NEW MEMBRANE FORMATION DURING CYTOKINESIS
IN NORMAL AND CYTOCHALASIN B-TREATED
EGGS OF XENOPUS LAEVIS

I. Electron Microscope Observations

JOHN G. BLUEMINK and SIEGFRIED W. DE LAAT

From the Hubrecht Laboratory, International Embryological Institute, Uppslalaan 1, Utrecht, The Netherlands

ABSTRACT

A method is described for measuring and calculating the preexisting surface in uncleaved Xenopus eggs and the rate of surface growth in cleaving eggs. Surface-marking experiments with cytochalasin B-treated eggs show that the unpigmented surface grows by de novo formation and not by expansion of preexisting pigmented surface. The onset of new surface formation during first cleavage was studied by using transmission electron microscope and scanning electron microscope techniques. At 3-4 min and at 7-8 min after the onset of cleavage the eggs were fixed in the presence of ruthenium red (RR). Evidence is presented that unpigmented surface representing new membrane comes into appearance between four and eight min. This surface has a selective binding capacity for RR. Concomitantly with the appearance of new membrane, endoplasmic reticulum (ER) cisternae are in continuity with, and dense cytoplasmic inclusions coalesce with, the membrane along the furrow. The latter give rise to liposome-like bodies. The possibility is discussed that the ER cisternae transport a surface exudate (a carbohydrate complex), that the dense cytoplasmic inclusions represent pools of membrane precursor, and that membranogenesis takes place by direct insertion of pooled precursors into the cell surface. In a second paper, these findings will be correlated with electrophysiological measurements.

INTRODUCTION

In an attempt to find the structural basis of the driving force underlying cytoplasmic cleavage, transmission electron microscope (TEM) studies of cytokinesis have so far been focused on the localization and orientation of filaments (Arnold, 1969, 1971; Berendsen, 1971; Bluemink, 1970, 1971a, 1971b; Fullilove and Jacobson, 1971; Kalt, 1971; Schroeder, 1968, 1970, 1972; Scott and Daniel, 1970; Selman and Perry, 1970; Szollosi, 1970; Tilney and Marsland, 1969). Dividing cells which were attached to their neighbors or eggs which had additional structures attached to their outer surfaces have provided strong evidence that surface growth also takes place during cytokinesis (Buck and Kristan, 1964; Dan et al., 1938; Dan and Dan, 1940; Dan, 1954a, 1954b; Pasteels and de Harven, 1962). In the amphibian egg Schechtmann (1937), using vital staining, observed areal growth in a narrow cortical zone along the upper edges of the furrow. Later studies by Selman and Waddington (1955) and by Zotin (1969) have provided addi-
tional evidence for new membrane formation during cytokinesis of the amphibian egg. The coalescence of membrane-bound bodies with the furrow surface, as observed in a variety of cells, has been interpreted as a possible mechanism for membrane growth (Ap Gwynn and Jones, 1972; Arnold, 1969, 1970; Fullilove and Jacobson, 1971; Kalt, 1971; Thomas, 1968). The direct insertion of precursor material into the cell surface during membrane growth has been suggested by Bluemink (1970, 1971b) who observed cytoplasmic pools of myelinic material in amphibian eggs.

A rapid fall in electrical membrane resistance and a concomitant rapid rise in electrical membrane potential occur in the course of the cleavage process (Woodward, 1968; de Laat et al., 1973). Taking the changes in electrical membrane characteristics as a criterion for the incorporation of new membrane surface (Woodward, 1968), it may be stated that the new membrane is produced during, not before or after, cleavage.

Luft (1971a, 1971b) introduced ruthenium dyes as contrast-producing and stabilizing agents in TEM microscopy. In combination with osmium tetroxide these dyes form complexes with phospholipids, and by doing so may also prevent phospholipids from being completely extracted during preparation. Using ruthenium violet, Szubinska (1971) noticed certain phenomena not hitherto observed during cell surface repair in wounded amebae. Ultrastructural changes in amebas which Szubinska regarded as a manifestation of membrane growth were later also observed during cortical wound healing in the amphibian egg (Bluemink, 1972).

In Xenopus eggs the membrane permeability characteristics change at ca. 7 min after the onset of cleavage (de Laat et al., 1973). Such a change could be brought about by the incorporation of new membrane surface. To analyze this, eggs were fixed in the presence of ruthenium red (RR) at 3-4 min and at 7-8 min after the onset of cleavage, i.e., just before and during the expected initiation of new membrane formation, respectively.

