

The Nature and Origin of the Cortical Morphogenetic Field in *Limnaea*

CHR. P. RAVEN

Zoological Laboratory, University of Utrecht, The Netherlands

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INTRODUCTION

In a recently delivered lecture Paul Weiss (1961) remarks: "I proposed long ago that the basis for the localization of the first differential steps in embryonic development is to be sought in a surface mosaic of areas with specifically different molecular populations, blocked out in the cortex of the egg"; and again, "There are major regional differences in the molecular composition of the egg surface, which, in being parceled out directly among the cleavage cells, leaves them, right from the start, with specifically different endowments in accordance with the particular parcel of egg surface each has acquired."

In our long-term analysis of the development of the pond snail, *Limnaea stagnalis*, we have been confronted at an early stage with the problem of the nature and structure of the cortical morphogenetic field. The results of our experiments on treatment of the eggs with lithium chloride (Raven, 1942), in which we obtained a roughly continuous series of cyclocephalic malformations, ranging from a slight mediad displacement of the eyes and tentacles, via synophthalmia, cyclopia, anophthalmia, to complete acephaly of the embryos, were interpreted by the assumption that the cortical field in *Limnaea* has the character of a gradient field with high point at the animal pole (Raven, 1949, 1952). Lithium, by depressing the apex of the gradient field, was supposed to suppress the differentiation of the most animal structures, the ciliary cells of the so-called apical plate. The cephalic plates, which in normal development arise at some distance on either side of the animal pole, now originate from cells lying nearer to this pole, eventually from a median cell group adjoining the animal pole.

With further intensification of the effect the cephalic plate differentiation is suppressed in its turn, leading to complete acephaly.

This hypothesis received further support when it appeared that a more or less opposite effect could be obtained by a heat-shock treatment of the eggs. Embryos with supernumerary eyes and tentacles in the dorsal midline of the head were produced by this treatment. This could be explained by the assumption that a strengthening of the gradient field, with subsequent reduplication of its apex, had taken place (Raven *et al.*, 1955).

Recent (unpublished) observations by our collaborator, N. H. Verdonk, have shown, however, that our original interpretation of the results is incorrect. Verdonk has made a detailed study of the cell lineage in normal and lithium-treated embryos of *Limnaea*. It appeared that the expected distortion of the head pattern after lithium treatment, the various primordia being outlined in the "wrong" places from the start, did not occur at all. The deviations from the normal course of development are of a much more subtle nature. Up to a certain stage, the cleavage mosaic in normal and lithium-treated embryos is identical. The first visible deviation is an abnormal direction of the cleavage spindle in one particular cell, lying at the base of the dorsal arm of the cross. Its daughter cells, which normally undergo no further divisions, in lithium embryos continue to divide, in this way giving rise to a transverse bridge of small cells connecting the two cephalic plates. Similar changes may occur in the lateral arms of the cross, some blastomeres undergoing more divisions, other cells fewer divisions, than in normal development. After heat-shock treatment the primary effects are of a similar nature, but located at other places.

These results can hardly be explained on the basis of a mere gradient-field hypothesis. The very localized effects of certain treatments, sometimes being confined to one or a few blastomeres, argue in favor of equally local variations in the cortical field. We therefore have come to the conclusion that this field has a mosaic character in the sense that there must be, besides continuous variations, also discontinuous, stepwise variations in the structure and properties of the cortex. In other words, our results have led us to a concept closely resembling the view advocated by Weiss.

