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Original Article

Surface polarization in loach eggs and two-cell embryos: correlations between surface relief, endocytosis and cortex contractility

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ABSTRACT The aim of this study was to examine the reorganization of the microfilamentous cortical layer (MC) accompanying ooplasmic segregation in loach eggs. Using scanning (SEM) and transmission electron microscopy (TEM), we found that the MC is thicker in folded areas. Prior to fertilization, surface microvilli are distributed more or less uniformly throughout the egg. A similar, more or less uniform, distribution of endocytotic events was observed in the eggs 5-15 min after insemination using fluorescence microscopy of Lucifer yellow CH uptake. During ooplasmic segregation, the surface is progressively polarized so that before the first cleavage onset (50-60 min after insemination) only the blastodisc surface is folded and undergoes endocytosis, whereas the vegetal surface is smooth and does not show internalization. In two-cell embryos, the blastomeric surface is also regionalized according to its relief and endocytosis. When surface tension was lowered by sucking most yolk granules out of the egg, we observed contractile responses only in the animal folded surface. These data suggest that a polar distribution of contractile structures is established in the loach egg undergoing ooplasmic segregation.

KEY WORDS: *loach eggs, surface relief, endocytosis, cortical contraction, ooplasmic segregation*

Introduction

In unfertilized fish (teleostean) eggs the ooplasm is located among numerous yolk granules (e.g. zebrafish, *Brachydanio rerio* - Hisaoka and Firlit, 1960; loach, *Misgurnus anguillicaudatus* - Iwamatsu and Ohta, 1977), or surrounds one huge central yolk sphere (medaka, *Oryzias latipes* - Iwamatsu, 1966). During ooplasmic segregation, the ooplasm (which is the cytoplasm free of yolk granules) moves to the animal pole of the egg and forms the blastodisc. Later the blastodisc undergoes cleavage and forms the cells of the embryo (Roosen-Runge, 1938; Hisaoka and Firlit, 1960; Svetlov *et al.*, 1962; Iwamatsu, 1973). Little is known about the mechanisms underlying polarized ooplasmic movements. Cortical contractile structures were presumed to play the leading role in ooplasmic segregation (Hisaoka and Firlit, 1960; Iwamatsu, 1973; Katow, 1983). Katow (1983) showed that the formation of the blastodisc in *Brachydanio rerio* was prevented by incubation in cytochalasin B. The MC, a subplasmalemmal network of actin filaments, becomes disorganized and detached from the plasma membrane. It has been proposed that ooplasmic segregation is mediated by the cortex, which contains cytochalasin B-sensitive microfilaments (Katow, 1983). We recently demonstrated that cortical microfilaments in the animal and vegetal parts of the loach eggs seem to play different roles in ooplasmic segregation. Although injections of cytochalasin D or DNase I under the animal pole surface suppressed blastodisc formation, the disintegration of MC in the vegetal part of the egg did not affect ooplasmic segregation (Ivanenkov *et al.*, 1987). In addition to fishes, the participation of the cortical cytoskeleton in the processes of ooplasmic segregation was reported in *Tubifex* (Shimizu, 1982, 1984, 1985, 1986), mollusks (Conrad, 1973; Conrad and Williams, 1974), and ascidians (Sawada, 1988).

Since it was still unclear exactly how the cortical cytoskeleton directs the ooplasm to the animal pole, we have, in the present work, studied the animal/vegetal changes in MC organization in the loach egg undergoing ooplasmic segregation. The results of our preliminary experiments confirmed the data of Bozhkova *et al.* (1983) that the surface of the yolky part of the loach egg has a much smoother relief than the blastodisc surface, and showed that MC is the main cytoplasmic component of surface folds. The present

Abbreviations used in this paper: MC, microfilamentous cortical layer; SLC, "star-like" complex.

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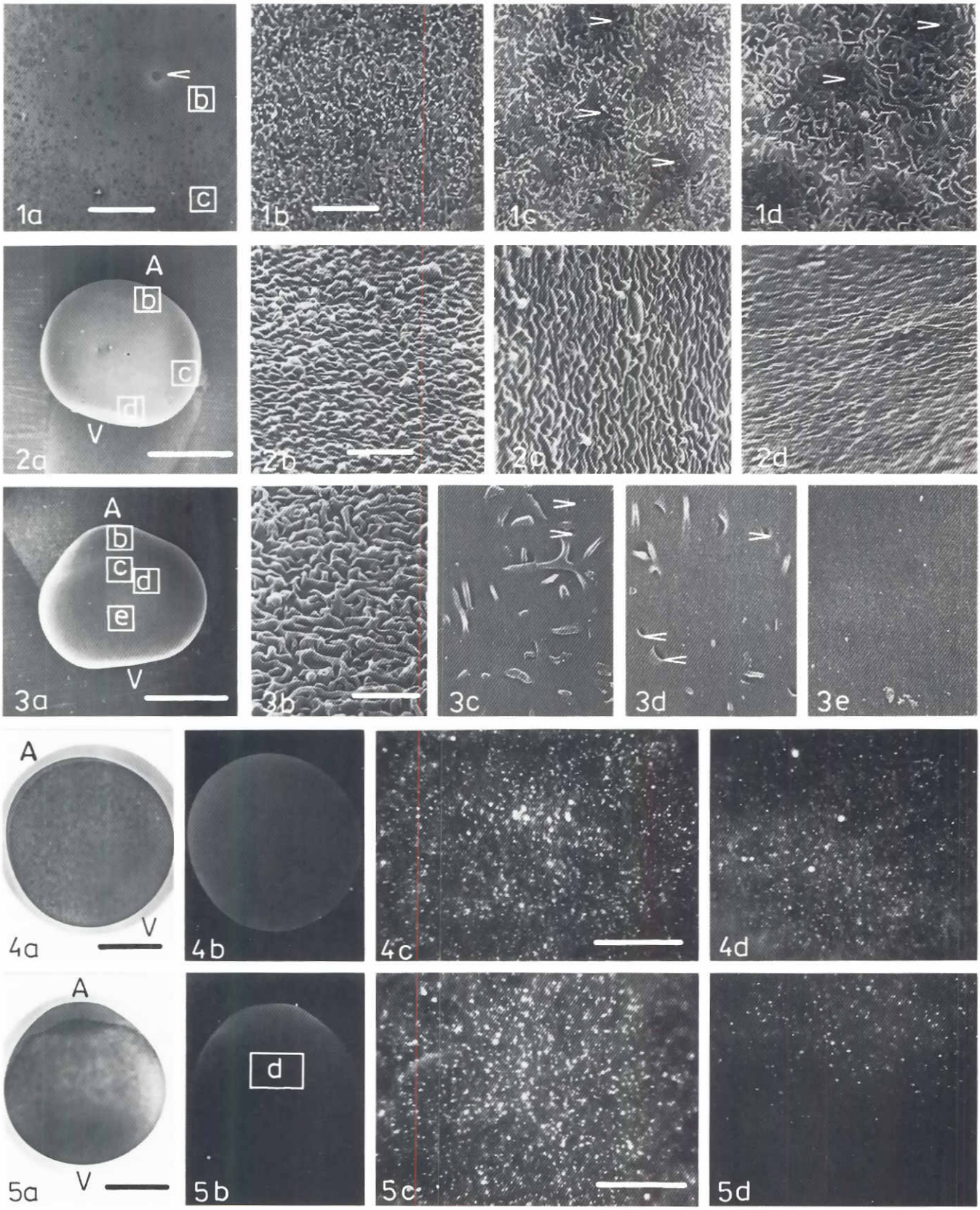
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study was aimed at: (1) investigating animal/vegetal changes in surface relief and the thickness of MC from insemination to the two-cell embryo stage; (2) studying animal/vegetal changes in endocytosis, since surface relief depends partly on membrane internalization by endocytosis, which was proved to accompany cortical granule exocytosis (Donovan and Hart, 1982, 1986); (3) elucidating whether animal/vegetal differences in surface relief and the mean thickness of MC correlate with differences in cortex contractility.

