The structure of a morphogenetic cytoplasm, present in the polar lobe of Bithynia tentaculata (Gastropoda, Prosobranchia)

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SUMMARY

In the first polar lobe of the egg of Bithynia tentaculata a cup-shaped mass of small vesicles is described, which fills the greater part of the lobe. It is named the 'vegetal body'. With methyl green-pyronin the vegetal body stains clearly, but after treatment with RNase no staining occurs, thus indicating the presence of RNA. The first polar lobe of Bithynia is of great importance for further development of the embryo and it is argued that the vegetal body could be a morphogenetic cytoplasm, responsible for the developmental effects of the polar lobe.

INTRODUCTION

In the eggs of many annelids and molluscs the first cleavages are characterized by the appearance of polar lobes. The contents of these lobes are of great importance for further development, as is shown by experiments involving removal of the lobe or deletion of cells receiving the lobe contents. In Ilyanassa, for instance, lobe-dependent structures are: foot, eyes, operculum, statocysts, shell, heart and intestine (Crampton, 1896; Clement, 1952, 1956, 1962; Cather, 1967; Atkinson, 1971). The successively appearing lobes do not all have the same effect. In Dentalium (Wilson, 1904), Sabellaria (Hatt, 1932; Novikoff, 1938a, b) and Mytilus (Rattenbury & Berg, 1954) development of the apical tuft of the larva is dependent on the presence of the first polar lobe, but not on the second one.

Attempts to identify polar lobe factors have given very poor results up till now. In many cases differences in composition can be demonstrated between the cytoplasm of the polar lobe and the rest of the egg (Pitotti, 1947; Clement & Lehmann, 1956; Pasteels & Mulnard, 1957; Reverberi, 1958, 1970; Berg & Kato, 1959; Collier, 1960a, b; Dalcq & Pasteels, 1963; Crowell, 1964). But centrifugation experiments (Clement, 1968; Verdonk, 1968) have established that in Ilyanassa and Dentalium the morphogenetic factors are not located in the displaceable components of the polar lobe cytoplasm. Unfortunately ultrastructural and histochemical studies on displacement of cytoplasmic components in polar lobes of centrifuged eggs are lacking.

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Accumulation of morphogenetic substances in special areas of the egg and segregation of these accumulations into special blastomeres are not restricted to polar-lobe-forming organisms, but can be demonstrated in many other animals. In spite of such a widespread occurrence of the segregation phenomenon, identification of the morphogenetic substances in the segregated cytoplasm has not been successful. Even in the polar granules of insect eggs, 'the only case known where developmentally significant information is localized in organelles that can be seen and followed during development' (Mahowald, 1971), it is not known whether it is the protein or the RNA component which is developmentally significant. We describe a structure in the polar lobe of *Bithynia tentaculata* which could be a second case of an organelle storing morphogenetic substances.

**METHODS**

*Bithynia tentaculata* is a freshwater prosobranch snail which can be collected in ditches around Utrecht, Holland. In aquaria in the laboratory, egg masses are soon deposited on plant leaves. These egg masses consist of capsules which cannot be separated from each other. The tough capsule membrane is first perforated with a very sharp knife in order not to compress the egg. Then the egg can be removed from the capsule with a hair-loop and the viscous capsule fluid can be washed off in tap-water.

*Fixation and staining for light microscopy.* Eggs fixed in Zenker's fluid were stained with Heidenhain's iron haematoxylin and eosin for general study or with methyl green–pyronin for nucleic acids (Brachet, 1942, 1953). For the latter stain eggs usually were fixed in ethanol–acetic acid (3:1) for a more vivid stain. For the Feulgen method, eggs were fixed in ethanol–acetic acid (3:1) and either stained as sections or *in toto* according to the method of van den Biggelaar (1971).

*Fixation and staining for electron microscopy.* Eggs were fixed for 3 h at 4 °C in a mixture of equal parts 2 % glutaraldehyde and 2 % osmium tetroxide, both in 0.1 M Na-cacodylate buffer at pH 7.4. The eggs were then washed in buffer, oriented in agar, dehydrated in a graded series of ethanol, followed by propylene oxide, and embedded in Epon 812. Sections were stained for 10 min in a saturated solution of uranyl-acetate in 70 % methanol, followed by 1 min in a lead solution according to Reynolds (1963).