In an attempt to obtain corroborating evidence for such a view, I have turned to a renewed study of ooplasmic segregation in uncleaved

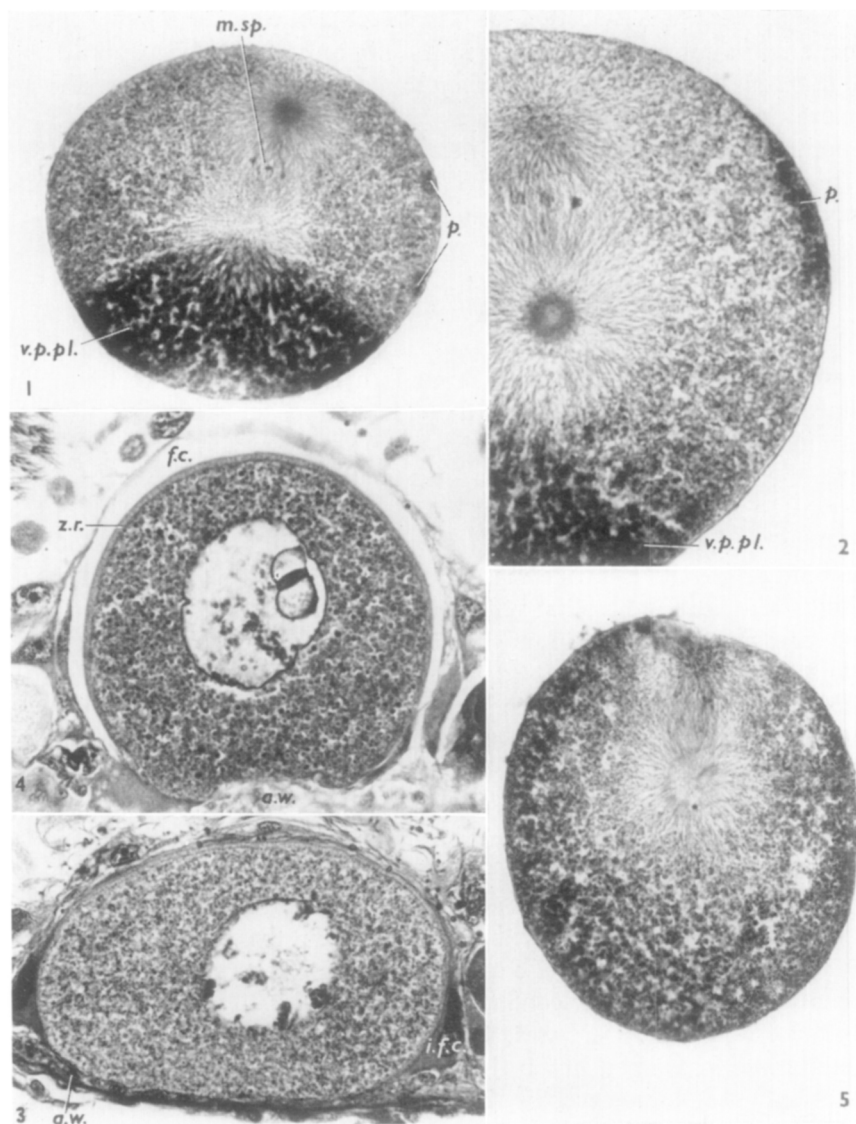


FIG. 1. Egg of *Limnaea stagnalis* fixed immediately after oviposition. Staining: azan. *m.sp.* first maturation spindle; *p.* "patches" of blue cytoplasm; *v.p.pl.* vegetative pole plasm. Magnification: $\times 525$.

FIG. 2. Same stage and staining as for Fig. 1. Longitudinal section through one of the "patches." Magnification: $\times 840$.

eggs. Furthermore, an attempt has been made to throw some light on the problem of the way in which the presumed cortical mosaic comes into being. In previous papers (Raven, 1958a, 1961) I have put forward the view that the structure of the cortical field reflects the mutual positions of the elements surrounding the egg cell in the gonad. The observations dealt with in this paper give further support to this supposition.