Taken together, our results demonstrate spatial differentiation of the loach egg with respect to cortical contractility, the ability to contract being associated with endocytotic activity and complicated relief of the plasma membrane.

Results

Animal/vegetal changes in surface relief and endocytosis during ooplasmic segregation

The location of the animal pole in unfertilized eggs is identifiable by the presence of the conical depression which represents the sperm entry site (Fig. 1a). In the intact egg the conical depression is situated beneath the micropyle. Numerous microvilli are distributed on the egg surface both in the animal and vegetal hemispheres (Fig. 1b - 1d). Cortical granules under the surface are seen as dark, circular areas (Fig. 1c, 1d). Near the conical depression, cortical granules are absent (Fig. 1a, 1b).

Fifteen min after insemination, the egg surface has numerous random folds, though polar differences in relief can be seen (Fig. 2a - 2d). The animal pole surface (Fig. 2b), and the equator surface (Fig. 2c) have deeper folds as compared with the smoother vegetal pole surface (Fig. 2d). The egg looks like a sphere.

Fifty min after insemination, the ooplasm accumulates at the animal pole and forms the blastodisc. The eggs become pear-shaped (Fig. 3a). Polar differences in relief become more pronounced.The surface of the blastodisc has numerous crest-shaped folds (Fig. 3b), whereas the surface of the vegetal hemisphere is quite smooth (Fig. 3e). At the boundary of the blastodisc, numerous surface pits (0.2 - 0.5μm in diameter) are observed. The pits are often located close to scarce surface folds (Fig. 3c, 3d).

The eggs incubated in Lucifer yellow CH (LY) from the 5th to the 12th min after insemination contain numerous fluorescent vesicles both in the animal and vegetal hemispheres (Fig. 4c, 4d). The eggs show slight polar differences in endocytosis, with fluorescence at the animal pole higher than at the vegetal pole (Fig. 4a, 4b).

In the eggs incubated in LY from the 35th to the 45th min, fluorescent vesicles are observed exclusively in the animal hemisphere (Fig. 5c, 5d). No fluorescent vesicles were found in the vegetal hemisphere (data not shown). The boundary of the area populated by fluorescent vesicles coincides with that of the blastodisc folded surface (Figs. 3a - 3e, 5a, 5b, 5d).

Regionalization of the surface of two-cell embryos with respect to relief and endocytosis

At the final stage of the 1st cleavage division, the surface of the yolky part of the egg remains quite smooth. At the boundary of the blastodisc, many surface pits are observed and are located very close to rare surface folds (Fig. 6d). Prominent regional differences in the blastomere surface relief were observed (Fig. 6a - 6g). The surface area away from the cleavage furrow has numerous crest-shaped folds (Fig. 6b). These folds are tightly clustered in an order that forms macrofolds, visible under low magnification (Fig. 6b). At the top of the blastomere, the surface has many shallow folds (Fig. 6c), and is smoother than in regions further from the cleavage furrow. The lamellar formations (ruffles) (according to the terminology of Bozhkova *et al.*, 1983) were observed along the furrow (Fig. 6f); these can also be seen under low magnification (Fig. 6e). Near the ruffles, the surface is much smoother (Fig. 6f) than further away from the cleavage furrow. The surface bordering the cleavage furrow is characterized by the presence of numerous spherical protrusions (0.07 - 0.2 μm in diameter - Fig. 6g).

Eggs incubated in LY during the 1st cleavage division contain fluorescent vesicles in the cortex of blastomeres exclusively (Fig. 7a, 7b). The boundary of the area populated by fluorescent vesicles coincides with that of the blastodisc folded surface (Figs. 6a, 6d, 7a, 7b). At the beginning of the 2nd cleavage division, the eggs (incubated in LY throughout the 1st cleavage division) expose surface areas containing rare fluorescent vesicles. These areas are

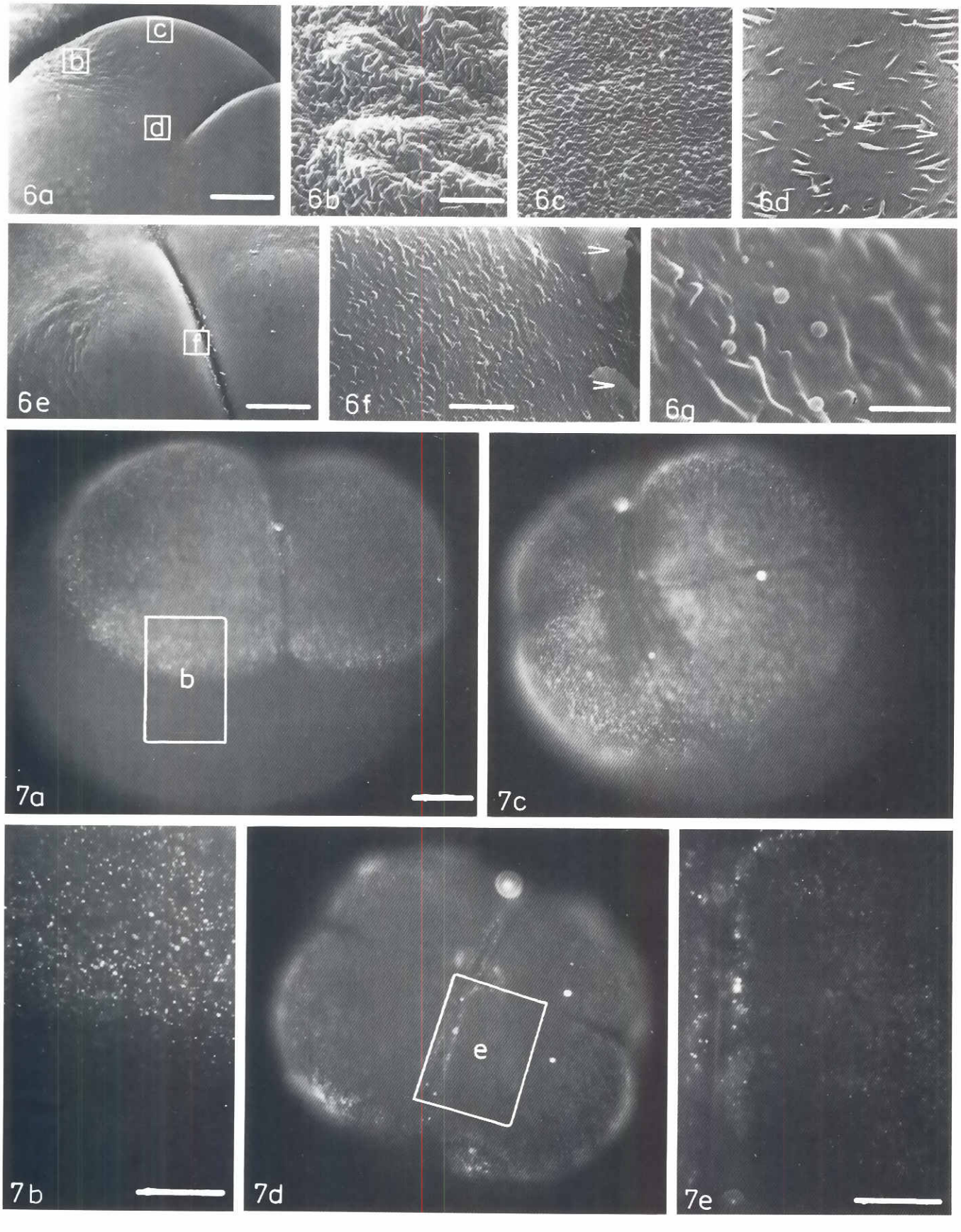
Fig. 1. SEM of the unfertilized egg. (1a) *Low magnification view of the animal pole area showing the conical depression (arrowhead) in the center of the animal hemisphere. Location of cortical granules beneath the surface are seen as numerous dark circlets. Rectangles (b) and (c) denote the areas shown in panels (1b) and (1c), respectively. Bar, 100 μm.* **(1b)** *Area of the egg close to the conical depression is free of subjacent cortical granules. Bar, 5 μm.* **(1c)** *Animal pole area. Arrowheads indicate location of cortical granules beneath the egg surface. Magnification is the same as in (1b).* **(1d)** *Vegetal pole area. Arrowheads indicate location of cortical granules. Magnification is the same as in (1b).*

