The Distribution of Special Cytoplasmic Differentiations of the Egg during Early Cleavage in *Limnaea stagnalis*

Chr. P. Raven

Zoological Laboratory, University of Utrecht, The Netherlands

INTRODUCTION

In an earlier paper (Raven, 1963), I described the distribution of cytoplasmic components in newly laid eggs of *Limnaea stagnalis*. At the vegetative pole of eggs fixed immediately after oviposition there is a vegetative pole plasm, occupying a sector of about 110 degrees with its apex near the center of the egg. It contains a dense mass of β-granules of the protein yolk, in contradistinction to the rest of the egg, which contains mainly α- and γ-granules. The vegetative pole plasm is situated somewhat obliquely with respect to the longitudinal axis of the first maturation spindle.

In the equatorial region of the eggs, there is a circle of six subcortical "patches" of cytoplasm whose staining differs from that of the surrounding cytoplasm. These "patches," which will further on be denoted by the term "subcortical accumulations" (SCA), are not evenly spaced around the equator of the egg. Four or five of them are to be found on that side where the boundary of the vegetative pole plasm is highest, one or two on the opposite side. Some of them are situated nearer the animal pole than others. Altogether, the SCA, together with the obliquity of the vegetative pole plasm with respect to the maturation spindle, define a pattern which is at the same time polar, dorsoventral, and asymmetric.

This pattern of cytoplasmic differentiations of the oviposited egg arises by ooplasmic segregation during the passage of the egg cell through the female genital tract of the parent. It was argued that it probably reflects a preexistent mosaic pattern in the egg cortex. Since the arrangement of the SCA of the oviposited egg shows a great resemblance to the configuration of the follicle cells surrounding the oocyte in the gonad, while the position of the vegetative pole plasm

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corresponds to the part of the egg surface formerly applied to the gonad wall, this cortical pattern probably arises during oogenesis by interactions between the oocyte and the surrounding structures of the gonad. The hypothesis was put forward that the "blue-print information" (Raven, 1958) in the egg cortex is transmitted from the parent to the offspring by way of the follicle, whose structure is, so to speak, "imprinted" upon the egg during oogenesis.

If, on the one hand, the visible cytoplasmic differentiations of the newly laid egg cell reflect a mosaic pattern of the invisible, molecular structure of the egg cortex, and, on the other hand, this cortical pattern is the carrier of the "blue-print information," necessary for the establishment of the spatial structure of the future embryo (Raven, 1958), it follows that these cytoplasmic differentiations must, in some way or other, be related to the ultimate pattern of cellular differentiations of the embryo. At the least, one may expect that there are fixed relationships between the pattern of cytoplasmic differentiations and the axes of symmetry of the later embryo.

In my paper of 1963, I stated that it is not possible to follow up the SCA throughout early cleavage, as they are already in the uncleaved egg overlaid by the vegetative pole plasm extending beneath the cortex toward the animal pole, and in this way become indistinguishable. A later study of numerous eggs, both at the uncleaved stage and during early cleavage, has demonstrated, however, that, contrary to my previous statement, the SCA remain recognizable, although with some difficulty, not only in azan-stained sections, but also in slides stained with iron hematoxylin and eosin. This enabled me to study the relationship between the pattern of cytoplasmic differentiations of the uncleaved egg and the axes of the future embryo.

MATERIALS AND METHODS

A preliminary study was made of 58 eggs from various batches, ranging in age from immediately after laying to a late 8-cell stage. Most of them had been sectioned at 7.5 μ and stained either with azan or with iron hematoxylin and eosin.

When it appeared that the SCA not only could be recognized in these eggs, but that they seemed to be displaced in a regular way during early cleavage, it was decided to study their evolution through a closely spaced series of stages, each represented by a sufficient number of eggs. To this end, 5 egg masses were used. Samples of eggs were fixed in Bouin's fluid at intervals of 10 minutes. With the first

egg mass, fixation began immediately after laying; with the second, 1 hour after oviposition, and so on, the last sample of the fifth egg mass being fixed about 30 minutes after the third cleavage. The eggs were cut into sections of 6 μ, special care being taken that no sections got lost. They were stained with iron hematoxylin and eosin.

Altogether, this series consists of 225 complete eggs. As the eggs belonging to one sample show slight differences in age, and, moreover, samples from different egg masses overlap, the eggs were regrouped according to the stage of development reached. Table 1 gives a survey of the groups distinguished and of the number of eggs belonging to each group.

TABLE 1

SURVEY OF EGGS STUDIED

Group No.	Stage of development	Number of eggs
1	Metaphase of 1st maturation division	11
2	Anaphase of 1st maturation division	16
3	Extrusion of 1st polar body	9
4	Telophase of 1st maturation division; formation of 2nd maturation spindle	11
5	Prometaphase of 2nd maturation division	12
6	Metaphase of 2nd maturation division	9
7	Late anaphase of 2nd maturation division; extrusion of 2nd polar body	6
8	Karyomere stage	19
9	Fusion of karyomeres to prophase of 1st cleavage	10
10	Prometaphase to anaphase of 1st cleavage	13
11	Stages 1–5[a]; early 2-cell stage	9
12	Stage 6; middle 2-cell stage	7
13	Stage 7; middle 2-cell stage	13
14	Stage 8; late 2-cell stage	10
15	Stage 9; late 2-cell stage	9
16	Stage 9/10; immediately preceding 2nd cleavage	9
17	Stages 11–13; early 4-cell stage	13
18	Stage 14; late 4-cell stage	13
19	Stage 15; late 4-cell stage	10
20	Stage 16; early 8-cell stage	7
21	Stages 17–19; later 8-cell stage	9
		225

[a] Stages 1–19 correspond to the photographs in Raven (1946a, Fig. 1).

In order to study the positions and displacements of the SCA, graphical reconstructions were made of all eggs. The outlines of all sections of an egg were drawn by means of a drawing prism, and the parts of the egg surface occupied by the SCA were established under oil immersion and marked in the outline drawings. As the eggs had been sectioned in arbitrary directions (orientation of these small eggs in paraffin before sectioning being hardly possible), it was necessary, in order to be able to compare different eggs, to transpose the SCA positions observed to a standard plane. To this end, these positions were projected, by means of a graphical method, onto an idealized equatorial plane of the egg. In the final figures, the parts of the SCA extending into the vegetal hemisphere are reproduced as they would be seen by looking at the egg from the direction of the vegetative pole (Fig. 1).

If one compares the graphical reconstructions from eggs of a same age group, it appears that they resemble each other in essential points, but, on the other hand, show a great deal of variation. For instance, all uncleaved eggs of groups 1 to 8 have six SCA arranged in an about equatorial girdle. But their sizes, shapes, distances apart, and positions with respect to the equator and the vegetative pole vary considerably from egg to egg. These differences among the eggs are perhaps partly due to the normal variability inherent in all biological structures. However, it can hardly be doubted that they are to a great extent to be ascribed to errors in the procedure employed. The following sources of error may be mentioned: (1) distortions of the eggs during fixation and sectioning; (2) errors in drawing, and in marking the positions of SCA in the drawings (as we will see, the boundaries of the SCA are often not very sharp, and arbitrary decisions have to be taken in settling them); (3) errors in the determination of the equatorial plane (this is not distinguishable as such in the egg, and has to be deduced from the position of the animal pole, which is itself not clearly marked at all stages); (4) errors in the projection of the SCA positions upon this plane. This last source of error is probably the most important one, particularly as in this procedure all eggs had to be treated as perfect spheres.

Fortunately, notwithstanding the uncertainties arising in this way, the eggs belonging to the same group showed sufficient agreement so that they could be compared. This made it possible to get rid of the greatest part of the variability by calculating the median configuration

of each group. To this end, it was necessary to bring all eggs of a group into a corresponding position. In the cleaved eggs of groups 11–21, the position of the cleavage furrows provided sufficient land-marks to do this. In the uncleaved eggs, however, a common system of reference had to be established. It was a lucky circumstance that in nearly all eggs of groups 1–10 the pattern of SCA already indicated in my previous paper (1963) was clearly recognizable: on one side of the vegetative pole there were 4 SCA, together occupying about 180° of

Fig. 1. Egg N 1–3, metaphase of first maturation division. Diagram of vegetal hemisphere; vegetative pole in center. *a–f*, the six SCA. Measuring points on out-line of SCA are indicated.

the egg circumference; on the opposite side there were only two of them, rather far apart and usually larger than the 4 on the other side (Fig. 1). The SCA were denoted by the letters *a* to *f*, beginning with the left one of the group of four, and continuing in a clockwise direction (in Fig. 6 of my 1963 paper, this notation runs in a counter-clockwise direction, but there the egg is viewed from the animal pole).

