Neritina*. During cleavage they become localized in two cells of the second quartet situated at left and right, presumably the cells $2a^1$ and $2c^1$.

In *Physa* (Wierzejski, 1905) and *Limnaea* (Raven, 1946; Minganti, 1950) dark granules become visible during early cleavage in the vegetal asters of the cleavage spindles. They fuse to a number of dark bodies very rich in RNA. At the 24-cell stage these bodies are shifted towards the central ends of the macromeres 3A–3D; at the next division they pass into the cells of the fourth quartet.

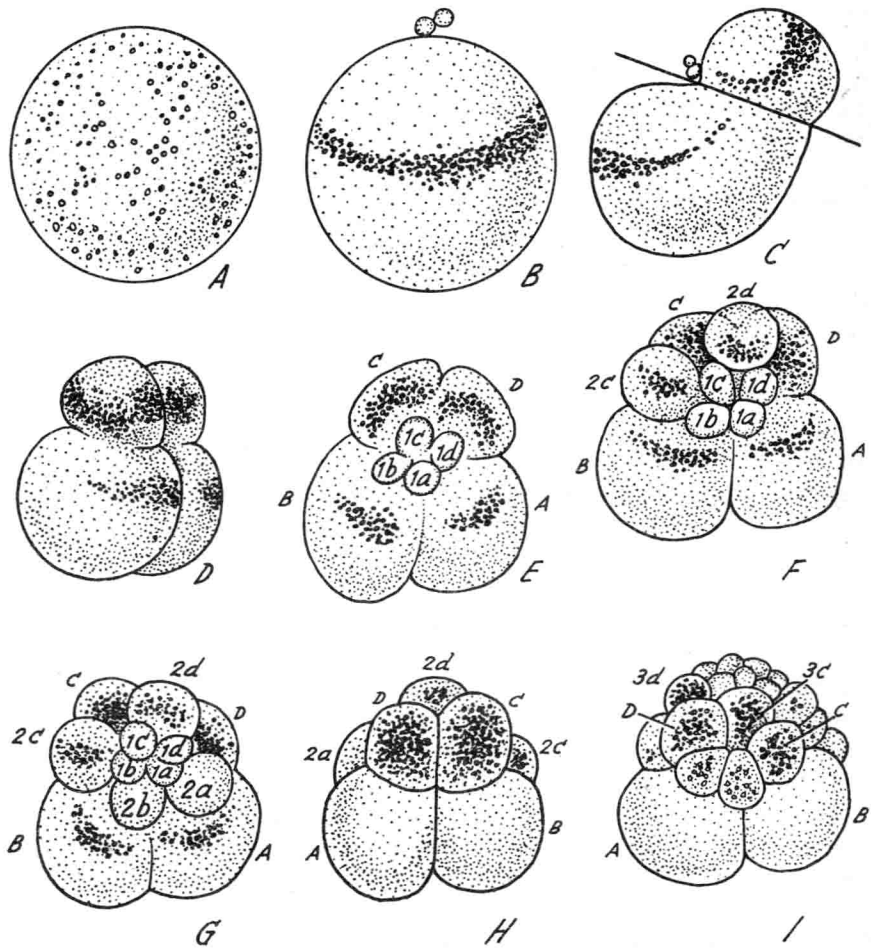

FIG. 2. Ooplasmic segregation in *Aplysia limacina*. Distribution of vitamin C (presumably bound to Golgi bodies) during early cleavage. *A*. Random distribution in immature egg. *B*. Annular concentration in mature egg. *C*. First cleavage. *D*. Four-cell stage. *E*. Eight-cell stage. *F*. Ten-cell stage. *G–H*. Twelve-cell stage. *I*. Somewhat later stage. After Ries, 1937.

In *Physa* (Mancuso, 1953) and *Succinea* (Jura, 1960) glycogen is at first more or less evenly distributed, but shifts during maturation to the vegetal part of the egg. At cleavage most of it passes into the macromeres; later it is found in the inner germ layer. In *Limnaea*, the cells 4a, 4b and 4c (but not 4d) are rich in glycogen. Their descendants form a horseshoe-shaped area surrounding the anterior border of the blastopore (Raven, 1946).

Collier (1960a) found in the polar lobe of *Ilyanassa* a concentration of

phospholipids about twice as high as in the rest of the egg; after its fusion with CD the concentration of phospholipids in the CD blastomere was 1·27 times greater than in the AB blastomere.

In *Limnaea* (Raven, 1945, 1946) and *Succinea* (Jura, 1960) the pole plasm substance is richer in RNA than the rest of the cytoplasm. Therefore the micromeres exhibit a stronger pyroninophily than the macromeres. In *Ilyanassa* (Collier, 1960b) the concentration of RNA is greater in AB than in CD.

Ascorbic acid in *Aplysia* is bound to the Golgi bodies, and shows the same segregation as the latter (Ries, 1937) (Fig. 2). In *Succinea* it shows at first a uniform distribution, then becomes concentrated in the animal pole plasm, and follows the latter in its distribution (Jura, 1960).

The benzidine peroxidase reaction in *Aplysia* is at first positive in the whole egg. With the vegetal concentration of the protein yolk, the reaction becomes restricted to the vegetal material. Its further distribution parallels that of the protein yolk, though the enzyme is not bound to the yolk granules (Ries, 1937, 1938).

Cytochrome oxidase in *Aplysia* is at first uniformly distributed, but then it concentrates in the zone of fat droplets in the animal half of the egg and passes mostly into the micromeres (Ries, 1937; Attardo, 1957). In *Bithynia* the enzyme is restricted to the animal pole plasm, and passes into the first and second micromeres (Attardo, 1955a). In *Physa* likewise the cytochrome oxidase is more abundant in the micromeres than in the vegetal part of the egg; later it is mainly found in the ectoderm (Mancuso, 1955b). Presumably in all these cases the enzyme is bound to the mitochondria, and follows their distribution.