Fig. 2. SEM of the egg 15 min after insemination. (2a) *Low magnification view. A, animal pole; V, vegetal pole. Rectangles (b), (c), (d) denote the areas shown in panels (2b), (2c) and (2d), respectively. Bar, 0.5 mm.* **(2b)** *Animal pole area. Bar, 5 μm.* **(2c)** *Equator area.* **(2d)** *Vegetal pole area. Magnification in (2c) and (2d) is the same as in (2b).*

Fig. 3. SEM of the egg 50 min after insemination. (3a) *Low magnification view. A, animal pole; V, vegetal pole. Rectangles (b), (c), (d), (e) denote the areas shown in panels (3b), (3c), (3d) and (3e), respectively. Bar, 0.5 mm. (3b) Animal pole area. Bar, 5 μm.* **(3c-d)** *Areas at the boundary of the blastodisc. Arrowheads indicate the pits.* **(3e)** *Area of the vegetal part of the egg. Magnification in (3c), (3d) and (3e) is the same as in (3b).*

Fig. 4. Fluid-phase endocytosis of LY during the time interval from the 5th to the 12th min after insemination. *Eggs were photographed 18-20 min after insemination.* **(4a)** *Bright field micrograph. A, animal pole; V, vegetal pole. Bar, 0.5 mm.* **(4b)** *Fluorescence micrograph of the same egg.* **(4c)** *Fluorescence micrograph of the animal pole area. Bar, 100 μm.* **(4d)** *Fluorescence micrograph of the vegetal pole area. Magnification is the same as in (4c).*

Fig. 5. Endocytosis of LY during the time interval from the 35th to the 45th min after insemination. *Eggs were photographed ca. 50 min after insemination.* **(5a)** *Bright field micrograph. A, animal pole; V, vegetal pole. Bar, 0.5 mm.* **(5b)** *Fluorescence micrograph of the same egg. Rectagle denotes the area shown in panel (5d).* **(5c)** *Fluorescence micrograph of the animal pole area. Bar, 100 μm.* **(5d)** *Fluorescence micrograph of the area at the boundary of the blastodisc. Magnification is the same as in (5c).*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located along the 1st cleavage furrow (Fig. 7c). As the 2nd cleavage division proceeds, the contacts of the first blastomeres become partially broken (Bozhkova *et al.*, 1983), and two rows of bright fluorescent vesicles (formed during the 1st cleavage division) can be seen along the bottom of the 1st cleavage furrow (Fig. 7d, 7e). The blastomere surface shows a complex non-uniform distribution of fluorescent vesicles (Fig. 7e).

Structure of the subplasmalemmal cytoskeleton

MC in loach eggs was revealed as an electron-dense layer separating the plasma membrane from cell organelles - mitochondria, membranous vesicles, particles (25 - 30 nm diameter) which are probably ribosomes (Figs. 8a, 10a, 10b, 11a, 11b).In the fertilized eggs, the MC underlying the smooth surface of the vegetal hemisphere (Fig. 10a, 10b) and the areas between folds in the animal hemisphere (Fig. 11a, 11b) is 0.15 - 0.25 μm thick. MC is the main cytoplasmic component of surface folds (Fig. 11a) which vary in height from a small protrusion of the surface to about 0.8 μm. Since the surface folds are filled with electron-dense MC, the mean MC is thicker in folded areas of the egg than in smooth ones (cf. Figs. 10a and 11a).

MC represents a network of microfilaments that are visible under high magnification (Figs. 9a, 10b, 11b). The density of the microfilamentous network appears to be roughly equal in both the vegetal and animal parts of the fertilized egg (cf. Figs. 10b and 11b). The areas with the lowered density of the microfilamentous network were observed in the central regions of many folds (Figs. 9a, 11b). In these areas the ribosome-like particles (Figs. 9a, 11b) and, sometimes, vesicles were observed.

The majority of the cortical microfilaments are randomly arranged so that only short pieces or cross sections can be seen (Figs. 9a, 10b, 11b). In the unfertilized eggs the microfilaments filling the microvilli are occasionally oriented parallel to the microvillus axis (Fig. 8b). In the fertilized eggs some of the microfilaments are aligned parallel to cortical tension, *i.e.*, perpendicular to the egg's radius (Figs. 9a, 11b). The aligned microfilaments were frequently observed in the blastodisc cortical areas at the base of many surface folds (Figs. 9a, 11b). The characteristic feature is that the aligned microfilaments are usually located at the boundary between the MC and the ooplasm (Figs. 9a, 11b), with these areas of the MC showing increased electron density in low magnification electron micrographs (Fig. 11a). The aligned microfilaments were not observed in the areas of MC adjoining the plasma membrane.

In the animal hemisphere of the fertilized egg the coated vesicles located in the MC (Fig. 9b) as well as in the deeper ooplasm can be seen sometimes.

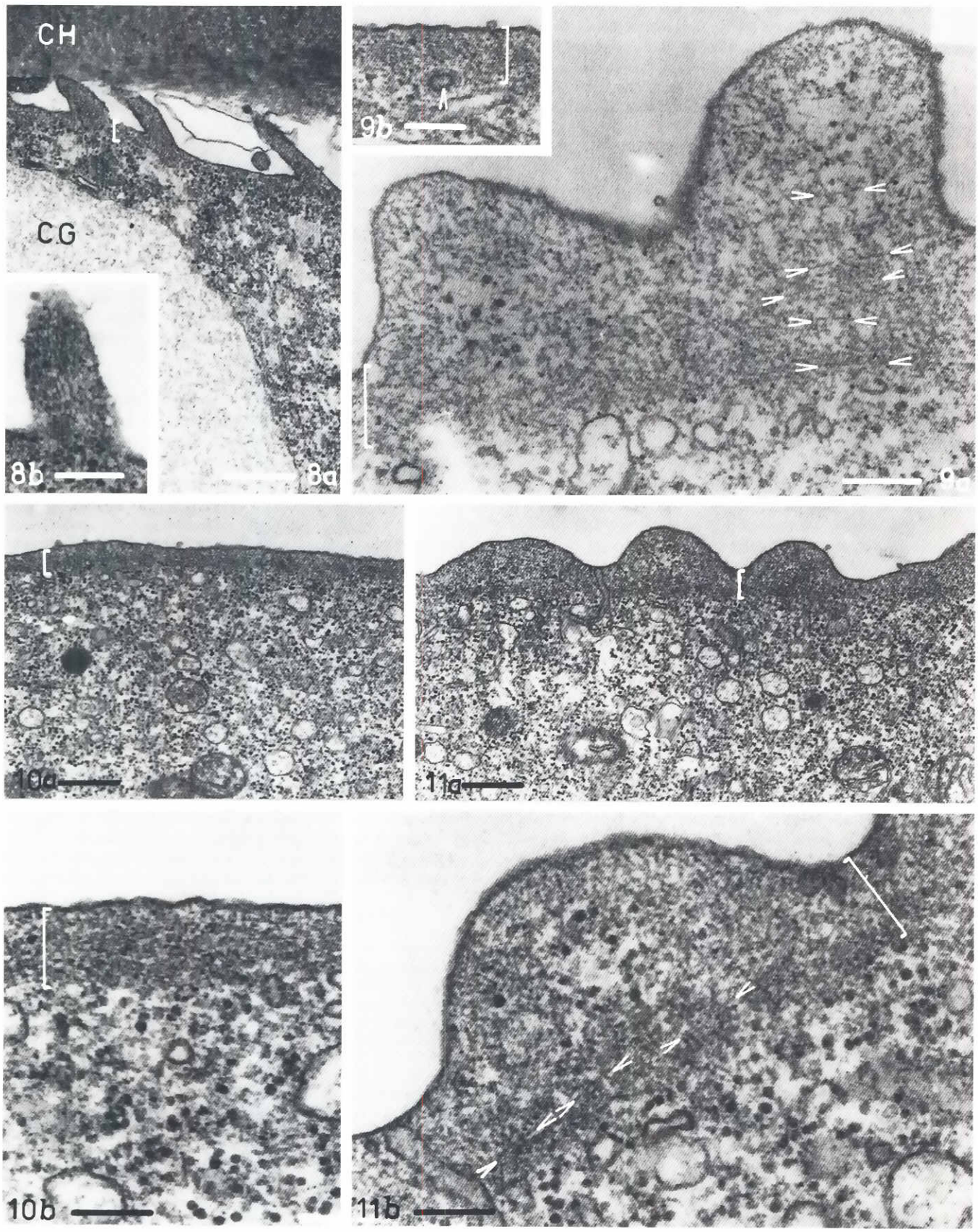
Contractile responses of eggs provoked by the decrease in surface tension