Now a system of common meridians could be drawn. As the starting
point, I chose a point that could as a rule easily be located, lying on
the egg equator (i.e., the circumference of the projection figures)
halfway between SCA *a* and *b*. The meridian of 0° was drawn through
this point, the other meridians following in a clockwise direction
(Fig. 1). Now each point in the field could be characterized by two
variables: its longitude, and its radial distance from the circumference
of the projection (from the latter value its real latitude could have
been calculated if necessary, but for the sake of simplicity the distance
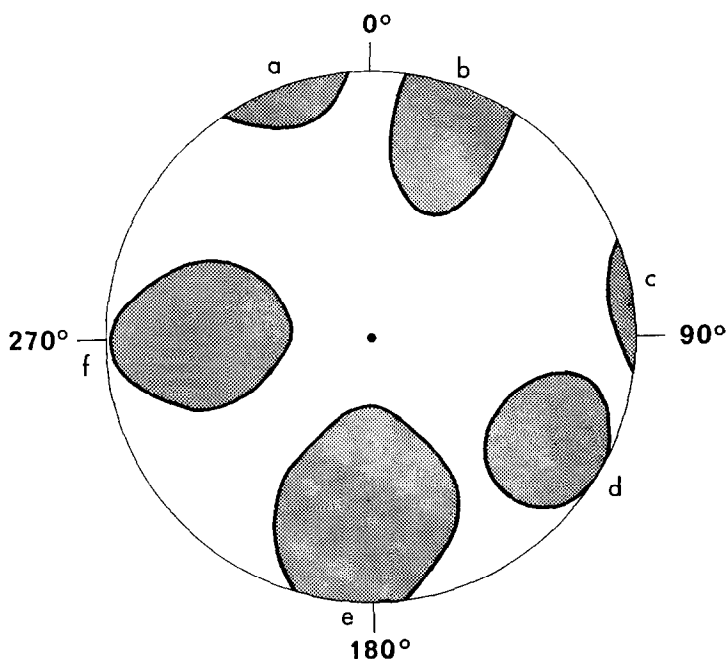measured in the projection was used as such).

FIG. 2.   Median projection of group 1 (11 eggs). Metaphase of first maturation
division.

The position of each SCA was now determined for each egg of a
group by measuring these "coordinates" at four extreme points of its
outline: inner, outer, left, and right; the latter two points are the
points of contact with tangent meridians. These points are indicated in
Fig. 1. In a group with *n* eggs, for every measuring point therefore *n*

longitude and *n* "latitude" values were obtained. The median of every *n* numbers was taken as a group characteristic (the median was preferred above the arithmetic mean, in order to minimize the influence of greatly deviating values). From these median values a "median projection" was constructed, which was considered to characterize the group as a whole (Fig. 2) (it must be noted that the term "median projection," as used in this paper, does *not* mean "projection on a median plane"). The great resemblance among the "median projections" of successive groups obtained in this way is a proof of the reliability of the method.
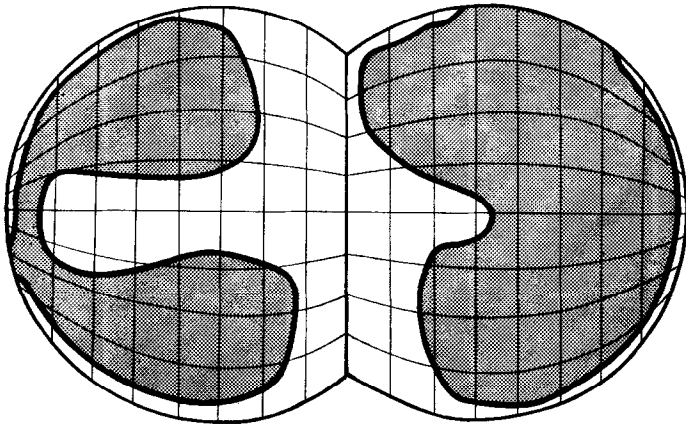

FIG. 3.   Egg N 62–3, stage 6. Diagram of vegetal hemisphere. Measuring-grid indicated.

For eggs in the 2-cell stage (groups 11–16), another procedure had to be adopted. Each projection figure of an egg was covered by a grid of intercrossing lines, adapted to the changing outline of the cells at successive stages (Fig. 3). For each of the 149 points of intersection it was noted whether it fell within or outside the outlines of SCA. In the "median projection" of a group those points, which in at least half of the cases were covered by an SCA, were surrounded by common outlines. The difficulty that each egg could be combined with any other in two opposite positions, in most cases could be solved owing to obvious differences in the distribution of SCA in the two blastomeres.

Finally, in 4- and 8-celled eggs (groups 17–21) the positions of the interblastomeric furrows and of the vegetative cross furrow provided

sufficient reference points for comparison. As in 2-cell stages, clear differences in the localization of SCA in different cells as a rule made it easy to determine in what way the eggs had to be combined.

OBSERVATIONS

Cytology of the SCA

Generally, the SCA, apart from certain exceptionally favorable cases, are not easily recognizable. The fact that we have discovered them only after having studied sections of *Limnaea* eggs for more than 25 years, is significant in this respect. Only if one is thoroughly acquainted with the material, and knows where to look, can he with any degree of accuracy determine their localization and their displacement throughout early development.

In all stages considered here, the SCA occupy more or less lens-shaped regions immediately beneath the plasma membrane in the equatorial and vegetative regions of the egg (Figs. 4 and 6). Their boundary against the internal cytoplasm, although rather sharp, is often more or less irregular owing to the fact that the vacuoles surrounding γ-granules in places indent this boundary. Sometimes these vacuoles may even nearly extend to the plasma membrane, so that an SCA in a section (Fig. 5) appears divided into two or more parts; comparison with the adjacent sections then shows the continuity between these parts. Toward the periphery the SCA often thin out and away beneath the plasma membrane (Fig. 7); in those cases the establishment of their peripheral boundary is rather difficult. Elsewhere, however, they may end rather abruptly, often bounded by a γ-vacuole (Fig. 6).

The main characteristic of the SCA is the fact that they consist of a "dense" cytoplasmic matrix with a great affinity for cytoplasmic stains. This makes them visible even with rather low magnifications, at least when the general staining of the sections is not too heavy. However, it does not suffice to characterize them, since other cytoplasmic differentiations, for example, the animal pole plasm, have the same property.

I mentioned already in my 1963 paper that the SCA contain granules that are smaller than the ordinary yolk granules (β-granules). As a rule these granules are densely packed together in the SCA. In iron hematoxylin-eosin preparations they are either red or blue black, depending on variations in the staining procedure. In azan preparations

they are blue, often more or less slate blue, and it is evident that they represent a kind of granule that is either sporadic or lacking altogether in the rest of the cytoplasm. They are apparently not mitochondria, as these have other staining properties. Originally, these special granules make up the bulk of the granular contents of the SCA; during cleavage stages, however, more and more β-granules appear among them.

A further component of the SCA is represented by rather coarse fibrils or lamellae. Until now we mainly observed them in uncleaved eggs. At some stages they appear to be arranged more or less parallel to the surface, in wavy and slightly intertwined bundles. However, at other stages (especially during the formation of the second maturation spindle, and at metaphase-anaphase of this division) one finds more or less lens-shaped bodies of coiled threads, which occupy nearly the whole of the SCA. At the same time the small granules present at other stages are lacking in the SCA. The most plausible explanation seems to be that there is some genetic relationship between the granules and fibrils (or lamellae), one being transformed into the other and back, at certain stages of the cell cycle. But it is clear that the real nature of these "granules" and "lamellae," and of the connection between the two, can be elucidated only by electron microscopy. I may add that similar knots of coiled fibrils or lamellae can at the same stages be observed in the immediate neighborhood of the maturation spindle. Whether they represent a similar kind of structure as that found in the SCA remains to be established.

As regards cytochemical characteristics of the SCA, we have made some observations indicating that they are rather rich in RNA and in lipids, but further investigations in this direction are needed.