Cleaving eggs exposed to cytochalasin B undergo furrow regression and expose their interblastomeric surface to the outside (Bluemink, 1971a; de Laat et al., 1973; Hammer et al., 1971). This treatment creates conditions which facilitate the direct demonstration and rating of new membrane formation. In a second paper, the findings presented here will be correlated with changes in membrane permeability properties.

MATERIALS AND METHODS

Eggs of Xenopus laevis were collected in the laboratory from hormonally stimulated couples, decapsulated, and handled further as described earlier (Bluemink, 1972; de Laat et al., 1973).

I. Method for Measuring Total Egg Surface

The following method was used for measuring the total egg surface and its rate of expansion under experimental conditions. Eggs within the vitelline membrane were kept for 40-45 min in 1/40th strength Steinberg solution (Steinberg, 1957) and fixed just before the onset of cleavage. From the same batch, eggs without vitelline membrane were placed in dishes provided with a 5% agar bottom containing 1/40th Steinberg solution, either with or without 10 μg cytochalasin B (CCB) per ml and 1% dimethyl sulfoxide (DMSO). These eggs were also fixed just before the onset of cleavage. During fixation the diameter of the eggs was measured and found to remain constant within ±1%.

The eggs were accurately cut into halves vertically with a silicone-coated razor blade. Each half egg was positioned in a dish with a wax bottom having a small depression in such a manner that the cut surface was horizontal. Using a Zeiss 45° drawing attachment fixed to a dissecting microscope, the outline of the cut surface was accurately drawn at a magnification of × 65. Fig. 1 a and b present two such outlines: Fig. 1 a that of an egg within the vitelline membrane and Fig. 1 b that of an egg without the vitelline membrane.

As shown in the figures, the outline was then divided by a horizontal line (x axis) into an upper and a lower curve. The shape of the curve can be defined by five height measurements in various positions as indicated (see Fig. 1 a and b). Arrows h1 and h’1 divide the egg diamete (X) into two, while each of the successive levels (h2 and h2 etc.) divide the resulting parts into two again, so that the separations between the various levels are 1/4 d, 1/8 d, and 1/16 d. For the lower and the upper curve of each drawing the heights and the respective horizontal distances were measured with an accuracy of 0.1 mm using vernier callipers. Since in none of the eggs was the right half identical to the left one, the heights measured on either side were averaged. The values of the averaged heights together with the values of their relative positions define theoretical upper and lower curves for each egg measured. For both the upper and the lower curves a fourth degree polynomial was fitted to the five values obtained for the interval defined between the midpoint (h = maximal, x = 0) and the periphery (h = 0, x = maximal).

The surface formed by 360° rotation of the upper curve about the vertical axis would describe the...
idealized three-dimensional upper part of the egg surface and likewise for the lower part. The rotational surface was approximated by choosing interpolated values for an increasing number of equally spaced points along the x axis till the result showed no further change. The area that would arise by 360° rotation about the vertical axis (x = 0) was calculated for both the upper and the lower curve separately and summed. The actual egg surface area was obtained by dividing the total by the square of the magnification.

The principle for performing the measurements and the computer program for calculating the rotational surface areas were provided by Ir. J. J. Bezem of the Rijksuniversiteit Utrecht.

II. Method for Measuring New Membrane Surface in CCB-Treated Eggs

The following procedure was adopted to measure the unpigmented surface area formed during first cleavage in CCB-treated eggs. Eggs without vitelline membrane were exposed to 10 μg CCB per ml at 40-45 min before the onset of first cleavage and fixed at the onset of second cleavage. The fixed eggs had a girdle of unpigmented surface and they were very flat. The large upper and lower surfaces were fairly parallel, whereas the surface along the side had a small radius of curvature. The borderline between preexisting and new, unpigmented surface is well-defined (see Figs. 4-6). These conditions allow one to draw the outline of the unpigmented new surface and to calculate its area.

The outline of the new, unpigmented membrane on the upper surface was drawn according to the technique described above. Two punctures were made at the edges on either end of the upper unpigmented surface to demarcate two imaginary limits for this area (area A in Fig. 2). The outline of the new membrane area on the lower egg surface was likewise demarcated and drawn (area B in Fig. 2). Then the egg was turned with one side facing upwards. The projection was readjusted so that the two pairs of punctures on the upper and lower surfaces coincided with those on respective drawings, after which the outline of the intervening area of new membrane could be drawn (area C in Fig. 2). The whole procedure was then repeated for the opposite side (area D in Fig. 2).