In order to determine whether the polar differences in surface relief and the mean thickness of MC correlate with the differences in contractability, we decreased the tension of the egg surface and studied the provoked contractile responses. The experiments are depicted schematically in Fig. 12. During blastodisc formation, streaks of ooplasm distributed among yolk granules move toward the animal pole. When the blastodisc is fully developed, the yolky part of the egg is represented by tightly packed yolk granules surrounded by a peripheral layer of ooplasm (Fig. 12a). To decrease the tension of the egg surface, a micropipette was introduced into the yolky part of the egg (in the animal pole, vegetal pole, or the equator), and a portion of yolk granules (1/2 - 2/3 of the initial volume, approximately) was sucked out of the egg (Fig. 12b). The highly viscous consistency of the yolk granules did not allow for rapid suction. Usually, suction continued for about 1.5 - 2 min. As the egg volume diminished and the surface tension decreased, the egg assumed an irregular flattened shape (Fig. 12c). The typical pear-shaped form of the egg was usually regained 3 - 10 min after suction (Figs. 12d, 13a). Rough comparisons between the original (Fig. 3a) and the resulting (Fig. 13a) egg volumes suggest approximately a 3-fold decrease during the experiments. This means that the area of the egg surface soon after the suction was about twice that necessary to cover the residual volume of yolk granules in the pear-shaped egg.

Eggs with the restored pear shape showed polar differences in the surface relief similar to those in intact eggs (Fig. 13a - 13d). The folded surface of the blastodisc only produced contractile responses provoked by the decrease in surface tension: numerous clusters of tightly packed folds appeared (Fig. 13b), and local contractions of the egg surface resulted in the formation of "star-like" complexes (Fig. 13e - 13h). This term - "star-like" complex (SLC) - was used by Roubaud and Pairault (1980), who observed similar structures in the intact blastulas of *Brachydanio rerio*. The position of the boundary between the folded and smooth surfaces was displaced towards the vegetal pole as compared with the intact egg. At the boundary, numerous surface pits were seen (Fig. 13c). A few surface pits were also found in the vegetal pole area (Fig. 13d).

Fig. 6. SEM of the embryo 70 min after insemination, at the stage of the 1st cleavage division completion. (6a) *Low magnification side view. Rectangles (b), (c), (d) denote areas shown in panels (6b), (6c) and (6d), respectively. Bar, 100 μm.* **(6b)** *Area distant from the cleavage furrow. Bar, 5 μm.* **(6c)** *Area at the top of the blastomere.* **(6d)** *Area at the boundary between the blastodisc and the yolky part of the egg. Arrowheads indicate the surface pits. Magnification in (6c) and (6d) is the same as in (6b).* **(6e)** *Low magnification top view of the same embryo as the one shown in (6a). Rectangle (f) denotes the area shown in panel (6f). Bar, 100 μm.* **(6f)** *Area in the proximity of the cleavage furrow. Arrowheads indicate lamellar formations. Bar, 5 μm.* **(6g)** *Part of panel (6f) at higher magnification. Bar, 1 μm.*

Fig. 7. Fluorescence microscopy of LY endocytosis during the 1st cleavage division. (7a) *The embryo incubated in LY from the 50th to the 60th min, and photographed ca. 65 min after insemination. Rectangle (b) denotes the area shown in panel (7b). Bar, 0.2 mm.* **(7b)** *Area at the boundary of the blastodisc. Bar, 100 μm.* **(7c)** *The embryo incubated in LY from the 60th min (beginning of the 1st cleavage furrow formation) to the 75th min (completion of the 1st cleavage division), and photographed ca. 80 min after insemination (onset of the 2nd cleavage division). The 1st cleavage furrow is vertical in the picture. Magnification is the same as in (7a).* **(7d)** *The embryo incubated in LY from the beginning to the completion of the 1st cleavage division (i.e. from the 60th to the 75th min after insemination), and photographed during the 2nd division completion (ca. 100 min after insemination). The 1st cleavage furrow is roughly vertical and decorated with fluorescent vesicles in the picture. Rectangle (e) denotes the area shown in panel (7e). Magnification is the same as in (7a).* **(7e)** *Distribution of fluorescent vesicles in the blastomere of the four-cell embryo. Bar, 100 μm.*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In the control experiments, local contraction was observed only at the site of puncture (data not shown).

To study the local surface contraction, we investigated the formation of SLCs. Forty seconds after suction, the least developed SLCs consisted of a huge central fold and numerous straight radial folds (Fig. 14a). Two minutes after suction, the huge central fold in all SLCs was surrounded by crest-shaped folds from which straight folds radiated (Fig. 14b). In the developed SLCs (3 - 10 min after suction) the central fold was less prominent, the number of surrounding tightly packed folds increased (Fig. 14c). The structure shown in Fig. 13f appears to represent a few fused SLCs.

Movement of the egg surface during ooplasmic segregation and the 1st cleavage division

Experiments with labeling present difficulties related to the removal of the chorion and cortical granule exudate soon after insemination, and the very weak ability of the egg surface to adsorb carbon particles. Distances between carbon particles located near the animal pole decreased by a factor of 1.15 - 1.25 during the formation of the blastodisc (Figs. 15a, 15b, 16a, 16b). During the 1st cleavage division, carbon particles located on the surface of different blastomeres moved away from each other, and perpendicularly away from cleavage plane (Figs. 15b, 15c, 16b, 16c). Most dramatic was the poleward movement of the particles, which lay in the future furrow (cf. Fig. 16c and 16b, particles 2 and 5).

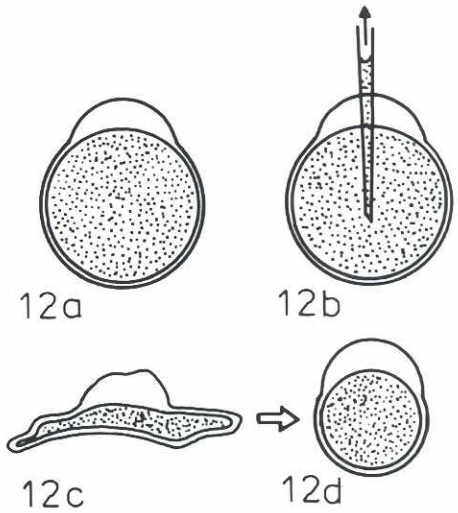


Fig. 12. Schematic representation of experiments on suction of yolk granules out of eggs. (12a) *The egg with formed blastodisc 40-45 min after insemination. Yolky part of the egg is marked with dots.* **(12b)** *The egg was punctured in the animal pole area, and suction of yolk granules was started.* **(12c)** *The egg after suction was completed, and micropiprette was withdrawn.* **(12d)** *The egg rapidly regains a pear-shaped form.*

Discussion

Our data show that ooplasmic segregation in the loach egg correlates with a remarkable polarization of the cell surface, as regards its contractility, relief and the intensity of fluid-phase endocytosis. Complex relations between these phenomena could be partly explained by our observations concerning the MC structure and changes in membrane folds.