Leucomethylene blue oxidoreductase in *Aplysia* is mainly found in the animal protoplasmic area of the uncleaved egg, then in CD. After the next cleavage the main activity is localized in C; finally, it is especially the micromeres of the C-quadrant that give the reaction (Ries and Gersch, 1936; Ries, 1937).

In the evaluation of these results, one of the inherent limitations of cytochemical methods has to be taken into account. There is often no strict proportionality between the concentration of the substance in question and the intensity of the reaction. Presumably a certain reaction does not, as a rule, occur at all when the intracellular concentration of the substance is too low. These methods therefore give an exaggerated picture of the differences in concentration. These differences generally are relative rather than absolute, the various cells differing in the proportions among several common substances. When this is borne in mind, however, the results of cytochemical observations can be used as a further clear illustration of the ooplasmic segregation taking place in these eggs.

III. The Importance of Ooplasmic Segregation

When one considers the extensive displacements and selective accumulations of cytoplasmic components during early development in gastropods, it is obvious that these processes are important for further development. It is evident that the determination of the cells, somehow or other, must be ultimately dependent upon the material composition of the cytoplasm. This composition, as we have seen, becomes divergent in different cells by a differential distribution of substances present in the egg.

A number of experiments support the view that ooplasmic segregation is important for cell determination.

In the first place we may mention the experiments on the morphogenetic role of the polar lobe in *Ilyanassa* (Crampton, 1896; Clement, 1952, 1956). As we have seen above (Section II), the substance of this lobe is first taken up in CD, and in the next cleavage in D. In later development the D-quadrant plays an important part; for instance, it produces the mesentoblast cell 4d, from which the primary mesoderm is derived.

When the AB and CD blastomeres are isolated at the 2-cell stage, both halves gastrulate and develop velar cilia, pigment, muscle tissue and a ciliated enteron. But whereas CD halves may develop such structures as shell, foot, operculum, otocysts, heart and eyes, these organs are entirely lacking in AB halves. After isolation at the 4-cell stage, A, B and C quarters produce partial larvae resembling those from AB; only D quarters develop foot, shell and eye.

Removal of the polar lobe at the first cleavage results in larvae having a disorganized velum and an indistinct dorsoventral polarity. There is no primary mesoderm, but mesenchyme and muscles, probably derived from ectomesoderm, may be present. The endoderm may show some differentiation, but shell, foot, otocysts, eyes and heart do not develop.

From these experiments the conclusion may be drawn that the presence of the polar lobe is necessary for normal development of the velum, and for the formation of the primary mesoderm. In the absence of the lobe substance, moreover, such organs as shell, foot, heart, otocysts and eyes do not develop; apparently the formation of these organs is dependent, directly or indirectly, on a normal distribution of ooplasmic substances among the cells by way of the polar lobe.

Further data on the importance of ooplasmic segregation for development results from centrifugation experiments. When eggs are centrifuged during early cleavage, the normal distribution of substances among the cells may be interfered with, and it may be expected that abnormal development will be the result. This is confirmed by the experiments.

If the eggs of *Physa* or *Limnaea* are centrifuged at the 2- or 4-cell stage, many abnormal embryos are produced (Clement, 1938; Raven and van Egmond, 1951). In *Limnaea* centrifuging immediately prior to third cleavage is much more harmful than at earlier or later stages. The effects are the more pronounced, the more closely centrifugation precedes third cleavage (Raven and Tates, 1961) (Fig. 3). This is

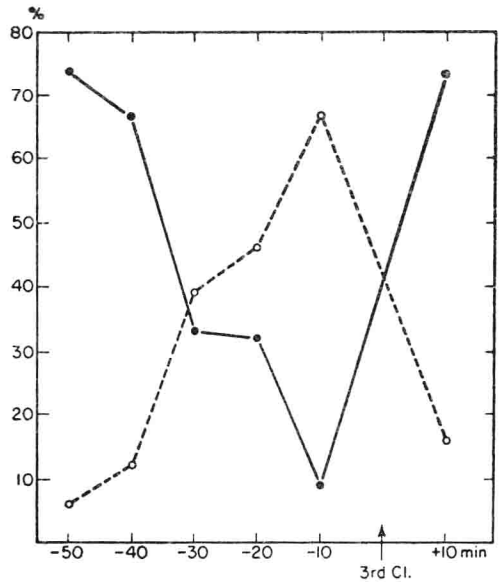

FIG. 3. Per cent normal embryos (solid line) and embryos with disturbed gastrulation (dashed line) in relation to time of centrifugation (50 min before to 10 min after 3rd cleavage) of eggs of *Limnaea stagnalis*.

probably due to the increase in segregation and the diminishing possibility of readjustment of the egg substances as the time interval decreases between centrifugation and third cleavage. It should be remembered that just at this stage extensive displacements of cytoplasmic substances take place in the normal egg, in preparation for the differential distribution of the pole plasm substance at third cleavage.

The deviations of development found in *Physa* and *Limnaea* after centrifugation at early cleavage stages are rather variable: exogastrulation, formation of bladder-like outgrowths at various places, eversion of the foregut, reductions, reduplications and dislocations of eyes and tentacles, malformations and dislocations of the shell, median splitting of the foot, inverse asymmetry (Clement, 1938; Raven and van Egmond, 1951; Raven and Koevoets, 1952; Paris, 1953; Raven and Beenakkers, 1955; Raven, 1958a; Raven and Tates, 1961). Raven and Beenakkers