OOPASMIC SEGREGATION IN UNCLEAVED EGGS

In an earlier paper (Raven, 1945), I have described the distribution of cytoplasmic components in normal uncleaved eggs. In eggs fixed immediately after oviposition and stained with azan, two major portions of the cytoplasm can be distinguished (Fig. 1). At the vegetative pole a special plasm is situated, staining blue with azan. This vegetative pole plasm (called "ectoplasm" in my 1945 paper) in a meridional section occupies a sector of about 110 degrees having its apex near the center of the egg. It is demarcated by a sharp line from the rest of the egg, which is orange-red in azan-stained sections. The vegetative pole plasm contains a dense mass of β -granules of the protein yolk. They stain dark indigo blue with azan. At the vegetative pole there is a cap of finer granules, staining light grayish blue. The rest of the egg contains a few scattered blue β -granules, but is rather characterized by its abundance of orange-red α -granules (mitochondria) and large, deep red γ -granules. The difference in staining between the two regions is due not only to the granules, however, but also involves the intergranular ground substance of the cytoplasm. When the deep aster of the first maturation spindle is situated at the boundary between the two plasms, it shows a sectorial coloration: that part of the aster that is situated within the vegetative pole plasm is blue, the rest is orange red; the line of demarcation between the differently colored parts within the aster is as sharp as it is elsewhere.

FIG. 3. Growing oocyte in the gonad. Staining: azan. *a.w.* connective tissue wall of acinus. *i.f.c.* inner follicle cell. Magnification: $\times 490$.

FIG. 4. Full-grown oocyte in the gonad. Staining: azan. *a.w.* acinus wall; *f.c.* follicular cavity; *z.r.* zona radiata. Magnification: $\times 470$.

FIG. 5. Egg fixed about 1 hour after oviposition, shortly before extrusion of first polar body. Vegetative pole plasm has spread beneath egg cortex toward animal pole. Staining: azan. Magnification: $\times 525$.

A reinvestigation of eggs at this stage brought to light some additional details that previously had escaped observation. In the first place, it appeared that in all eggs the position of the vegetative pole plasm is not strictly symmetric with respect to the longitudinal direction of the first maturation spindle. If one assumes that the latter coincides with the main animal-vegetative axis of the egg, the vegetative pole plasm is situated somewhat obliquely with respect to this axis. If, on the other hand, it is assumed that the center of the vegetative pole plasm represents the vegetative end of the main egg axis, it follows that the first maturation spindle at this stage regularly makes a certain angle with this axis.

In the equatorial zone of the eggs, there are immediately beneath the cortex some small lenticular patches of cytoplasm with about the same staining properties as the vegetative pole plasm (Figs. 1 and 2). However, the granules in these regions are, on an average, somewhat smaller than the β -granules in the vegetative pole plasm. Though I had noticed these "patches" at the time of my earlier investigation (Raven, 1945), I then paid no further attention to them, thinking that they were due to slight irregularities in staining. During my reinvestigation of the eggs, however, I was struck by their regular occurrence. This was substantiated by making a number of wax reconstructions of eggs at this stage. They showed that there are generally six of these "patches." They are not evenly spaced around the equator of the egg, but distributed more or less asymmetrically. Four or five of them are to be found on that side of the egg where the boundary of the vegetative pole plasm is highest, one or two on the opposite side. Moreover, some of these "patches" are situated nearer the animal pole than other ones (Fig. 6). Altogether, a certain pattern in their distribution is clearly indicated, though there are slight differences between individual eggs. This pattern, together with the obliquity of the vegetative pole plasm (or maturation spindle) suggests a certain dorsoventrality of the egg structure. On the other hand, the pattern is not strictly bilaterally symmetric, but asymmetric.

In a full-grown oocyte in the gonad, ready to ovulate, neither the vegetative pole plasm nor the equatorial ring of subcortical "patches" are visible. Its cytoplasm is filled with numerous granules of different size and stainability. In azan-stained sections, blue, red, and orange-red granules can be distinguished. As they are distributed almost at random throughout the cell (though a basoapical gradient in their