**RESULTS**

I. *Observations with the light microscope*

At the vegetal pole of freshly laid eggs of *Bithynia*, in which the germinal vesicle is still present, a densely staining cytoplasm is present. At this stage the dense plasm is intimately connected with the surface of the egg (Fig. 1). After the germinal vesicle has disappeared, the dense cytoplasm rises from the surface (Fig. 2) and soon assumes the shape of a cup with the open side towards the vegetal pole. Because of its position we call it the 'vegetal body'. It stays at the
The polar lobe of Bithynia

Figs. 1–5. Light micrographs of eggs of Bithynia tentaculata stained with iron haematoxylin and eosin. x 350.

Fig. 1. Egg just after oviposition with the germinal vesicle still present. Arrow indicates dense cytoplasm at the vegetal pole.

Fig. 2. Egg after breakdown of the germinal vesicle. Dense cytoplasm (arrow) free from the surface.

Fig. 3. Egg at first cleavage. Arrow indicates dense cytoplasm now present as cup-shaped vegetal body in the polar lobe.

Fig. 4. Two cell stage with the vegetal body (arrow) at the vegetal side of the CD-blastomere.

Fig. 5. Section through the CD-blastomere at the beginning of second cleavage. Arrow indicates the second polar lobe, filled with clear cytoplasm but missing the vegetal body.

Fig. 6. Light micrograph of an egg at first cleavage, stained with methyl green–pyronin. The vegetal body (arrow) is densely stained.
vegetal pole, surrounded by clear cytoplasm, until first cleavage, when a polar lobe is formed. Compared with other polar lobes, described in molluscs, this lobe is remarkable in several respects: it is extremely small (diameter 20–30 μm) and it is usually nearly free from yolk granules.

Fig. 7. Electron micrograph of the first polar lobe with vegetal body. Arrow points to a dense body. AZ, Attachment zone; L, lipid; M, mitochondrion. × 9700.
The polar lobe of Bithynia

The vegetal body is incorporated in the first polar lobe (Fig. 3). After first cleavage the vegetal body is transferred with the lobe to the CD-blastomere. During the whole 2-cell stage it remains at the vegetal side of the CD-blastomere (Fig. 4). At second cleavage the vegetal body suddenly disappears at about the start of anaphase. A second polar lobe is formed at this stage, which, however, is never as completely separated as the first polar lobe and remains broadly connected with the CD-blastomere. The second lobe contains a clear cytoplasm but the vegetal body is no longer present (Fig. 5). In order to study the chemical nature of the vegetal body, we stained it with the Feulgen method for the presence of DNA. Both in sections and in whole mounts the staining was negative. With methyl green–pyronin the body stains clearly (Fig. 6). After pre-treatment of the sections with RNase (Worthington Biochem. Corp.) no staining of the vegetal body occurs, indicating the presence of RNA.

II. Observations with the electron microscope

The vegetal body is a cup-shaped mass of small vesicles and among them some ribosomes, mitochondria, large vesicles and dense bodies can be found (Fig. 7). The vesicles have a diameter of 50–100 nm and most of them are filled with a dark-staining substance (Fig. 8), probably RNA, as RNase treatment of thick sections makes the vegetal body almost completely disappear. The vesicles are not homogeneously distributed, but they are arranged in an irregular network, with vesicle-free areas in between (Fig. 7). The same type of vesicles also forms a thin layer under the membrane of the stalk which connects the polar lobe to the egg and this layer extends to a limited distance (about 15 μm) under the neighbouring egg-membrane (Fig. 9). Occasionally a small cluster of vesicles can be found outside the polar lobe in the egg cytoplasm, close to the stalk. In view of their distribution it seems possible that a few vesicles will come to lie in the AB-blastomere at first cleavage.

Preliminary centrifugation experiments indicate that the vegetal body is hard to displace. Even when part of the uncleaved egg is centrifuged off, the vegetal body stays in most cases in its original place and when the egg is left to cleave after centrifugation, the vegetal body is found in the polar lobe at first cleavage. These results indicate that the vegetal body is firmly attached to the vegetal pole and that there is a strong cohesion of the body itself. Still, no special devices responsible for attachment and cohesion can be found. As attachment is likely to take place by means of the cytoplasmic zone lying between the vegetal body and the vegetal pole, we called this zone the ‘attachment zone’ (Fig. 7).

In the polar lobe there is also to be found a concentration of multivesicular bodies. They often contain densely coated vesicles, which can easily be distinguished from the vesicles making up the vegetal body. The multivesicular bodies are often ruptured and their coated vesicles may be seen lying in strings in the attachment zone (Fig. 10).
Fig. 8. Detail of the vegetal body, showing the small vesicles of which it consists. Most vesicles are completely or partly filled with a dark-staining substance, probably RNA. ×105000.