Localization of the SCA at Various Stages

Figure 1 shows the graphical reconstruction of the vegetal hemisphere of one of the eggs belonging to group 1, which had been fixed immediately after oviposition at the metaphase of the first maturation division. Figure 2 gives the corresponding "median projection" of the 11 eggs of this group.

In the center of this figure we see the vegetative pole of the egg, while the circular outline represents the egg equator. The six SCA, *a–f*, are arranged in a circle around the vegetative pole. Four of them, *a–d*, are smaller and nearer together, occupying about 180 degrees of the circumference of the egg (situated between the meridians 325°

and 145°). Of these, *a* and *c* extend only partly into the vegetal hemisphere, their main area lying above the equator; *b* reaches farther down, while *d* lies almost entirely in the vegetal hemisphere. The other two SCA, *e* and *f*, are larger and farther apart; *e* is localized for the greater part, *f* is entirely in the vegetal hemisphere.

If we compare with the configuration of Fig. 2 the "median projections" of groups 2–8, we find the same pattern in all of them. Small variations among the groups in sizes, shapes, positions, and distances
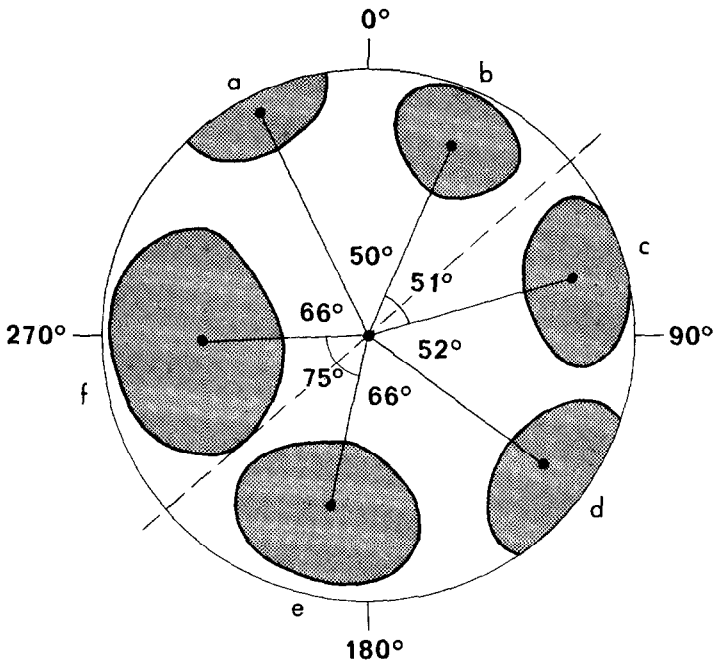
Fig. 8. Median projection of the 93 eggs of groups 1–8, ranging from metaphase of first maturation division to the karyomere stage. The interrupted line indicates the approximate plane of symmetry of the pattern.

of the SCA occur, but they show no general trend and are probably not significant. Partly they may represent a remainder of the variance not yet eliminated by our averaging procedure, partly they may be due to slight changes in shape of the egg in the course of the maturation divisions. However this may be, it is evident that during the uncleaved stage, from oviposition to the karyomere stage after the

extrusion of the second polar body, no essential changes in the positions of the SCA occur. This entitles us to take the 93 eggs of groups 1–8 together, and construct a median diagram characterizing this whole stretch of development. This diagram is represented in Fig. 8.

This figure emphasizes the essential characteristics of the SCA pattern of the uncleaved egg of *Limnaea stagnalis*. We find once more the SCA *a–d*, occupying about 180° of the vegetal hemisphere. *a* and *d* extend across the equator upon the animal half of the egg; *b* and *c* are situated wholly below the equator. In the opposite half we find the
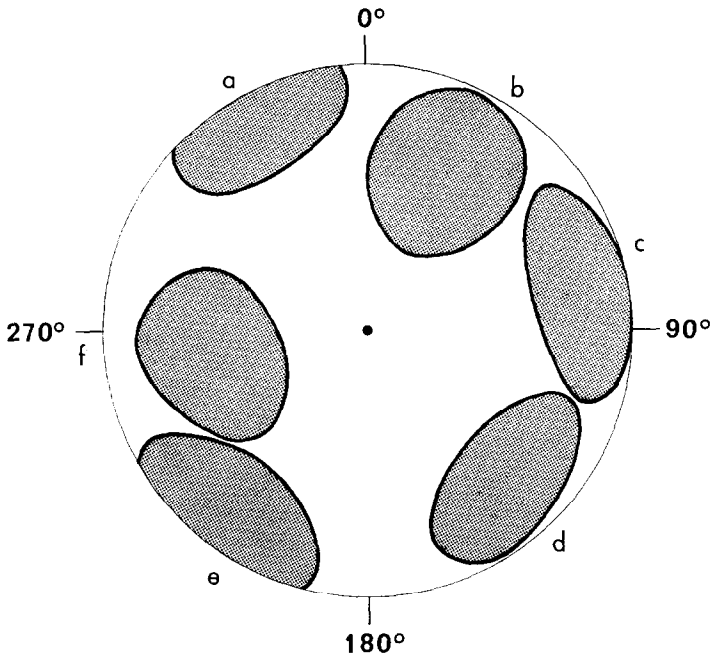

Fig. 9.   Median projection of group 9 (10 eggs). Fusion of karyomeres to prophase of first cleavage.

SCA *e* and *f*, both entirely situated in the vegetal hemisphere; *e* is somewhat smaller than *f*, and lies a little farther from the vegetative pole. If we measure the angular distances between the midpoints of individual SCA, a striking regularity emerges: *a–b*, *b–c*, and *c–d* are all between 50 and 52°; *d–e* and *f–a* both at 66°; *e–f* at 75°. It is evident that the SCA are arranged according to a nearly bilaterally

symmetrical pattern. The plane of symmetry makes an angle of about
50° with our (arbitrarily chosen) 0°–180° meridians. The main asym-
metry with respect to this plane is in the sizes and positions of SCA
e and f.

From the end of the karyomere stage on, important changes begin
to occur. While the egg begins to prepare for cleavage, the SCA show
a tendency to extend in a latitudinal direction. When two adjacent
SCA meet in the process, they coalesce. In individual eggs of group 9,
several such fusions have already taken place, but they do not yet
preponderate and hence do not find expression in the "median projec-
tion" of this group (Fig. 9), which clearly shows, however, the general
lateral extension of the SCA. In the next stage (Fig. 10), however,
immediately preceding first cleavage, SCA a to d have fused to a
common subequatorial band encircling somewhat more than 180 de-
grees of the egg. SCA e and f are still separate (in the "median projec-
tion"), but in immediate contact. The asymmetry in their position has
become somewhat more pronounced. The wide gap now separating f
and a is worth noticing. The figure also indicates the median position
of the first cleavage spindle, as found in a number of the eggs of this
group (since the spindle is situated in the animal hemisphere, and the
eggs are often sectioned in an unfavorable direction, only 5 eggs of
group 10 could be used for this purpose; therefore, the accuracy of
this estimate of the spindle position is not high). The spindle makes
only a slight angle with the 90°–270° meridian, suggesting that the
first cleavage furrow will come in quite near our arbitrary 0–180°
meridian. This means that it will probably separate, on one side of the
egg, the areas of SCA a and b, while it cuts in almost between d and e
on the other side.