The structure of MC

In the loach egg, MC is a three-dimensional network of randomly oriented microfilaments (Ivanenkov *et al.*, 1987), spaced 15 - 20 nm apart (Figs. 10b, 11b). The MC separates cell organelles - vesicles, mitochondria and ribosome-like particles - from the plasma membrane. These facts may indicate that MC is a gel, where special proteins link microfilaments to each other and bind them to the plasma membrane. A similar structure of the subplasmalemmal cytoskeleton has been described for the other cell types (Stossel *et al.*, 1981; Hartwig *et al.*, 1985).

In the loach egg the MC is almost equally thick (about 0.2 μm) in regions with smooth surface, e.g. vegetal areas (Fig. 10a, 10b) or between folds observed in animal areas (Fig. 11a, 11b). Within surface folds, the depth of the fold adds to the MC thickness (Fig. 11a). To understand this MC structure, let us first consider possible determinants of MC thickness in smooth surface areas. Since microfilaments appear to be involved in a continuous assembly-disassembly in living cells (Wang, 1985; Korn *et al.*, 1987), the MC thickness may be determined by the dynamic equilibrium between polymerization and depolymerization of the cortical actin. The nucleating centers are confined to the plasma membrane, whereas the depolymerizing factors are mainly located in the cytoplasm. There are several pieces of evidence to support this suggestion. First, we could make MC thinner or thicker by locally applying depolymerizing (DNase I) or stabilizing (phalloidin) agents (Ivanenkov *et al.*, 1987, 1990). Second, in a preliminary study we have isolated two proteins from the loach egg cytoplasm, which decrease the viscosity of F-actin solutions *in vitro*. One of them proved to be Ca^{2+}-dependent (unpublished data). These proteins are likely to affect

Fig. 8. TEM of the cortical area of the unfertilized egg. (8a) *Low magnification view. Bracket denotes the subplasmalemmal MC. CH, chorion; CG, cortical granule. Bar, 0.5 μm.* **(8b)** *Microvillus at higher magnification. Note the aligned microfilaments. Bar, 0.2 μm.*

Fig. 9. Electron micrographs showing features of the MC in the animal hemisphere of the egg. *The egg was fixed 45 min after insemination.* **(9a)** *TEM of the blastodisc cortical area. Individual microfilaments aligned parallel to cortex tension (i.e. perpendicular to the egg's radius) can be seen (indicated with arrowheads) in the thin section. Bracket denotes the MC between folds. Bar, 0.2 μm.* **(9b)** *Coated vesicle (indicated with arrowhead) in the cortical area at the boundary between the blastodisc and the yolky part of the egg. Bracket denotes the MC. Bar, 0.2 μm.*

Fig. 10. TEM of the cortical area in the vegetal part of the egg. *The egg was fixed 45 min after insemination.* **(10a)** *Low magnification view. Bracket denotes the MC. Numerous ribosome-like particles are separated from the plasma membrane by the MC. Electron-dense body is probably a primary lysosome. Bar, 0.5 μm.* **(10b)** *Cortical area at higher magnification. Bracket denotes the MC. Bar, 0.2 μm.*

Fig. 11. Electron micrographs of the blastodisc cortical area (taken from the same section as Fig. 10). (11a) *Low magnification view. Bracket denotes the MC between folds. Bar, 0.5 μm.* **(11b)** *Part of panel (11a) at higher magnification. Arrowheads indicate microfilaments aligned parallel to cortex tension. Bracket denotes the MC. Bar, 0.2 μm.*

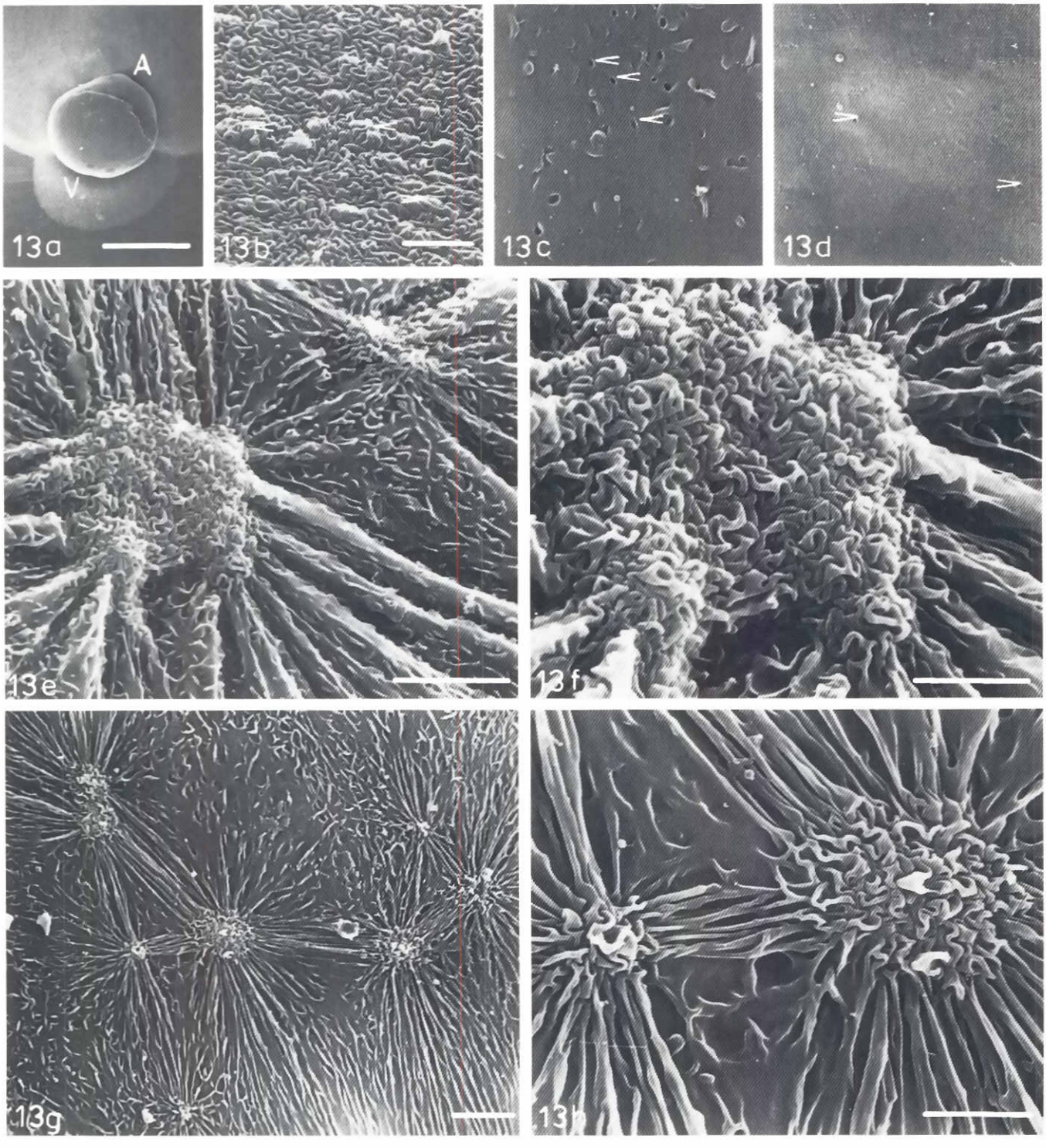
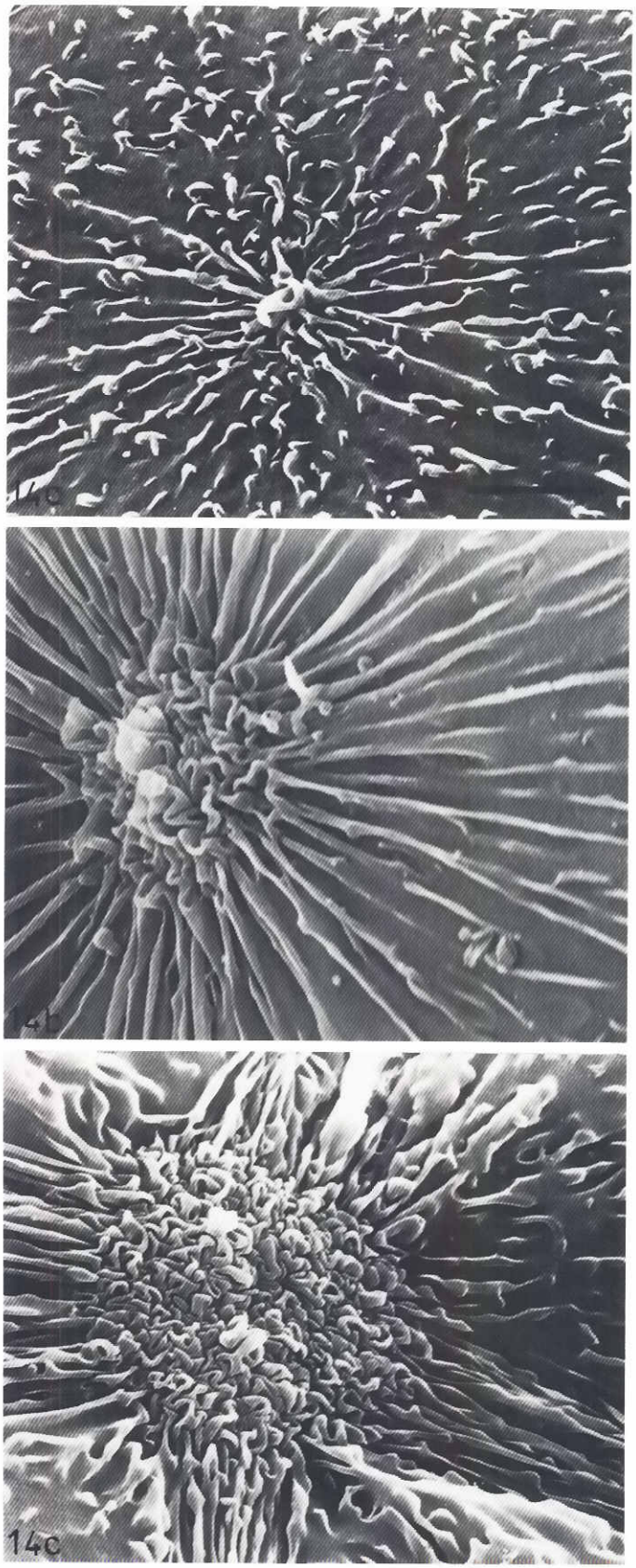