The total area (areas A + B + C + D in Fig. 2) was measured planimetrically. 12 eggs were drawn and measured in this manner. The new surface area for each egg was calculated by dividing the value obtained by the square of the magnification rate.

CCB-treated eggs within the vitelline membrane had a band of unpigmented surface on their flat upper surface with small extensions at both ends lying on the lateral surface. The outline of the unpigmented band on the upper surface was drawn according to the method described above. Two punctures were then made at the edges on either end of the upper unpigmented surface to demarcate two imaginary limits for the area drawn. The remaining small extensions at either end were drawn after the egg was repositioned in order to allow these in their turn to be viewed in the horizontal plane. The total area was then measured and calculated as described above.

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III. Method for Measuring Interblastomeric Surface

To obtain an estimate for the rate of new membrane growth during normal first cleavage it was necessary to measure the interblastomeric surface area after two blastomeres were formed. The interblastomeric surface exists in the form of a double sheet of membrane. *Xenopus* eggs which had cleaved within the vitelline membrane were fixed at the onset of second cleavage. The vitelline membrane was removed and the two blastomeres were gently split apart. In eggs split successfully, the separated blastomeres showed an unpigmented inner surface with a well-defined outer perimeter (see Fig. 3). All eggs examined showed incomplete separation of the two blastomeres and had an area of continuous cytoplasm in an eccentric position (the shaded area in Fig. 3).

Each half egg was positioned in a dish with a wax bottom having a small depression in such a manner that the interblastomeric surface was horizontal. By using a Zeiss 45° drawing attachment fixed to a dissecting microscope, the outline of the split surface was accurately drawn at a magnification of X 65.

IV. Method for Measuring New Membrane Surface in Cleaving Eggs without Vitelline Membrane

Eggs without vitelline membrane were fixed at the onset of second cleavage. The two blastomeres were gently split apart. The unpigmented surface area of each half egg was drawn according to the procedure described above (method III). The area of the drawings was measured planimetrically, hereafter divided by the square of the magnification.

V. TEM

For routine TEM analysis eggs were prepared using two different aldehyde fixation mixtures as the primary fixative (see Bluemink, 1972). For eggs fixed in the presence of RR the following procedure was used. Freshly laid eggs were collected in groups at 5-min intervals. Groups of eggs of the same age devoid of their vitelline membrane were kept in 4 ml 1/16th Steinberg solution in Falcon plastic petri dishes (Falcon Plastics, Div. of B-D Laboratories, Los Angeles, Calif.) on a bed of 5% agar. In order to avoid the risk of fixing damaged eggs, the eggs were not manipulated for a period of 30 min before fixation. In one group all the eggs which entered cleavage within 1 min after the first one had started to cleave were fixed together with the latter, the other eggs being discarded. The eggs were fixed by adding 4 ml of double-strength fixing fluid to the culture dish. Fixation was at two different times: 3–4 min and 7–8 min after the onset of cleavage.

The normal strength fixing fluid used for routine fixation contained 2.5% glutaraldehyde, 1% acrolein (freshly distilled) in 0.05 M cacodylate buffer (pH 7.2–7.4), and 500–550 ppm RR. The RR solution was prepared following the salt precipitation method described by Luft (1971a). The purity and concentration of the stock solutions were checked by measuring the density and the spectrum with a Zeiss spectrophotometer PMQ II following the criteria given by Luft. The stock solutions had concentrations varying between 1,548 and 1,625 ppm when measured (after dilution) at a wavelength of 5,330 Å. The solutions did not contain appreciable amounts of ruthenium brown or violet; by using 1.0-cm quartz cells, the absorptions at wavelengths 3,600 and 7,500 Å of a solution containing 6 ppm RR wt/vol were determined to be 0.04 and 0.01.
respectively. The eggs were fixed for 18 h at room temperature. Without touching the eggs the fixation fluid was then replaced carefully by 0.05 M cacodylate buffer (pH 7.2–7.4). The latter was renewed several times. The eggs were postfixed for 3 h at room temperature in 2% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.2–7.4) containing 500–550 ppm RR.

To improve the conditions for dehydration and plastic impregnation, we cut away a small cap from the egg on either side laterally from the furrow. For easy orientation and to prevent damage during manipulation the eggs were embedded in small cubes of 5% agar. Dehydration was performed with cello-solve (without block staining), and for embedding a Dow epoxy resin mixture (Bluemink, 1971 b) was used. Untained sections were examined at 60 kV with a Zeiss EM-9 S2 electron microscope fitted with a 30-µm objective aperture.