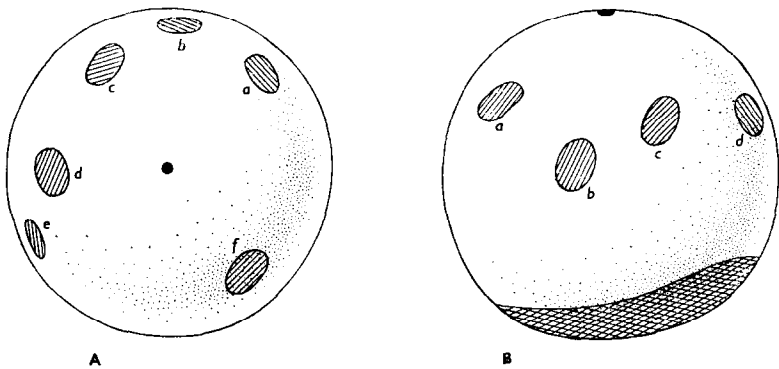


FIG. 6. Diagram of *Limnaea* egg immediately after oviposition (stage of Figs. 1 and 2). A. From the animal pole. B. From the side. Black dot: animal end of first maturation spindle. Crosshatched: surface area of vegetative pole plasm; a-f: subcortical "patches" of different staining.

density can be observed), the latter shows a uniform violet-red staining at low magnifications (Fig. 4). Apparently, the ooplasmic segregation giving rise to the cytoplasmic differentiations of the recently laid egg takes place during the passage of the eggs through the genital duct of the parent, which takes about 3-5 hours (Bretschneider, 1948), by a local accumulation of cytoplasmic constituents which were almost uniformly distributed in the oocyte. This has been confirmed by the study of some egg samples taken from various parts of the genital tract. They indicate that a gradual separation of the granular inclusions of different staining takes place as the egg passes down the genital tract. The boundaries between regions of different staining become progressively more distinct. A well-formed vegetative pole plasm has not been observed until the eggs have reached the pars nidamentaria of the oviduct.

It is evident, therefore, that the characteristic distribution of cytoplasmic constituents found in the recently laid egg comes about by the selective accumulation of particular components of the cytoplasm beneath certain parts of the cortex. Hence, this distribution probably reflects local differences in the properties of the cortex, or, in other words, makes visible certain aspects of the presumed cortical mosaic which itself is invisible, being based on differences in molecular composition of the plasma membrane. I may emphasize in this connection that the visible pattern of cytoplasmic differentiations agrees

with the hypothetical morphogenetic field to be assumed in gastropods in that it is at the same time polar, dorsoventral, and asymmetric (cf. Raven, 1958b, p. 194).

We have of course tried to relate this pattern with particular cellular determinations at a later stage, but have not yet succeeded in doing so. The distribution of the six equatorial "patches" is vaguely reminiscent of certain particulars of the cleavage mosaic; it might have something to do with the fate of particular cells of the molluscan cross. But it is not possible to follow up the cortical regions in question throughout early cleavage. The vegetative pole plasm already in uncleaved eggs begins to extend beneath the cortex toward the animal pole (Fig. 5), and in so doing overflows the equatorial "patches," which further on are no longer distinguishable. We are now trying to characterize the early cytoplasmic differentiations by means of cytochemical methods. It is possible that these methods will enable us to follow their further fate.

THE ORIGIN OF THE CORTICAL MOSAIC

The selective accumulation of particular cytoplasmic components beneath certain parts of the egg cortex during the passage of the eggs through the genital duct points to a preexistent mosaic pattern in the cortex. This raises the question of the origin of this pattern. If it exists at the very beginning of embryonic development, it must have been established in an earlier period, i.e., during the formation of the egg cell in the gonad.

The hermaphroditic gonad of *Limnaea* consists of numerous acini enclosed within the hepatic gland. The acini are arranged in a cluster around the spermoviduct. They are often branched, and they open by means of short vasa efferentia into the spermoviduct.

The wall of the acini and efferent ducts consists of an outer fibrous layer of connective tissue with small nuclei. On the side of the lumen it is covered in the efferent ducts with a cuboid epithelium of ciliated cells. This extends into the proximal third of the acinus proper, but it is greatly flattened here. The distal two thirds of the acinus are lined on their inside with the germinal epithelium. This consists of two regions: (1) the oogenetic zone, occupying the most apical part of the acinus, and encircled on all sides by (2) the spermatogenetic zone, in which the various stages of spermatogenesis follow each other in a

proximodistal direction, the oldest stages (Sertoli cells with sperm bundles) being found adjacent to the oogenetic zone.