Fig. 13. Contractile responses of the egg to the decrease in surface tension caused by suction of yolk granules. (13a) *Low magnification view of the egg fixed 10 min after suction. The egg cracked when it was placed in an electron microscope. A, animal pole; V, vegetal pole. Bar, 0.5 mm.* **(13b)** *Surface of the blastodisc. Arrowheads indicate clusters of bulging folds. Bar, 5 μm.* **(13c)** *Vegetal hemisphere area near the equator. Arrowheads indicate numerous surface pits. Magnification is the same as in (13b).* **(13d)** *Vegetal pole area. Arrowheads indicate scarce surface pits. Magnification is the same as in (13b).* **(13e)** *Local contractions of the blastodisc surface resulted in the formation of SLCs. Bar, 10 μm.* **(13f)** *Part of panel (13e) at higher magnification. Bar, 5 μm.* **(13g)** *SLCs at the boundary of the blastodisc. Bar, 10 μm.* **(13h)** *Part of panel (13g) at higher magnification. Bar, 5 μm.*



MC thickness. Third, the plasma membrane appears to contain several factors, favoring actin polimerization and anchoring microfilaments to the membrane (Niggli and Burger, 1987). If polymerizing and depolymerizing factors are balanced and equally distributed under the smooth surface areas, this could explain the uniform MC thickness (about 0.2 μm).

Are these considerations valid for interpretating MC thickness in folded areas? In seeking an answer, one cannot exclude the possibility that fold formation, in general, may be accomplished through several rather different mechanisms.

Mechanisms of surface folding and cortical contraction

Dealing with the formation of lamellipodia, Oster and Perelson (1987) have proposed a mechanism by which cell protrusion is driven by osmotic forces, coordinated with the polymerization of the actin network. The latter fills such protrusions and undergoes remodeling due to reversible severing of microfilaments by Ca^{2+}-sensitive proteins. This model predicts a higher amount of actin nucleating factors in areas of prospective folds compared to smooth areas of MC. Another mechanism of protrusions must operate, if both folded and smooth membrane regions do not differ in this respect.

Our morphological observations indicate that "folding" is the most probable way for surface fold formation in the loach egg. The fold width is, as a rule, twice the MC thickness in smooth areas, *i.e.* 0.3 - 0.5 μm (Fig. 3b, 3c). In addition, the dynamics of SLCs contributes much to the understanding of a possible mechanism of fold formation. SLC initially forms as a huge multilobed fold (Fig. 14a), probably from a pre-existing single membrane fold. Randomly oriented radial folds 0.3 - 0.5 μm wide appear around the central fold, so that neighboring surface patches protrude and approach each other until the distance between them corresponds to the double MC thickness (Fig. 14b, 14c). Since ATP is required for *in vitro* contraction of cortical fragments, and contractility is lost after the cortical microfilamentous gel is destroyed by DNase I (Ivanenkov *et al.*, 1987), acto-myosin interactions (e.g. of sliding-filament type) can be considered as a molecular basis of cortical contractility in the loach egg.

Taking into account correlations between contractility, folding and the described details of MC structure, we can put forward a hypothesis that fold formation may serve as a major type of cortical contraction during the precleavage period of loach embryogenesis. There must therefore exist a single molecular mechanism underlying both contraction and folding (Fig. 17).

TEM revealed that various states of cortical behavior did not greatly affect the density of the microfilamentous network and interfilament distance. We can infer from this observation that the cortical microfilamentous gel lacks the ability to contract rapidly and cause further diminishing of network interstices. Such a property might arise from the critical concentration of cross-links within MC in the loach egg, which makes the rapid myosin-dependent sliding of microfilaments impossible (e.g. in AA' direction in Fig. 17a). There is, however, some possibility of interaction with myosin and mutual sliding for free microfilament ends at the border between the

Fig. 14. Development of SLCs. *SLCs were observed 40 sec* **(14a)**, *2 min* **(14b)**, *and 10 min after suction* **(14c)**. *Bar, 5 μm.*