VI. Scanning Electron Microscopy

For scanning electron microscope (SEM) analysis eggs were fixed in aldehyde and postfixed in osmium tetroxide in the presence of RR as described above. After dehydration in increasing concentrations of acetone, the eggs were dried according to the critical-point drying technique, as introduced by Anderson (1951), by using a Polaron critical-point drying apparatus, type E 3,000 (Polaron Equipment Limited, England). The dried specimens were fixed onto stubs, gold-coated, and examined at 5 kV with a Cambridge Stereoscan microscope. The SEM facilities were made available by the Centrum voor Submicroscopisch Onderzoek van Biologische Objecten, Rijksuniversiteit Utrecht, Dr. P. F. Elbers, director.

RESULTS

Surface-Marking Experiments

In most cleaving *Xenopus* eggs devoid of their vitelline membrane the unpigmented surface becomes manifest about 7–9 min after the onset of cleavage (de Laat et al., 1973). This unpigmented surface is considered to be the new membrane area (Selman and Waddington, 1955; Dan and Kuno-Kojima, 1963; Selman and Perry, 1970). If an egg is exposed to 5 or 10 μg CCB per ml during this phase of cleavage, the indented furrow smooths out and, while the unpigmented surface becomes part of the egg outer surface, it steadily increases in area (Bluemink, 1971 a). At the end of first cleavage the unpigmented surface forms a wide girdle around the egg (Figs. 4–6). The upper surface in such eggs is very flat which allows one to visualize the process of growth.

*Xenopus* eggs without vitelline membrane were transferred at the onset of first cleavage to a 5% agar bed in a dish containing 1/10th normal Steinberg solution with 5 or 10 μg CCB per ml and 1% DMSO. By using a capillary-braking pipette, tiny iron oxide particles were deposited on the surface in selected positions. The marked eggs were photographed at regular time intervals. From these photographs the areal changes of the pigmented and the unpigmented surface as well as the movements of the particles could be detected. The changes in relative positions of the particles, together with areal changes in pigment pattern, permit conclusions to be drawn with regard to surface growth. In Figs. 4–6 sequences are presented which show the areal changes recorded from three different eggs. Note the increase in egg diameter within each sequence and also that the area of unpigmented surface in two eggs represented in Figs. 5 and 6 differs greatly. For further details, see the legends of these figures.

In theory it is possible that a CCB-induced smoothing-out of the preexisting rough egg surface during cleavage contributes to the total increase of the surface area. If so, this may occur independently of the process of new surface formation. In TEM micrographs of the rough egg surface of uncleaved eggs an area measured between two points following the contour of the membrane yields a length of about 30% in excess of the linear distance. This means that if the surface irregularities would be smoothed out completely the membrane area available would be sufficient to cover 69% more area. The increase in distance between two particles having only unpigmented surface between them can be as much as ca. 185% (Fig. 5). A comparison with the maximal possible increase in linear distance resulting from smoothing-out (30%) leads to the conclusion that at least the major part of the unpigmented surface is newly formed surface which grows in area during cleavage. In addition, this experiment shows that the unpigmented surface does not grow at the expense of pigmented surface.

Total Surface Area of Uncleaved Eggs under Various Conditions

Viewed from the side, a *Xenopus* egg inside its vitelline membrane has the shape of a somewhat
The figures show three different *Xenopus* eggs (−VM), from three experimental series, during first cleavage in the presence of 10 μg CCB per ml. The dark dots are iron oxide particles deposited on the egg surface. Fig. 4 a, 9 min; b, 13 min; c, 17 min after the onset of cleavage. Particles along the midline of the furrow. During the first minutes of furrow regression the surface of the unpigmented zone greatly expands along its own length. The particles move apart at a speed of ca. 0.05 mm per min, while remaining aligned along the midline of the furrow. X 26.5.

Figure 5 a, 10 min; b, 18 min; c, 30 min after the onset of cleavage. Four particles are arranged in a line across the furrow (arrow, see Fig. 5 a). Two particles on the pigmented area, while two others are situated at the border between the pigmented and the unpigmented surface. During the increase in width of the unpigmented area the distance between each lateral pair of particles remains constant. It shows that unpigmented surface does not grow at the expense of pigmented surface. The distance between the two particles in the middle, having only unpigmented surface between them, increases by ca. 185% in 20 min. The velocity of linear increase is 0.02 mm per min. X 26.5.