Fig. 9. Superficial layer of the stalk (ST) of the polar lobe and its implantation region on the egg, showing a layer of small vesicles under the membrane. ×27700.
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Fig. 10. Multivesicular bodies (MVB) in the polar lobe. One of them is ruptured (double arrow), releasing coated vesicles (arrow) into the attachment zone (AZ). ×27700.
DISCUSSION

The facts that are known at present about the vegetal body are all in agreement with the view that the vegetal body could contain the morphogenetic factors involved in determining the lobe-dependent structures.

In *Bithynia* the significance of the polar lobe for development has been established by removing the lobe at first cleavage. No adult organs like eyes, tentacles, shell, etc., develop in lobeless embryos (Verdonk & Cather, 1973). The vegetal body fills the greater part of this polar lobe so it is obvious to assume that the developmental information is located in the vegetal body and most probably inside the vesicles. After first cleavage the vegetal body is transferred with the polar lobe to the CD-blastomere. In isolation experiments (Verdonk & Cather, 1973) the AB-blastomere forms hardly any adult structures, whereas the CD-blastomere, containing the polar lobe, forms most adult structures. At second cleavage the vegetal body disappears rather suddenly between metaphase and anaphase, some time after the disappearance of the nuclear membrane. After second cleavage the C- and D-blastomeres have the same morphogenetic capacities (Verdonk & Cather, 1973). As the vegetal body disappears before the cleavage furrow separates the C- and D-blastomeres, the substances contained in the vegetal body can spread over both cells and in this way give them the same developmental potentialities.

Other arguments in favour of a morphogenetic role are the fact that the vesicles forming the vegetal body are typical storage vesicles, which could keep the morphogenetic factors inactive until their time has come to act, and the fact that the vegetal body contains RNA, which is an obvious candidate for the role of morphogenetic factor.

A large structure such as the vegetal body of *Bithynia* cannot easily be overlooked, still nothing like it has been found in thoroughly investigated species like *Ilyanassa* (Pucci-Minafra, Minafra & Collier, 1969; Gérin, 1972), *Mytilus* (Humphreys, 1964) and *Dentalium* (Reverberi, 1970). This probably means that the vegetal body is an exceptional structure, but the vesicles composing it could well be a general carrier of developmental information. If in other animals these vesicles should not be as numerous as in *Bithynia* and scattered over a larger area, they could probably escape attention.

In all species investigated thus far, several larval or adult structures are dependent on one lobe, so it is to be expected that this lobe contains several kinds of morphogenetic substances which become separated during cleavage until at last each one reaches its own target cell. Such a mechanism might be deduced from experiments with *Dentalium*. Here the apical tuft and the post-trochal region are both dependent on the first polar lobe. If 60% of the lobe is removed, however, larvae develop with an apical tuft and a reduced post-trochal region (Geilenkirchen, Verdonk & Timmermans, 1970). This result points to the possibility of at least two different factors: an apical tuft factor located in the
animal region of the lobe, which is not removed, and a post-trochal region factor in the vegetal region. The finding in Bithynia of a mass of apparently identical vesicles, whose contents probably determine several adult organs, points to another possible mechanism: a polar lobe might contain one kind of morphogenetic factor only, which in some way, maybe by unequal distribution over the cells, determines the development of several organs.

The lack of any visible mechanical structure holding together the vegetal body and attaching it to the vegetal pole is another problem. Special cytoplasms accumulated under a particular area of the cell periphery are a common feature in many eggs, but they do not seem to react in the same way to centrifugal force. The vegetal body of Bithynia and the polar lobe factors of Ilyanassa and Dentalium are not displaced by centrifugation, while the animal pole plasm and the subcortical accumulations of Lymnaea, for instance, are easily disrupted and dispersed (Raven, 1945; Raven & Brunnekreeft, 1951; Raven & van der Wal, 1964; Raven, 1967). In all cases there is probably some kind of attraction exerted by particular areas of the egg periphery upon certain cytoplasmic components, but we can only speculate about their nature.

The last problem to be discussed is the concentration of multivesicular bodies in the polar lobe of Bithynia. Very little is known about multivesicular bodies. In eggs they seem to be formed by transformation of yolk granules (Pasteels & de Harven, 1963) and they contain a high concentration of phosphatases (Dalcq, 1962; Dalcq & Pasteels, 1963). Reverberi noted multivesicular bodies in the polar lobe of Dentalium, and the polar lobe of Sabellaria shows a high concentration of phosphatase (Dalcq & Pasteels, 1963), which could also result from a high concentration of multivesicular bodies. No multivesicular bodies have been reported in the polar lobes of Ilyanassa and Mytilus. Too little is known as yet to enable us to suggest a function for the multivesicular bodies in polar lobes.

REFERENCES


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