The individual eggs of group 11 (stages 1–5, during the ingression
of the cleavage furrow) are no great help in the interpretation of the
changes that occur during this period. The SCA apparently are greatly
drawn out beneath the plasma membrane (cf. Fig. 20), and their
position in individual eggs shows great variations. The "median pro-
jection," constructed from 9 eggs, however (Fig. 11) allows certain
conclusions. In one blastomere, the vegetative region is occupied by a
single large SCA, leaving free a zone adjacent to the cleavage furrow
and beneath the equator. In the other blastomere, there are two SCA,
one of which has clearly arisen by the fusion of two smaller ones. The
two SCA are separated by a rather wide gap. If we identify this gap
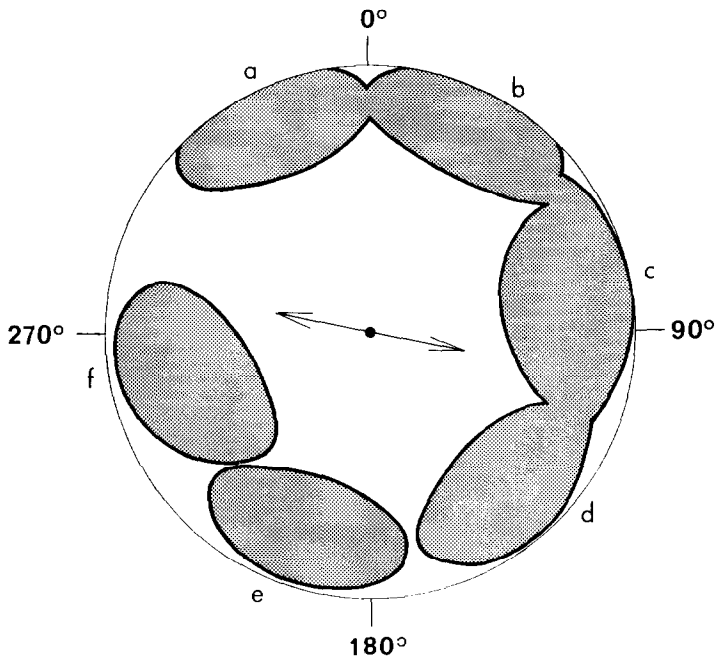with the one present in the previous stage between SCA f and a, we

FIG. 10. Median projection of group 10 (13 eggs). Prometaphase to anaphase of first cleavage. Median position of cleavage spindle is indicated.
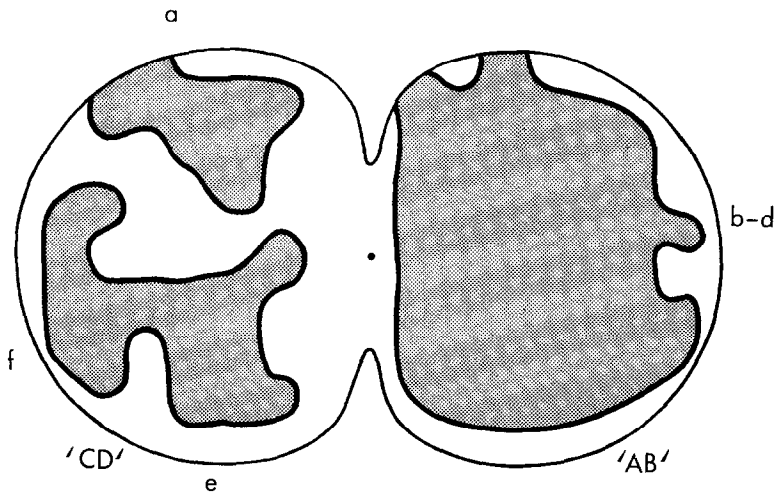

FIG. 11. Median projection of group 11 (9 eggs). Early 2-cell stage.

may conclude that one blastomere has got the original SCA *a* and the
fused *e* and *f*, the other one the product of fusion of *b*, *c*, and *d*. This
is just what one would expect from the position of the spindle in
Fig. 10. Failing other more direct arguments, this seems therefore for
the moment the most satisfactory interpretation of the displacements
of the SCA at first cleavage.

It is at present not yet possible to indicate with absolute certainty
which of the two blastomeres is AB and which CD. Some preliminary
observations at later stages make us believe, however, that the blasto-
mere containing the substance of SCA *e*, *f* and *a* is CD. For con-
venience' sake, and in order to save prolix circumscriptions, we will
denote the blastomeres by 'AB' and 'CD', within single quotation
marks, to indicate that these are only provisional attributions, and may
have to be interchanged in future.

At stage 6 (Fig. 12; cf. also Fig. 3) in one blastomere (presumably
'AB') we once more find a single SCA, occupying the vegetative side
of the blastomere. Its upper boundary is situated at some distance
below the equator. Moreover, it leaves free a rather wide zone along
the interblastomeric furrow; the middle of this boundary of the SCA
shows an indentation opposite the vegetative pole. In the other blasto-
mere ('CD') there is a crescent-shaped SCA, which has apparently
arisen by the coalescence of SCA *e* + *f* and *a* of the previous stage. Its
extremities remain at some distance from the interblastomeric furrow
also in this blastomere, while its middle part shows a deep indentation
facing the vegetative pole, whereas its upper boundary here ap-
proaches or even surpasses the equator.

In the following stages (stages 7 to 9/10, groups 13 to 16), no great
changes occur. The SCA in both blastomeres show some concentration
toward the vegetative side, especially in 'CD'. But in the main the
configuration remains the same throughout the latter part of the 2-cell
stage. This is illustrated by Fig. 13, which shows the "median projec-
tion" of the eggs of group 14 (stage 8).

With the commencement of the second cleavage a new period of
redistribution of the SCA occurs. Again the eggs of these stages show
great individual variations, but the "median projection" of group 17,
which summarizes the 13 eggs of stages 11–13, gives a clear picture
(Fig. 14). The vegetal cross furrow, which runs between blastomeres
B and D, allows us to orient the egg. In one of the blastomere pairs,
arisen from the cells of the 2-cell stage, the SCA are arranged in a

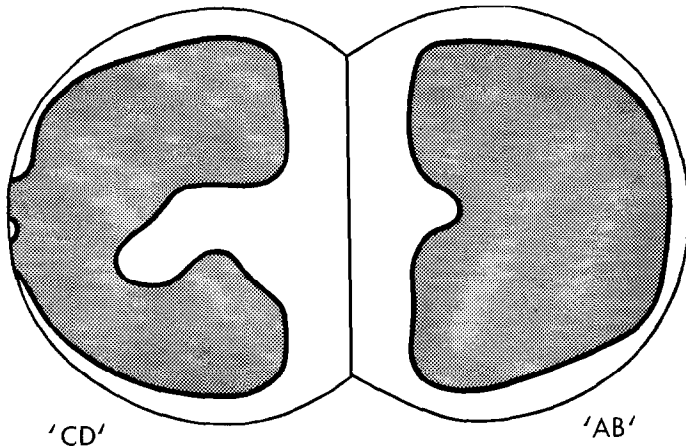'CD'   'AB'

Fig. 12.   Median projection of group 12 (7 eggs). Middle 2-cell stage.

distinct crescent, leaving free a wide area along the cross furrow and the adjoining parts of the furrows between neighboring cells. It is obvious to assume that this pair has arisen by the division of the blastomere showing this same disposition at the 2-cell stage, namely 'CD'. This leads to the notation indicated in Fig. 14. In blastomere 'B'

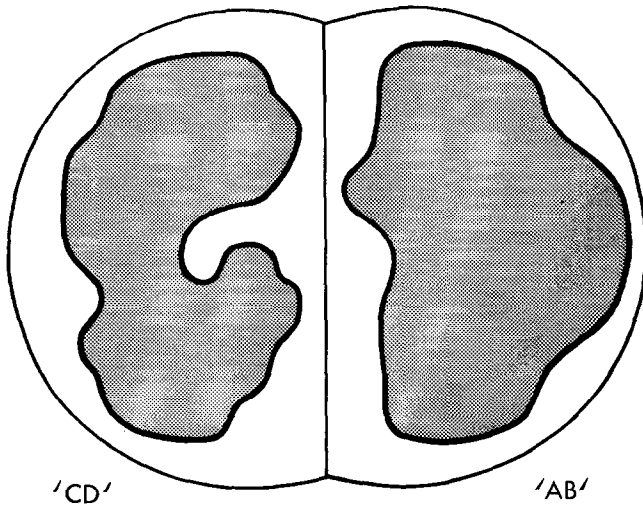'CD'   'AB'

Fig. 13.   Median projection of group 14 (10 eggs). Late 2-cell stage.

the SCA approaches the cross furrow, but in the majority of cases does not reach it. In blastomere 'A', on the other hand, a characteristic situation has appeared. The SCA has concentrated in the most vegetative part of the cell, in most cases entirely filling the angle between the furrows.