Figure 6 a, 8 min; b, 17 min; c, 26 min after the onset of cleavage. Four particles, two on either long side of the unpigmented surface, together roughly forming a square. In the course of time the distance between each lateral pair remains constant, but the distance across the unpigmented zone increases by ca. 160% in 18 min. The velocity of linear increase is ca. 0.04 mm per min. X 26.5.
flattened sphere (Fig. 1 a). After the removal of the vitelline membrane the heavy yolk-laden egg flattens against the substrate, attaining a bun shape (Fig. 1 b). The tendency of the egg to flatten is counteracted by the mechanical rigidity of the egg surface. The rate of flattening of the egg is directly related to the rate of smoothing-out of its surface.

To test the possibility that CCB causes the rough surface to smooth out, we measured the total surface area of eggs within the vitelline membrane (without CCB) and that of eggs devoid of the vitelline membrane (with and without CCB), using method I. The results are presented in Table I.

With Student's t test it can be shown that the mean geometrical surface area of eggs inside the vitelline membrane (series 1) is smaller than that of eggs outside the vitelline membrane not exposed to CCB (series 2): P < 0.001. The mean geometrical surface area of the eggs in series 1 is also smaller than the surface area of CCB-treated eggs devoid of their vitelline membrane (series 3): P < 0.001. By comparing the means of series 2 and 3 it is evident that CCB treatment does not lead to an extra smoothing-out of the surface: P > 0.5. It may be concluded that the exposure of eggs to CCB in the period before the onset of cleavage does not affect the rigidity of the rough preexisting cell surface.

When the eggs are devoid of their vitelline membrane the geometrical surface area increases by about 7-8% because of the process of flattening. The rate of linear expansion required to account for this increase in area would be 3.4-3.9%. Such a small rate of smoothing-out would be impossible to observe in TEM micrographs.

**New Membrane Formed during First Cleavage under Various Conditions**

The area of newly formed membrane (= unpigmented surface) was measured at the onset of second cleavage so that an estimate of the rate of new membrane formation during first cleavage could be obtained. The surface area was measured in eggs which had cleaved normally inside the vitelline membrane (+VM, -CCB by method III), in eggs which had cleaved outside the vitelline membrane (-VM, -CCB by method IV), and in such eggs which were then exposed to CCB (-VM, +CCB and +VM, +CCB by method II). The results are presented in Table II.

**Table I**

| Geometrical Surface Area (mm²) of the Uncleaved Egg under Various Conditions |
|---------------------------------|-----------------|-----------------|-----------------|
|                                  | Series 1        | Series 2        | Series 3        |
| +VM, -CCB                        | 5.11            | 5.61            | 5.53            |
| -VM, -CCB                        | 5.05-5.17       | 5.38-5.84       | 5.32-5.74       |

* X, mean surface area.  
† n, number of eggs measured.  
‡ SD, standard deviation.  
§ C.I., 0.95-confidence interval for the mean.

In eggs cleaving inside the vitelline membrane the new membrane area should be twice the interblastomeric surface when first cleavage is completed. One would expect this to be at the onset of second cleavage. However, all eggs examined showed incomplete separation of the two blastomeres and had an area of continuous cytoplasm in an eccentric position (the shaded area in Fig. 3). No correction was made for this area since its border is not well defined. However, an estimate can be provided. In the eleven eggs of series 1 the average area (X) not covered by membrane is 0.26 mm² ±0.18. If this value is subtracted from the average surface area given in Table II, it turns out that the significance of the differences between series 1, 2, 3, and 4 is not affected.

Upon application of the t test it is found that the surface area of new membrane formed during cleavage of eggs outside the vitelline membrane (-CCB in series 2) is significantly smaller than that of eggs cleaving normally inside the vitelline membrane (series 1, P < 0.001). The area of new membrane formed in CCB-treated eggs differs significantly from that formed in untreated eggs under the same conditions. When series 2 and 3 are compared, both involving eggs without vitelline membrane, it is clear that CCB-treated eggs have formed a significantly larger area of new membrane (P < 0.001). On the other hand, comparison of series 1 and 4 involving eggs without vitelline membrane shows that CCB-treated eggs have formed an area of new membrane that is significantly smaller than normal (P < 0.001). It must be concluded that the presence or absence of the vitelline membrane is a second factor influencing the size of the new membrane area ultimately formed at the external surface.
Normal Cleavage Furrow after Routine Fixation

The cytokinetic event in a cleaving zygote of *Xenopus laevis* can be subdivided into five stages by using the extension of the furrow as a criterion. These five stages have been indexed as the single stripe (SS), shallow groove (SG), half-advanced furrow (HAF), far-advanced furrow (FAF), and just-completed furrow (JCF) stages (see Bluemink, 1971a, 1971b and de Laat et al., 1973).