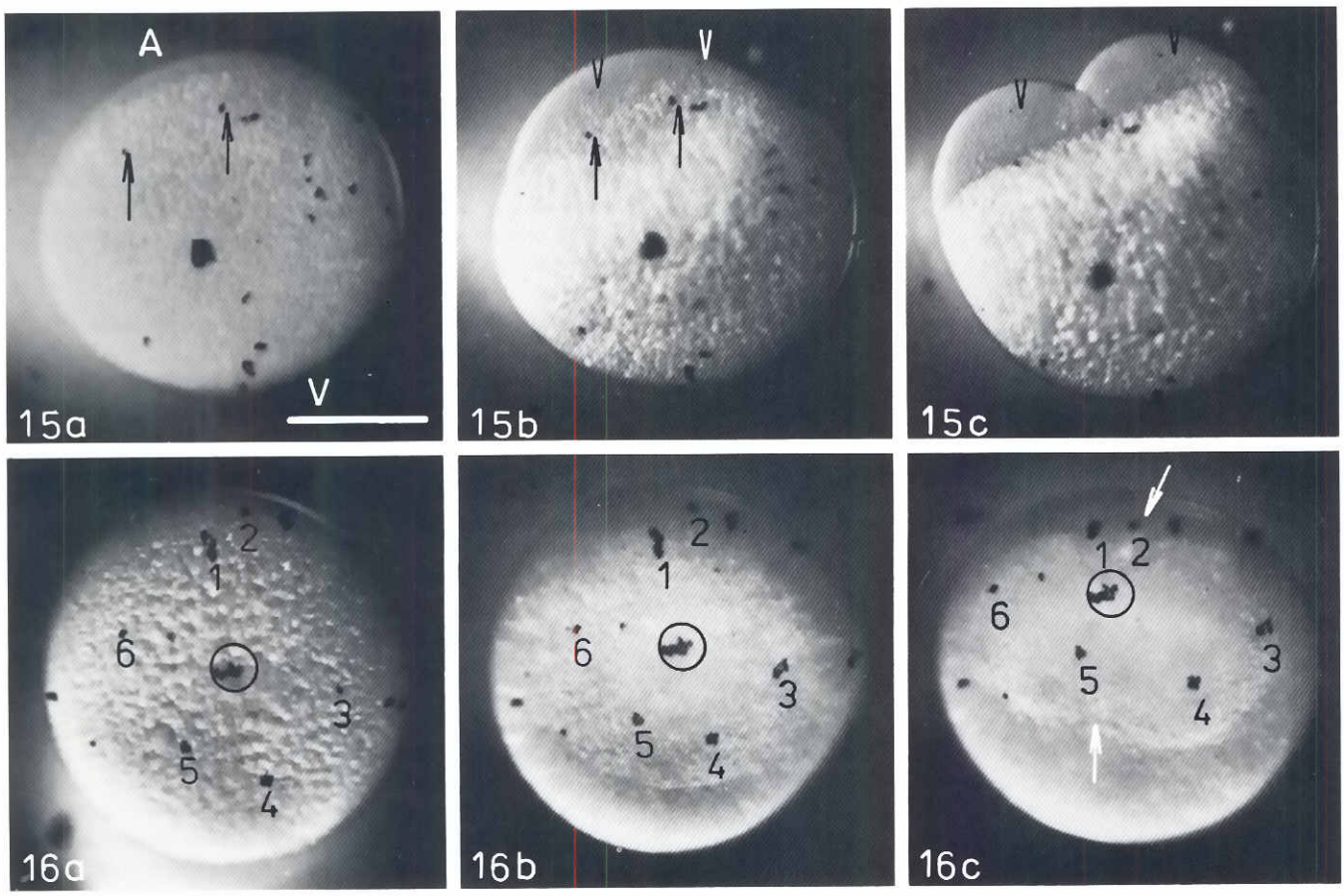


Fig. 15. Movement of the egg surface during ooplasmic segregation and the 1st cleavage division (side views). *The egg was photographed 20 min* **(15a)**, *50 min* **(15b)**, *and 70 min* **(15c)** *after insemination. Arrows indicate carbon particles which drew together during blastodisc formation. Arrowheads indicate carbon particles which moved away from each other during the 1st cleavage division. A, animal pole; V, vegetal pole. Bar, 0.5 mm.*

Fig. 16. Movement of the egg surface during blastodisc formation and the 1st cleavage division (top views). *The egg was photographed 20 min* **(16a)**, *50 min* **(16b)**, *and 70 min* **(16c)** *after insemination. The encircled carbon particle is located roughly at the animal pole. Numerals 1-6 designate carbon particles. Movement of particles (1-6) was towards the animal pole during blastodisc formation (cf. Fig. 16b and 16a). Particles 3 and 4 moved perpendicularly away from the cleavage plane (cf. Fig. 16c and 16b). Particles (2, 5) located in the cleavage furrow plane moved towards the animal pole (cf. Fig. 16c and 16b). Magnification is the same as in Fig. 15.*

MC and the endoplasm (Fig. 17a). A casual surface bending (Fig. 17b) promotes an interaction of cytoplasmic ends of microfilaments and the generating of a force, which acts upon the cortex along BB' direction (Fig. 17b), and tends to pull adjacent regions of MC to the midline of the surface bending. The interacting adjacent regions of MC eventually join each other, giving rise to a fold. The excess of surface would favor formation of more numerous deeper folds (Fig. 17c), as observed in eggs with experimentally reduced volume. We may conventionally designate this mechanism of folding as a "zipper-like" model.

Apart from the data already mentioned, there is additional support for this view. First, some vesicles and ribosomes could be seen in central regions of many folds (Figs. 9a, 11b), suggesting that during the fold formation, some endoplasmic organelles become trapped between adjoining regions of MC. These observations seem to be crucial for making a choice between the proposed "zipper-like" model of folding or the other conceivable model which regards folding as a result of the microfilamentous gel contraction. According to the latter model, the increase in density of the microfilamentous network should be expected in the central regions of folds due to contraction of the cortical gel. On the other hand, the "zipper-like" model suggests that some of the ooplasmic organelles may be trapped between the adjacent regions of MC during "fastening" of the "zipper". Thus, the lowered density of the microfilamentous network and the presence of ooplasmic organelles in the central regions of many folds support the "zipper-like" model.

Second, consistent with our idea is a correlation between surface relief and contractility: the folded animal surface contracted when the internal pressure was artificially lowered, whereas the smooth vegetal surface remained unchanged (Fig. 13). This suggests that surface roughness favors folding.

Third, the microfilaments aligned parallel to cortical tension were observed in the very areas of MC which were suggested in the

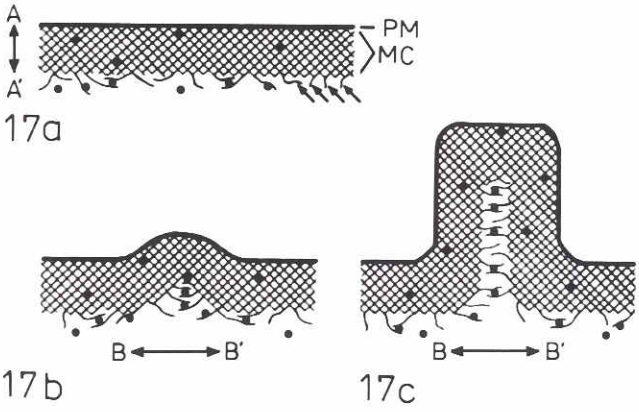


Fig. 17. Schematic representation of surface fold formation. (17a) *MC structure in the area with smooth surface. PM, plasma membrane; MC, microfilamentous cortex. Arrows indicate cytoplasmic ends of cortical microfilaments. Black circles represent myosin. Double arrow (AA') indicates direction perpendicular to the egg surface (see the text).* **(17b)** *Low surface fold. Interaction of cytoplasmic ends of cortical microfilaments with myosin creates tension in BB' direction.* **(17c)** *High surface fold. Symbols are the same as in (17b).*

"zipper-like" model, *i.e.*, at the boundary between the MC and the ooplasm, and at the base of many folds (cf. Figs. 9a and 17b).

Our hypothesis states that fold formation serves as an elementary event of cortical contraction. This line of thought corresponds well to the results of Roubaud and Pairault (1980), who observed SLCs in the intact blastulas of *Brachydanio*. These authors suggested that surface folds increase surface tension and reflect the organization of contractile microfilamentous structures. We would stress, however, that a putative "zipper-like" mechanism can provide, for the most part, rapid contractile processes not requiring major restructuring of MC gel, as opposed to slow extensive deformations due to continuous rearrangements of the cross-links described by Sato *et al.* (1987).