In the oogenetic zone younger and older oocytes lie side by side without any apparent regularity. The early oocytes show signs of ameboid mobility (Bretschneider and Raven, 1951); presumably, they move about, but do not leave the oogenetic zone in which they originated. Then they become sessile and a follicle is formed around them.

The growing oocytes lie with one side flat against the connective tissue wall of the gonad. The rest of the oocyte protrudes into the lumen and is surrounded by a follicle. This consists more or less clearly of two layers.

The inner layer of the follicle consists of cells, which are more or less wedge shaped in cross section (Fig. 3). They are attached with their wider basal part to the fibrous layer of the acinus wall around the oocyte, probably by means of micropodia penetrating into this layer (Recourt, 1961). The rest of the cell curves smoothly round the side of the oocyte toward its inner pole. The nucleus is large and flat, and often it lies close to the surface of the oocyte. The peripheral parts of the follicle cells are very thin and membranous and are difficult to trace in light-microscopic sections. Electron-microscopic observations have shown, however, that the borders of adjacent follicle cells probably meet and, by mutual interpenetration of folds, form imbricated complexes of double membranes (Recourt, 1961).

Originally the plasma membranes of the oocyte and of the inner surface of the follicle cells are smooth and closely applied against each other. In older follicles, however, a cleft appears between the oocyte and follicle cells, first at the apex, then gradually extending toward the base of the oocyte (Fig. 4). At the same time, microvilli are formed on the surface of the oocyte (Recourt, 1961).

The outer layer of the follicle is composed of cells which partly resemble those of the inner layer, partly are slightly different. It is rather difficult, however, clearly to delimit this layer from the surrounding cells of the gonad or from adjacent follicles. Although there are indications that it has a more or less regular structure, it is hard to make sure of this. Therefore, I will leave it out of further consideration, restricting myself to the cells of the inner follicle layer.

Apparently, after the ameboid oocyte has become sessile, the inner

follicle layer is gradually built up by recruitment of neighboring cells from the germinal epithelium. In a small oocyte still having an irregular outline I found only two of these cells. Slightly larger oocytes had four or five cells in the inner follicle epithelium. As soon as the number of six inner follicle cells has been attained, however, further recruitment stops. All larger oocytes have six cells in the inner follicle layer. Only once did I find an exceptionally large oocyte with seven inner follicle cells.

But not only is the number of these cells remarkably constant, the same holds for their arrangement. In order to test this, I made wax reconstructions of a large number of oocytes, on which I marked by different colors (1) that part of the surface of the oocyte lying against the basal membrane of the acinus wall, (2) the position of the nuclei of the inner follicle cells, and (3) the extension of the thicker central regions of their cell bodies, excluding their membranous peripheral portions.

The earliest sessile oocytes are rather flattened structures, about the shape of a segment of a sphere. Their flat side lying against the acinus wall takes about 40% of their surface. With further growth they bulge forth into the lumen, and that part with which they are applied to the acinus wall is gradually reduced to about 15–20% of their surface. Only immediately before ovulation a further reduction of this surface of attachment occurs until they become entirely free.

In all oocytes exceeding a certain size there are six inner follicle cells arranged in a characteristic pattern around the oocyte (Fig. 7). Looking from the free inner pole of the oocyte, there are on one side three follicle cells lying near together, their compact cell bodies immediately adjoining (cells 1–3). As a rule the middle of the three (2) is somewhat narrower than its neighbors. At a small distance in a counterclockwise direction, there is a fourth cell (4). As a rule, there is a clear gap between this cell and the left element of the first-mentioned triplet (3) (this gap is probably bridged by the membranous peripheral cell parts left out of the reconstruction), but the two cells may meet in their basal parts near the basal membrane. Together, these four cells occupy about 180 degrees of the circumference of the oocyte. In the other half of this circumference, one finds two more follicle cells (5 and 6). They are separated by wide gaps both from one another and from the adjacent cells (again disregarding their thin peripheral parts). In the one exceptional follicle

with seven cells, these two were replaced by three cells in a similar position.