Herewith the typical distribution of the SCA over the 4 quadrants of the egg has been established, and no great changes occur up to the end of the 8-cell stage (cf. Figs. 15–17, which show the configurations
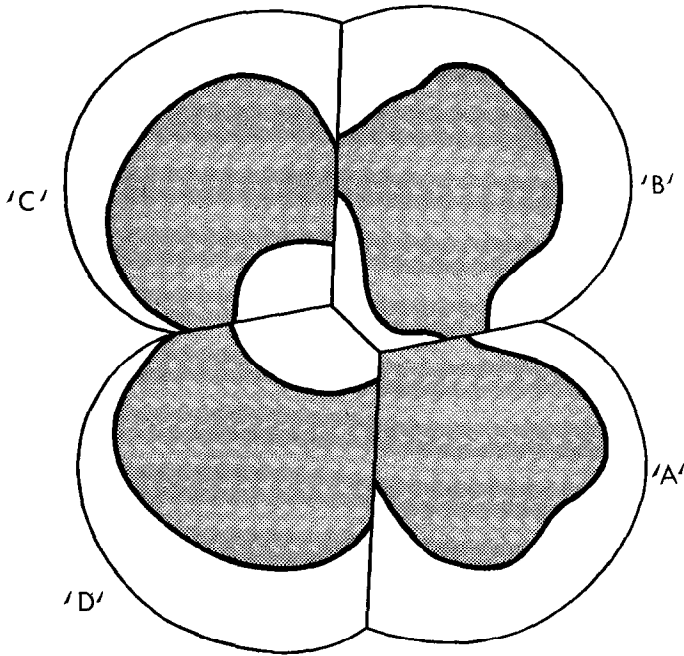

FIG. 14.   Median projection of group 17 (13 eggs). Early 4-cell stage.

found in these groups. In these figures, the eggs have been oriented in the usual way with the cross furrow horizontally and blastomere 'D' upward). Only some further concentration of the SCA toward the vegetative pole takes place. The opposite blastomeres 'A' and 'C' can be easily distinguished. In 'A' the SCA is strongly concentrated in the angle between adjacent furrows; up to stage 16 a small area remains free, but afterward the SCA entirely fills the angle. In 'C', on the other hand, the SCA arches from the interblastomeric furrow 'C/D' to

furrow 'B/C', leaving free an extensive area in the intervening angle. This area becomes smaller with further development, and may become obliterated in individual eggs of stages 18–19. In blastomere 'B', the SCA mostly extend up to the cross furrow. In 'D', on the other hand, an extensive area along the cross-furrow and the interblastomeric furrow 'C/D' is originally left free; only toward the end of the 8-cell stage does this area begin to fill up. It is further noticeable that the
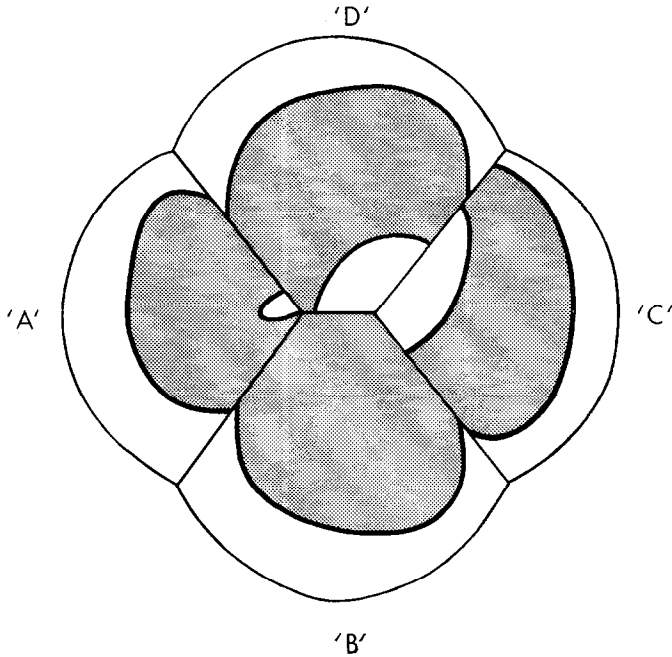

FIG. 15.   Median projection of group 19 (10 eggs). Late 4-cell stage.

upper margin of the SCA in this blastomere rises gradually from interblastomeric wall 'A/D' to 'C/D'; this is evident in all stages from stage 11 to stage 19 (cf. Figs. 14–17).

For the present, the evolution of the SCA has not been studied beyond stage 19.

The SCA in Centrifuged Eggs

The study of the SCA in centrifuged eggs is attended with particular difficulties. While it is already not very easy to recognize and localize

them in normal eggs, this is rendered well nigh impossible after dis-
turbance of the egg structure by centrifugation. I mentioned already
that the main criterion for their recognition is the opacity and stain-
ability of their cytoplasmic matrix. In the centrifugal region of
centrifuged eggs this is almost entirely obscured by the dense packing
of the protein yolk platelets. A further complication arises by the
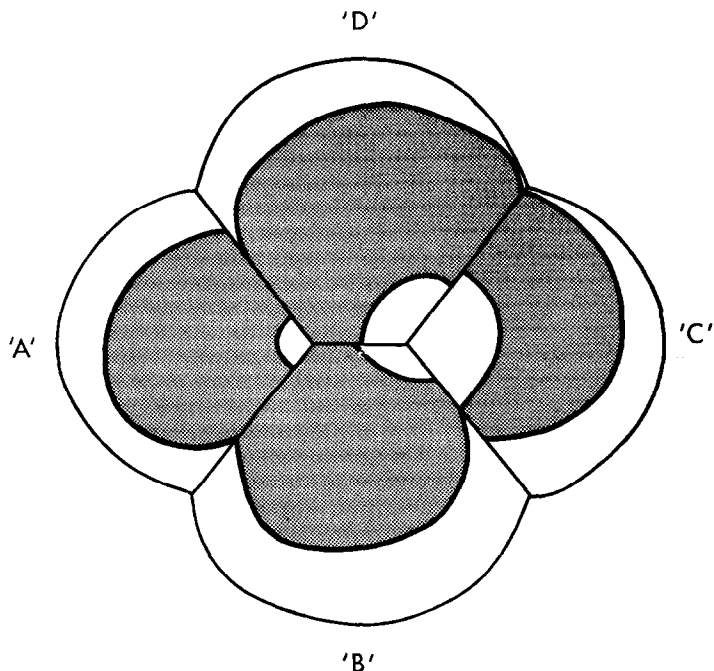tendency of the mitochondria to accumulate beneath the egg cortex,

FIG. 16.   Median projection of group 20 (7 eggs). Early 8-cell stage.

which leads to the formation of a "spurious pole plasm" in the mito-
chondria zone (Raven and Brunnekreeft, 1951; Raven and van der
Wal, 1964).

Notwithstanding these difficulties, a study of Limnaea eggs centri-
fuged at various moments during the uncleaved stage has provided
some interesting results, as the following example shows.

Experiment JJ. An egg mass was centrifuged for 5 minutes at 3800
rpm (1860 g) about 15 minutes before first cleavage. A first sample of

eggs was fixed 15 minutes later at the moment of cleavage. The eggs showed a clear stratification into 3 layers: (1) a centripetal zone of fat and frothy alveolar protoplasm, with γ-granules in the vacuoles; (2) a layer of mitochondria; (3) a centrifugal dense yolk mass, mainly consisting of β-granules. An egg of this sample has been illustrated by Raven (1946b, Fig. 1). A reexamination of the sections shows that no SCA can be distinguished. Owing to the circumstances mentioned above, this is not surprising as regards the mitochondria and yolk
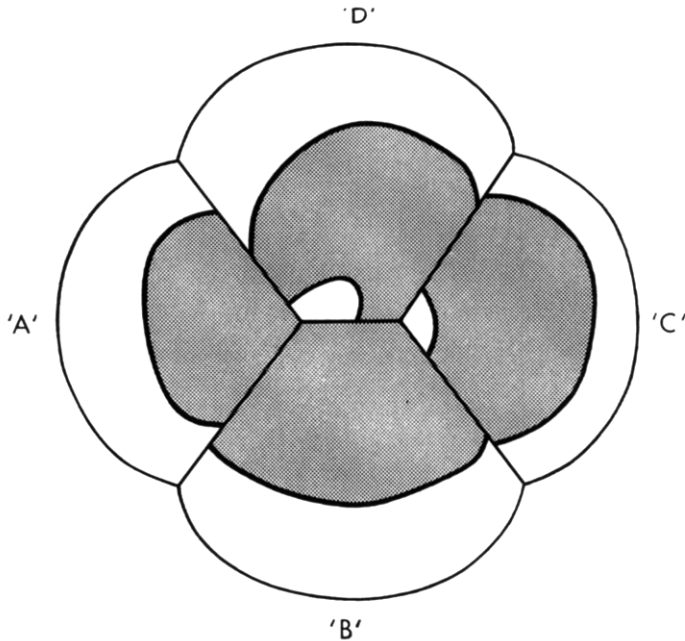

FIG. 17. Median projection of group 21 (9 eggs). Late 8-cell stage.

zones. However, in zone 1, which occupies about half of the egg, the frothy cytoplasm extends everywhere up to the plasma membrane, and it is beyond doubt that SCA are lacking here. The obvious conclusion must be that the components of the SCA have been disrupted and displaced by centrifugation, and arranged themselves according to their density in the stratification pattern.