It is worth noting that microvilli in the mature unfertilized egg (Figs. 1b - 1d, 8a, 8b) are narrower than surface folds in the activated egg. The microvilli differ from the surface folds with respect to their cytoskeleton organization, representing in some cases the orientation of microfilaments parallel to the microvillus axis (Fig. 8b). In the mature egg, microvilli are derivatives of oocyte microvilli, by means of which a growing oocyte makes contact with follicle cells (Iwamatsu and Ohta, 1977). Oocyte microvilli probably arise in another way as do folds in the fertilized egg (Oster *et al.*, 1985).

Correlation between the surface relief and endocytosis

In the egg of *Brachydanio rerio*, as shown by Donovan and Hart (1982, 1986), the internalization of the plasma membrane through the coated and smooth vesicles closely follows the cortical reaction on activation. We confirmed this for the loach egg and found, moreover, that endocytosis occurs at least up to the four-cell stage. Five to ten minutes after activation, the uptake of LY is observed over the entire egg surface, whereas later, endocytosis is confined to the animal hemisphere, and restricted within the folded surface only.

At present it is unclear why endocytosis is intimately correlated with folding. Coated vesicles found in the MC (Fig. 9a) and in the deeper ooplasm suggest that membrane internalization is performed through coated pits and vesicles to a certain extent at least. Concave surface regions at the base of folds may be the sites of clathrin coat assembly (Rodman *et al.*, 1984) and coated pit formation. Betchaku and Trinkaus (1986) found a correlation between fluid-phase endocytosis and surface folding during epiboly in *Fundulus* embryos, and proposed that endocytic vesicles could arise when the opposed surface of adjacent folds fuse. Both suggestions are compatible with our finding of numerous pits 0.2 - 0.5 μm in diameter at the edge of the folded surface (Figs. 3c, 3d, 6d, 13c). Endocytosis appears to occur in these pits either by the pit closing into a vesicle, or by smaller vesicles budding off the bottom of pits.

According to Donovan and Hart (1986), at the early stages of fish egg development, endocytosis serves to take up the excessive plasma membrane. The latter was created during oogenesis in the form of numerous microvilli, and during activation of the egg, which results in the construction of a mosaic plasma membrane consisting of the original egg plasma membrane and membranes of cortical granules. We would ascribe to endocytosis in the loach egg at least one additional function. Within 50 min after insemination, the plasma membrane is involved in endocytosis so that the vegetal surface becomes completely smooth, whereas only the blastodisc surface remains folded and thus retains a higher capacity for contraction. Endocytosis thus enhances the polarized distribution of contractile cortical elements.

Possible significance of polar distribution of cortical contractile structures

Ooplasmic segregation in eggs of various animals seems to be associated with the polarized contractile activity of the egg cortex (Sawada, 1988). We also observed polarized cortical contraction in the loach egg. During the blastodisc formation (from the 20th to the 50th min after insemination) the surface area of the animal hemisphere was reduced by approximately a factor of 1.5. Assuming that there is a structural connection between the MC and subcortical ooplasm (Ivanenkov *et al.*, 1987), it may be concluded that polar contraction of the MC might contribute to the gathering of the ooplasm and formation of the blastodisc at the animal pole of the egg.

Like the activated egg, early blastomeres of the loach embryo are also markedly regionalized in surface relief and endocytosis. Three specific membrane domains could be distinguished. The first domain is a relatively smooth surface of lateral zones of the 1st cleavage furrow, which contain rare fluorescent vesicles (Figs. 6f, 6g, 7c). We suggest that the plasma membrane forms *de novo* in these zones, probably by local exocytosis. This view was supported by experiments showing the movement of carbon particles away from the furrow. In addition, numerous spherical blebs, 0.07 - 0.2 μm in diameter, were seen in these zones (Fig. 9g), which might reflect "new" membrane insertions. The formation of the new membrane in the lateral furrow walls was observed in cleaving eggs of various animals (Bluemink and deLaat, 1973, 1977; Byers and Armstrong, 1986; Pratt and George, 1989).

The second membrane domain is represented by intensively folded regions most distant from the furrow, which show an intense uptake of LY (Figs. 6b, 7c). Contraction of these MC regions appears to favor the stretching of new membrane areas, where endocytosis is hampered.

The third membrane domain is the bottom of the furrow, where the excess of membrane is probably created during contraction. We suppose that the redundant membrane in this region might be taken up by endocytosis. The latter does occur, since LY resides there after the completion of the 1st cleavage division, and can be revealed near the lamellar structures (Fig. 6f) as two rows of fluorescent vesicles (Fig. 7d, 7e).

Materials and Methods

Obtaining of gametes

Mature eggs of *Misgurnus fossilis* were obtained and artificially fertilized as previously described (Neyfakh, 1959). The fertilized eggs were cultivated in dechlorinated tap water at 21.5°C.

Fluorescence microscopy with LY

When the eggs reached a certain stage, they were transfered to 1.5% Lucifer yellow CH (Sigma, U. S. A.) solution in tap water. The low molecular weight of the dye (M.W. 457.3) allowed its penetration across the fertilization membrane. Within 10 min, the eggs were washed twice in tap water and placed for 1 - 2 min in 0.5% trypsin (Trypsin 1:250, Serva Feinbiochemica, Heidelberg/ New York) solution in tap water to soften the fertilization membrane. The eggs were then extensively washed in tap water, the membrane being removed with sharp needles, and were placed in an observation chamber. The chamber was made by glueing a plastic washer 0.9 - 1.0 mm thick with a hole 6 mm in diameter to a microscopic slide with epoxy resin. After the eggs were placed in the hole in a drop of tap water, the chamber was covered with a cover glass. The eggs were then examined under a LUMAM U-2 fluorescence microscope (USSR) with an excitation wavelength filter of 360 - 420 nm and a 480 - 700 nm barrier filter.

Labeling the egg with carbon particles

Experiments were performed in Petri dishes, the bottom of which was layered with 1% agar solution in tap water. Eggs without fertilization membranes were placed in holes made in the agar layer, and powdered with carbon particles (Norit A, Serva Feinbiochemica, Heidelberg/ New York). The eggs were photographed at selected intervals.

Micromanipulations

Suctions of yolk granules out of the eggs were made 40 - 50 min after insemination with a glass micropipette 20 - 30 µm in diameter, using a MM-1 micromanipulator (USSR). The egg was punctured either in the area of the animal pole, or the vegetal pole, or the equator, and the tip of the micropipette was moved to the center of the egg. The suction continued ca. 2 min. The fertilization membrane of the egg was then removed, and after a certain period the egg was fixed for electron microscopy. In the control experiments, the tip of the micropipette was allowed to stay in the punctured egg also ca. 2 min, but no suction was made.

Electron microscopy

Fertilized eggs deprived of fertilization membranes as well as unfertilized eggs were fixed in a solution containing 2% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, for 24 h at 4°C, and, after washing, postfixed in 1% OsO_4 for 2 h. To prepare unfertilized eggs for SEM, chorions were carefully removed with sharp needles before postfixation in OsO_4. In the eggs fixed 15 min after insemination, the animal pole was identified, prior to postfixation in OsO_4, by the presence of the thickest layer of segregated ooplasm. Fine notches were then made on the equator with sharp needles for the proper orientation of the eggs for SEM.

For TEM, the preparations were dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate, and examined under a JEM 100CX electron microscope (Japan).

For SEM, the preparations were dehydrated in acetone series and dried at the critical point of carbon dioxide in an EIKO Critical Point Dryer DX-1 (Japan). The eggs were then coated with ca. 100 A° of gold and examined under a TEMSCAN-100CXII microscope (Japan).

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