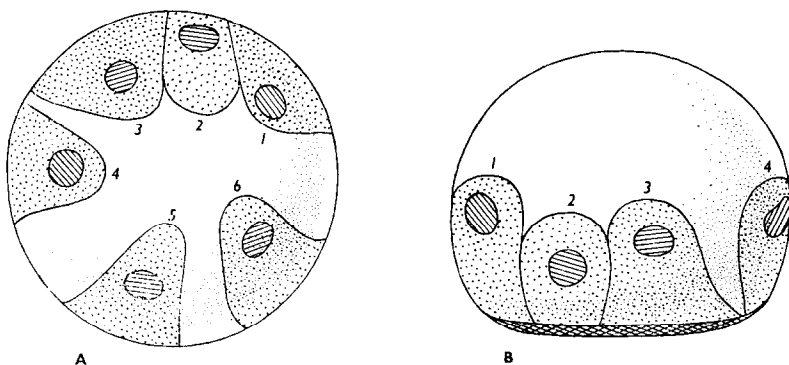


FIG. 7. Diagram of arrangement of the inner follicle cells around oocyte. A. From inner pole of oocyte. B. From the side. Crosshatched: area of contact with gonad wall; 1-6: inner follicle cells.

It must be emphasized that the observation of this pattern is no simple matter. It is based on very subtle differences in the mutual positions of the cells, which can be established only by means of accurate reconstructions. Even then the normal variability inherent in all biological structures makes it impossible to arrive at more accurate statements, e.g., in terms of angular distances between the elements. But my observations have convinced me that the above description of the structure of the inner follicle layer is essentially correct and can be applied to at least the great majority of older follicles in *Limnaea stagnalis*.

Considering the oocyte with its surrounding structures as a whole, it is clear that it forms part of an ordered pattern. This pattern is structured along three perpendicular directions. On the one hand, the opposition of the convex free part of the oocyte to its flattened basal surface determines a main axis. Secondly, the contrast between one half of the follicle with four closely crowded follicle cells and the other half with two widely spaced cells determines a second axis perpendicular to the first, which may tentatively be indicated as dorsoventral. Finally, the fact that the plane passing through these two axes does not represent a plane of bilateral symmetry, means that the structure in question also exhibits a left-right asymmetry.

DISCUSSION

Our observations have resulted in the conclusion that both the distribution of cytoplasmic constituents in the recently laid egg of *Limnaea*, and the position of the oocyte with its surrounding structures in the gonad, give evidence of an ordered pattern, which is at the same time polar, dorsoventral, and asymmetric. It is obvious to relate these two statements to each other, and to assume that the one pattern is a consequence of the other.

With respect to the polar axis of the two systems, there are strong arguments in favor of such a conclusion. Though there is no direct proof of it in *Limnaea*, a comparison with the relationships in other mollusks makes it probable that the axis connecting the free pole of the oocyte with the center of its attached basal surface corresponds to the later animal-vegetative axis of the fertilized egg, the free surface becoming the animal side, the attached surface the vegetative side of the egg.

If this be true, however, a further coordination between the two patterns can be established. It seems obvious in this case to homologize that part of the egg surface beneath which the vegetative pole plasm accumulates during the passage of the egg through the female duct, with the region formerly applied to the wall of the acinus. The extension of the two regions is about the same, as is their localization. Apparently, this part of the cortex is endowed, by its close association with the connective tissue wall and basement membrane of the acinus, with special properties differing from those of the remaining part of the surface. When ovulation occurs and the egg cell leaves the gonad and is fertilized in the spermoviduct, then becomes bathed by the secretions of the accessory glands in the oviduct, a thorough remodeling of the egg cytoplasm takes place, during which some of its constituents become heaped up in the egg sector covered by the different cortex, forming the vegetative pole plasm.