A second sample was fixed 1 hour after first cleavage, while the eggs were at prophase of second cleavage (stage 8). They had been selected from a group in which the distribution of substances between the two
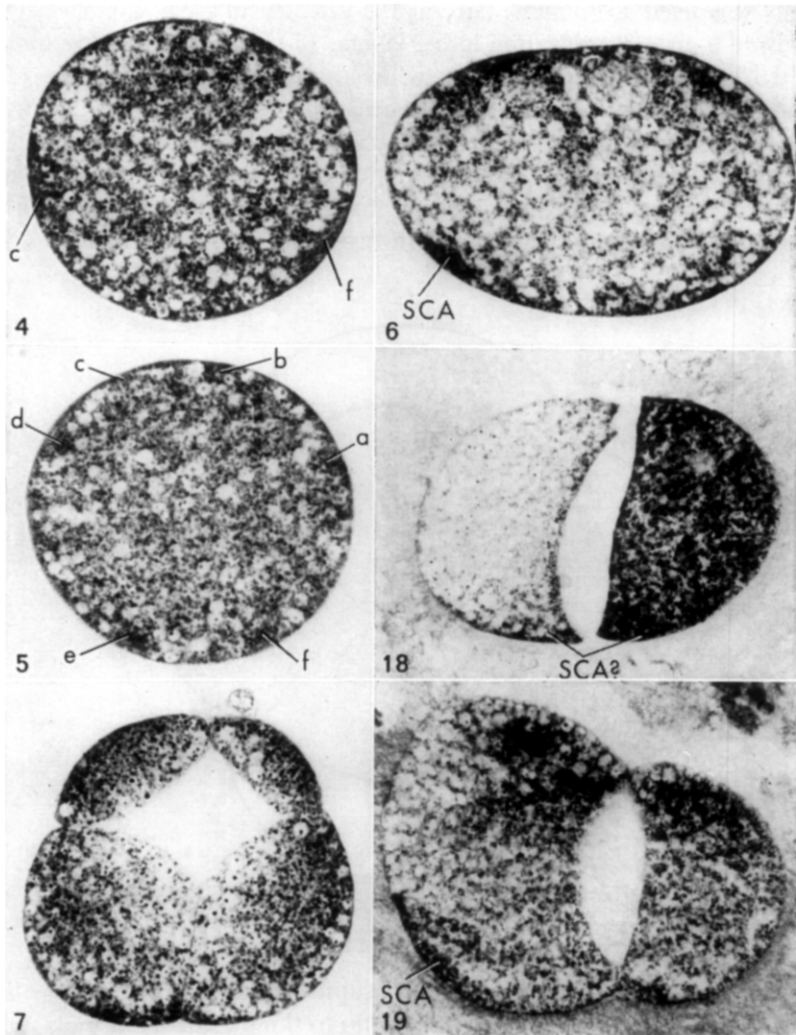
PLATE 1

FIG. 4. Egg N 24–3, prometaphase of 2nd maturation division. SCA c and f. Magnification: ×440.

FIG. 5. Egg N 22–3, prometaphase of 2nd maturation division. The six SCA a–f (c only marginally sectioned). Magnification: ×440.

FIG. 6. Egg N 55–1, stage 7. 'AB' at left, 'CD' at right. Magnification: ×440.

FIG. 7. Egg N 95–2, stage 17. Macromere 1A at left, 1D at right. SCA in vegetative part of macromeres. Magnification: ×440.

blastomeres was maximally unequal, one blastomere containing all the
β-yolk, and the other the frothy cytoplasm. In the vegetative part of
the latter blastomere, clear indications of the re-formation of SCA
can be observed (Fig. 18).

In a third sample fixed 25 minutes later, at anaphase of second
cleavage, in which the packing of the proteid yolk has become some-
what less dense by a beginning redispersion of the yolk platelets, also
in the yolk-rich portions of the egg new-formed SCA are indicated as
regions of somewhat greater density beneath the plasma membrane,
in the vegetative part of the blastomeres. Finally, in an egg sample
fixed 4 hours after centrifugation, distinct SCA have been formed in
the vegetative regions both of the yolk-rich and of the mainly cyto-
plasmic blastomeres (Fig. 19). As far as can be made out, their
localization corresponds to that in normal eggs of the same stages.

These observations seem to show that the SCA—disrupted and dis-
persed during centrifugation—after some time are re-formed beneath
those parts of the egg surface where they are situated in normal eggs
at the corresponding stages.

The Displacement of Spindle Remnants after First and
Second Cleavage

Though only indirectly related to our subject, some observations
made in this series of normal eggs on the fate of the spindle remnant
during early cleavage in *Limnaea* have to be reported here. The male
and female pronucleus meet immediately beneath the animal pole, and
the first cleavage spindle is formed in this region. It then sinks to a
somewhat deeper level, but remains in the animal hemisphere. At
anaphase of first cleavage we find it at a distance of about one-fourth
to one-third of the egg diameter beneath the animal pole (Fig. 20).
The first furrow begins to indent the egg at the animal pole, but soon
it extends around the egg. The cleavage spindle is pushed inward by
the advancing furrow. At stage 4 the furrow has cut maximally
through, the two blastomeres remaining in contact by a narrow stalk
only. The cleavage spindle lies in this stalk, still somewhat nearer the

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FIG. 18.   Egg JJ II, B. Centrifuged egg, 75 minutes after centrifugation. First
indication of SCA in both blastomeres. Magnification: ×350.

FIG. 19.   Egg JJ IV, A. Centrifuged egg, 4 hours after centrifugation. Distinct
SCA in both blastomeres. Magnification: ×350.

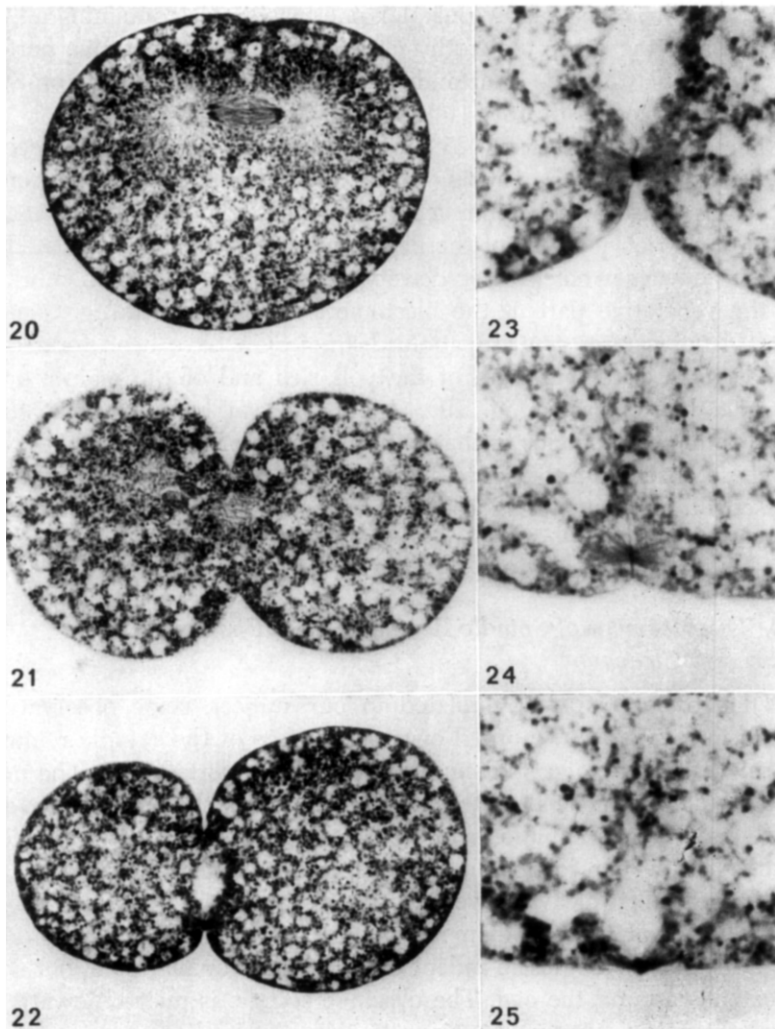PLATE 2

FIG. 20.   Egg N 46–2, stage 1. Anaphase spindle of first cleavage. Magnification: ×440.

FIG. 21.   Egg N 49–2, stage 4. Spindle remnant in connecting stalk beneath animal furrow. Magnification: ×440.

FIG. 22.   Egg N 53–1, stage 5. Spindle remnant displaced toward vegetal furrow, beneath cleavage cavity. Magnification: ×440.

bottom of the animal furrow than of the vegetative one (Fig. 21); the two groups of karyomeres at its extremities lie at the same level. Now the two blastomeres begin to flatten against each other, with the formation of an interblastomeric wall, in which soon a lens-shaped cleavage cavity appears. During this process the spindle remnant is displaced in a vegetative direction, while the two groups of karyomeres retain their position in the animal half of the blastomeres or are even shifted to a more animal level. Electron microscopic observations by W. Berendsen (personal communication) have shown that this displacement of the spindle remnant is due to the ingrowth of a duplicature of the plasma membrane from the bottom of the animal furrow towards the vegetative side, pushing the spindle remnant before it. At stage 5, which is reached on an average about 18 minutes after stage 4, we find the spindle remnant beneath the cleavage cavity, immediately adjacent to the bottom of the vegetative furrow (Figs. 22 and 23). With further recession of the vegetative cleavage furrow the spindle remnant is taken along, remaining immediately beneath the surface in the most vegetative part of the interblastomeric wall as a conspicuous mid-body, from which some coarse fibers extend on either side into the cytoplasm of the blastomeres (Fig. 24). At stage 7, when the furrow has completely leveled out, it lies in the surface at the vegetative pole; in some cases its mid-body even slightly projects above the surface (Fig. 25). In some eggs at stage 8 the mid-body may still be found in this position, but in most eggs of this stage the spindle remnant has been resorbed.

A similar sequence of events is found during the next cleavage. At stage 11, while the furrows of second cleavage are cutting through, the cleavage spindle lies immediately beneath the bottom of the animal furrow. During stage 12, the spindle remnant is displaced toward the vegetative furrow, while the karyomeres stay behind in the animal part of the blastomeres. At stage 13, we find the spindle remnants close beneath the surface in the vegetative parts of the furrows between A and B and between C and D, respectively, at some distance from the

FIG. 23. Higher magnification of vegetal region of same egg. Spindle remnant with midbody. Magnification: ×1100.

FIG. 24. Egg N 86–2, stage 12. Spindle remnant immediately beneath vegetative furrow. Magnification: ×1100.

FIG. 25. Egg N 55–1, stage 7. Spindle remnant projects above the surface at vegetative pole. Magnification: ×1100.

ends of the vegetative cross furrow. During stage 14, they still more approach the ends of the cross furrow. At stage 15, they are no more to be found, apparently having been resorbed.

DISCUSSION

If we compare the positions of the SCA in the uncleaved eggs of *Limnaea stagnalis*, as represented in Fig. 8, with the "patches" in Fig. 6 of my previous paper (1963), it is clear that we have to do with the same pattern. In this comparison it must be taken into account that the latter figure shows the "patches" as seen from the animal pole, whereas the drawings of the present paper represent them as seen from the vegetal side; therefore, the two configurations mirror each other.

As a matter of fact, there are some slight differences between the two drawings. In the first place, it appears that the positions of the SCA in my 1963 paper have been located somewhat higher up on the animal hemisphere. Moreover, the position of SCA e, as shown there, is inaccurate; actually, it lies nearer to f than indicated at that time. Since the positions of the "patches" had been deduced in my previous investigation from a restricted number of eggs only, it is clear that Fig. 8 of the present paper, which gives the median positions as calculated from measurements on 93 uncleaved eggs, is the more reliable of the two. By the way it must be noted that therewith the agreement with the follicle cell positions, as depicted in Fig. 7 of my 1963 paper, becomes even more striking. The areas covered by the SCA are somewhat larger than previously indicated. It must be remembered that the gradual thinning out of the SCA toward their periphery makes their delimitation somewhat arbitrary. In the present paper, not only the thicker central parts of the SCA have been taken into consideration, but as far as possible also their peripheral thin margins.

Anyhow, there appears to be little doubt that we have to do here with the same cytoplasmic differentiations as previously. We now have to examine whether they are related to the ultimate pattern of cellular differentiations of the embryo.

If we consider the pattern of SCA of the uncleaved egg, as represented in Fig. 8, one is struck by its regularity, and its clearly expressed "dorsoventrality" and near-bilateral symmetry. On one side there are four smaller SCA $(a–d)$, at regular distances apart. The two middle ones (b and c) lie subequatorially, whereas a and d extend upward

beyond the equator. In the other half of the egg we find the two larger SCA *e* and *f*, each at the same angular distance from the nearest member of the first-mentioned group (cf. also the photograph, Fig. 5). Thus we can draw a plane of symmetry, which makes an angle of about 50° in a clockwise direction with the plane of our arbitrary 0°–180° meridians.

It is obvious to ask whether this plane of symmetry of the pattern of cytoplasmic differentiations of the uncleaved egg has any relations with the median plane of the later embryo. The answer to that question is complicated by the fact that the relationship between the axes of the future embryo and the first cleavage furrows is not a simple one in molluscs. I have treated this problem at some length in my book (1966, p. 68). A consideration of later development shows that the median plane of the embryo is determined by the position of the cells 2d and 4d, these two blastomeres indicating the dorsal side of the future embryo. Therefore, our problem can be solved by establishing the position of these cells with respect to our "plane of symmetry."

We have seen that the first cleavage furrow apparently divides the egg along a plane which almost coincides with our arbitrary 0°–180° meridian (Figs. 10 and 11). Owing to the displacements of the SCA just preceding and during cleavage, some uncertainty remains, but it seems hardly probable that the deviation between the two planes amounts to more than 10°. This means that we can also state that the plane of symmetry of the SCA pattern of the uncleaved egg makes an angle of about 50° (to the right) with the plane of first cleavage.

Figure 26 gives a picture of the vegetative side of a *Limnaea* egg at a 25-cell stage, according to Verdonk (1965). At this stage, the dorsoventrality of the embryo becomes evident by the division of macromere 3D into 4D and 4d. From the notation of the cells, the parts formed by the descendants of AB and CD, respectively, and hence the position of the first cleavage furrow can be recognized in the figure. We have indicated it in the drawing by a line XX′, connecting the two points at the equator where a- and d-cells, on the one hand, and b- and c-cells, on the other, meet. We further have drawn a second line YY′, making an angle of 50° (to the right) with the former, and passing through the vegetative pole; it indicates the former position of the plane of symmetry of the SCA. It is evident that this plane just coincides with the column of 2d–4d cells marking the mediodorsal side of the future

embryo. This shows that the plane of symmetry of the SCA does indeed coincide with the median plane of the embryo.

It further follows that the contrast between the four SCA *a–d*, on one side of the egg, and SCA *e* and *f*, on the other side, corresponds to the dorsoventrality of the future embryo. It is not immediately evident which side is dorsal and which is ventral. For the sake of convenience, we have provisionally, on the basis of some preliminary but admittedly insufficient evidence, decided in favor of a notation which indicates the side of SCA *e* and *f* as dorsal. Future research must show whether our guess is right.
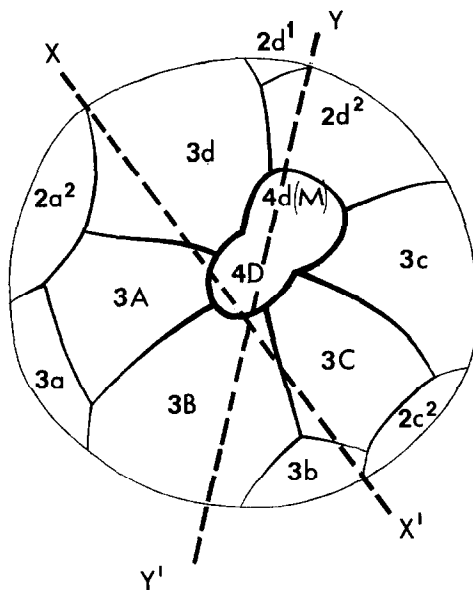

FIG. 26. Vegetal hemisphere of 24–25 cell stage. Division of *3D*; formation of the primary mesoblast *4d* or *M*. After Verdonk (1965). *XX'*: plane of first cleavage; *YY'*: approximate plane of symmetry of SCA pattern of uncleaved egg.