A similar explanation holds with respect to the equatorial "patches" of the oviposited egg. It hardly seems doubtful that these six "patches" can be related to the six inner follicle cells surrounding the oocyte. The configurations of these "patches," on the one hand, and of the follicle cells, particularly their nuclei, on the other, are pretty well superimposable, especially if one takes account of the rounding off of the egg cell which has taken place in the meantime. If one com-

compares the egg of Fig. 6 with the follicle of Fig. 7, the "patches" *a*, *b*, *c*, and *d* show a position similar to the nuclei of follicle cells 1, 2, 3, and 4. The "patches" *f*, and especially *e*, in this egg apparently do not exactly correspond to the positions of the nuclei of cells 6 and 5 in Fig. 7, but appear to be slightly shifted in a clockwise direction with respect to them. It seems doubtful, however, that this deviation is significant, as it is not found in all eggs. Anyhow, it hardly detracts from the striking similarity of the two configurations. The size of the "patches" also agrees well with the area of egg surface covered by a follicle cell nucleus. It is the simplest explanation also in this case to assume that the egg cortex facing the follicle cell nuclei has acquired special properties differing from those of other regions and, in consequence, has attracted or captured particular cytoplasmic constituents during the general rearrangement following ovulation and fertilization.

In some earlier papers (Raven, 1958a, 1958b, and 1961) I have put forward the view that the pattern of the cortical field is "imprinted" upon the egg during oogenesis by the surrounding structures and have adduced circumstantial evidence in support of such a view. This evidence mainly concerned the probability that the main directions of the field are established in conformity with the position of the egg in the gonad. The above observations in my opinion lend further support to this view, and in one respect give a valuable extension to it. As far as I know it is the first instance in which details of the cortical pattern of the fertilized egg have been shown to reflect peculiarities in the mutual positions of elements surrounding the oocyte.

It seems to me that this observation, if it can be confirmed in other species, is of considerable theoretical importance. As the future spatial pattern of the embryo is to a large extent dependent on the "blue-print information" in the cortex of the egg (cf. Raven, 1958a), it appears that the prodromes of this pattern are transmitted from the parent to the offspring through this channel.

Of course one can now go one step further back and ask in what way the structure of the egg follicle is established. This is a question that cannot be answered in detail at this moment. But since the follicle may be considered as a part of the adult organism, it is obvious that the establishment of its pattern has to be understood on the same lines as that of any other adult structure. In other words: the pattern of the egg follicle presumably arises "epigenetically" by the inter-

action of its constituent parts, the gonad wall, germinal epithelium cells, and oocyte, each of them taking part in this development by virtue of its own cell-specific properties. The special importance of this particular developmental process derives from its role as a link in the transmission of information from parent to offspring (Raven, 1961). This demonstrates once more the hardly extricable intertwinement of epigenetic and preformationist aspects in development.

SUMMARY

The vegetative pole plasm in recently laid egg cells of *Limnaea stagnalis* is situated somewhat obliquely with respect to the longitudinal axis of the first maturation spindle. In the equatorial zone of the egg there are six subcortical "patches" of cytoplasm staining differently from the surrounding cytoplasm. They are arranged according to an asymmetric pattern. These cytoplasmic differentiations of the oviposited egg arise by ooplasmic segregation during the passage of the egg cell through the female genital duct of the parent. They point to a preexistent mosaic pattern in the cortex, which is at the same time polar, dorsoventral, and asymmetric.

The growing oocytes in the gonad lie with one side flat against the acinus wall. They are surrounded by six inner follicle cells in a characteristic arrangement. The oocyte with its surrounding structures also embodies a pattern which is at the same time polar, dorsoventral, and asymmetric.

The patterns of the egg follicle and of the oviposited egg show a great deal of similarity. It is concluded that the mosaic pattern of the cortex of the fertilized egg cell reflects peculiarities in the mutual positions of elements surrounding the oocyte.

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