However this may be, it is clear that the symmetry and dorso-ventrality of the future embryo is already adumbrated in the arrangement of the cytoplasmic differentiations of the uncleaved egg. Since the SCA arise only after ovulation by the accumulation of particular cytoplasmic components beneath certain areas of the cortex, I have concluded in my previous paper (1963) that they probably reflect

particulars of the cortical morphogenetic field. Moreover, as the pattern of the SCA is identical with the pattern of the follicle cells around the growing oocyte, the hypothesis was put forward that the "blue-print information" (Raven, 1958) in the egg cortex is "imprinted" upon the egg during oogenesis by the structures surrounding the oocyte. Our present finding, that the arrangement of the SCA, and therefore of the follicle cells, indeed foreshadows the main directions of the future embryo, lends support to this hypothesis.

In my 1963 paper, I stated that the pattern of the SCA (and of the follicle cells) is not only dorsoventral, but also slightly asymmetric. In the arrangement of Fig. 8 of the present paper, this asymmetry is only very slight. It is mainly restricted to small differences in the size and position of SCA *e* and *f*. One may even ask whether it is significant. There are two circumstances which might argue for its reality. In the first place this asymmetry between *e* and *f*, in the same direction, is found in 6 of the 8 "median projections" of the single groups 1–8, of which Fig. 8 is a summary (group 1, Fig. 2, is one of the exceptions). Perhaps more significant is the fact that the asymmetry in the positions of *e* and *f* becomes accentuated in the eggs of groups 9 and 10 (Figs. 9 and 10). At further development, relationships become further complicated by the asymmetric way in which the cleavage furrows intersect the original pattern. For the moment, we must leave undecided whether the pattern of SCA does indeed reflect the asymmetry of the future embryo. If it does, the observation that this asymmetry is restricted to that half of the egg that we have considered as dorsal may be confronted with the fact that the asymmetry of the future animal is most pronounced in the dorsal part of the body (visceral sac and shell).

We must now discuss the further distribution of the SCA during early cleavage. We have seen that the pattern of cytoplasmic differentiations of the uncleaved egg is not merely intersected by the cleavage furrows and divided passively among the blastomeres, but that regular displacements and fusions of the SCA occur, by which they are distributed in a characteristic way among the four quadrants of the egg. These changes begin already some time before first cleavage with an extension of the SCA in a latitudinal direction, leading to their fusion. Extensive rearrangements occur during the first and the second cell division. In the period between two divisions the changes are less pronounced, and are mainly restricted to a gradual displacement in

a vegetative direction. As a result, the SCA, from their original equatorial or subequatorial position, have shifted at the 8-cell stage in each quadrant to a position in the most vegetative part of the macromere, together surrounding the vegetative pole and the vegetal cross furrow (cf. Figs. 4 and 7).

At all stages during these displacements, the SCA retain their localization immediately beneath the plasmalemma of the egg. We have ascribed their first accumulation, at the time when the eggs pass through the female genital tract after ovulation, to a specific attraction by certain areas of the plasma membrane carrying the cortical field. In view of the extensive cytoplasmic perturbations which are to be expected in the egg during the cleavage divisions, the fact that these plasms retain their close apposition to the plasmalemma argues in favor of their continuing attraction by the cortex. This supposition is corroborated by our observations in centrifuged eggs, demonstrating that the SCA, when they are disrupted and dispersed by centrifugation, are re-formed in the subsequent hours in their normal localization, even during early cleavage stages. This reassembling of the components of the SCA occurs simultaneously with the gradual redispersal of the substances accumulated in layers during centrifugation and can most easily be explained by the assumption that an attractive action is exerted upon these components by special areas of the egg cortex. In this respect the SCA resemble the "matrix" of the animal pole plasm which behaves in the same way (Raven and van der Wal, 1964).

If we accept this hypothesis that the components of the SCA are kept together (and, if necessary, reassembled) by cortical attractions, then the problem arises how we must visualize the displacements of the SCA during early cleavage. We have seen that the main displacements take place at the time of cell divisions. At each division, the cleavage furrow first indents the egg and deepens until the daughter cells are connected by a narrow stalk only. Then the blastomeres begin to flatten against each other, with concomitant formation of an interblastomeric wall, the furrows level out, and the egg contour as a whole rounds off more or less (cf. Raven, 1946a). Apparently, the total area of the egg surface undergoes rapid changes during the process, first increasing and then decreasing to a lesser extent. These changes have been extensively studied, e.g., in sea urchins (cf. Wolpert, 1960). Although such studies have not yet been made in *Limnaea*, we may

expect that similar relationships obtain here, some parts of the egg surface increasing, other parts decreasing in area in regular succession, while new egg surface is inserted at other places. It is obvious to relate the displacements of the SCA to this reorganization of the egg surface during cleavages. In view of the general direction of these displacements toward the vegetative pole, one might assume that the egg cortex as a whole shows a shift in this direction, taking the SCA along. Such a shift could be visualized by the assumption of a local new formation of egg surface near the animal pole, and a simultaneous disappearance of surface material at the vegetative pole, either by ingression or resorption. In case of an ingression of the egg cortex at the vegetative pole one could suppose that the invaginated surface material would be used in the formation of the interblastomeric wall. Our observations on the vegetative displacement of the spindle remnant taking place during these stages and W. Berendsen's electron microscopic observations mentioned above (page 429) are hardly compatible with such a view, however. On the contrary, both the vegetative displacement of the SCA and of the spindle remnants find an easy explanation if one assumes that new cell membrane is formed near the animal pole, while resorption of plasmalemma takes place simultaneously at the vegetative pole. The latitudinal extensions and fusions of the SCA, on the other hand, do not fit into such a simple scheme.

However this may be, it is clear that we must beware of a too static picture of the cortical morphogenetic field. The cortex is not merely a mosaic of areas of different molecular structure, given once and for all, and parceled out in a quite passive way among the cleavage cells. The cortical pattern shows alterations of a dynamic nature during early cleavage, probably connected in some way or other with reconstruction processes in the surface membrane at cell divisions.

In this connection may be mentioned Geilenkirchen's observation (1964) that the morphogenetic effects of centrifugation in *Limnaea* are most pronounced when the eggs are centrifuged at the very moment of first, second, or third cleavage. This is related by him to the strong elongation of the egg cells when centrifuged at this time, which is thought to interfere with the normal pattern of insertion and resorption of cell membrane during division. This could alter the position of areas of morphogenetic importance in the cell membrane. Our present results lend support to such a view.

A final question concerns the ultimate destiny of the substance of

the SCA. Nothing definite can be said about that at the moment. Some preliminary observations seem to show that the blastomere 4d gets a great amount of this material, but other vegetative cells also get part of it. I hope to be able to decide this by a study of cleavage beyond the 8-cell stage.

SUMMARY

Uncleaved eggs of *Limnaea stagnalis* show a pattern of cytoplasmic differentiations in which there are 6 "subcortical accumulations" (SCA) in the equatorial region. SCA consist of a dense cytoplasmic matrix, containing a special kind of small granules. At certain stages also lens-shaped bodies, consisting of coiled threads, are found in them. At the uncleaved stage the SCA form a regular pattern, which is dorsoventral and nearly symmetrical. Its plane of symmetry coincides with the median plane of the future embryo. Just before first cleavage, the SCA exhibit a latitudinal extension and fusion. They are distributed in a regular way among the 2, then 4, blastomeres. Their positions in the 4 quadrants of the egg exhibit characteristic differences. In each quadrant they show a gradual shift toward the vegetative pole.

In centrifuged eggs, the SCA are indistinguishable immediately after centrifuging. In a few hours, however, they are re-formed in their typical localization, even in cleaving eggs.

After first and second cleavage, the spindle remnants are displaced in a vegetal direction in the interblastomeric wall until they lie immediately beneath the surface in the most vegetative part of this wall.

The SCA probably reflect particulars of the cortical morphogenetic field. As their pattern in the uncleaved egg is identical with the pattern of follicle cells surrounding the growing oocyte in the gonad, these observations lend support to the hypothesis that the cortical field is "imprinted" upon the egg during oogenesis by the surrounding structures. Its pattern shows alterations of a dynamic nature during early cleavage, probably connected with reconstruction processes in the cell membrane at cell divisions.

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