Cytokinesis starts with contraction in a stripe-shaped zone at the animal pole surface (SS stage). As a result of progressive contraction during the next 4 min the stripe develops into a groove with a depth of approximately 100 µm (SG stage). Both in the stripe region and in the bottom of the groove a 0.2-µm thick layer of parallel 80 Å filaments is present below the membrane (Kalt, 1971; Selman and Perry, 1970; Bluemink, 1971a). Up until the SG stage the surface in the region of contraction is pigmented and folded. Apart from profiles of surface folds no other cellular components are seen external to the furrow surface.

Another 4 min later the groove has deepened into a furrow of about 150 µm deep (HAF stage). The surface along the slopes is taut (see Fig. 7). In the deepest part of the furrow the walls come close and run almost parallel. The 0.2-µm thick filament layer is uninterrupted at the bottom of the furrow and extends some distance up along the slopes. External to the furrow surface, clusters of membranous material are present in the furrow gap. Their profiles are either free or attached to the cell membrane. In the cytoplasm along the slopes of the furrow, cisternae are found, some of them in open communication with the furrow gap (Fig. 8).

Small clusters of membranous material occur as cytoplasmic inclusions, some of which show an investing membrane. The clusters are preserved much better after fixation in the presence of RR (see next section). Beside the clusters lumps of dense fibrous material are frequently seen at the outer furrow surface (Fig. 10). These structures are more numerous at the FAF stage and have previously been brought into relation with lanthanum-binding exudate which forms a coat present exclusively at the furrow surface (Bluemink, 1971b).

The cell membrane along the furrow bottom with the filaments beneath has a well-defined trilamellar appearance (Fig. 9). The spacing of the dense lamellae is ca. 70 Å. In sections cut perpendicular to the unpigmented surface the trilamellar spacing is less well defined (Fig. 10) when compared with the cell membrane of the preexisting pigmented surface.

RR Fixation

When fixed 3–4 min after the SS stage (= SG stage) in the presence of RR, the egg surface including the furrow surface (Fig. 11) has no RR-binding coat. TEM micrographs show that the RR dye has not penetrated into the egg and that no part of the cytoplasm shows an increase in electron density because of RR treatment (Figs. 11 and 12).

When fixed 7–8 min after the SS stage (= HAF stage) in the presence of RR, it is precisely the taut, unpigmented furrow surface that is seen to have acquired an even, deep red staining. The rest of the rough, pigmented egg surface has remained unstained. TEM analysis shows that the cell membrane along the deep part of the furrow is lined by an electron-dense layer (Figs. 13 and 14). The

**Table II**

|                  | Series 1 + VM, - CCB | Series 2 - VM, - CCB | Series 3 - VM, + CCB | Series 4 + VM, + CCB |
|------------------|----------------------|----------------------|----------------------|----------------------|
| X*              | 1.39                 | 0.33                 | 2.15                 | 0.65                 |
| n†              | 11                   | 11                   | 12                   | 15                   |
| SD§             | 0.29                 | 0.06                 | 0.48                 | 0.15                 |
| C.I.Ⅵ           | 1.20-1.58            | 0.29-0.37            | 1.85-2.45            | 0.57-0.73            |

* X, mean surface area.
† n, number of eggs measured.
§ SD, standard deviation.
Ⅵ C.I., 0.95 confidence interval for the mean.
FIGURE 7 Median cross section through the deep part of the half-advanced furrow (HAF stage). c = clusters of membranous material in the furrow gap; f = 0.2 µm thick filament layer. X 14,400.

FIGURE 8 Open ER cisterna at the furrow surface, containing membranous material. X 81,000.

FIGURE 9 Preexisting cell membrane of the pigmented rough surface. X 158,000.

FIGURE 10 Newly formed cell membrane of the unpigmented, smooth furrow surface. Small lumps of fibrous material (arrows). X 158,000.
pigmented rough surface outside the furrow region has not acquired a similar dense RR coat (compare Figs. 14 and 15). There is however a gradual transition between the coated and the uncoated cell surface.

Along the furrow surface cisternae are in open communication with the furrow gap (Fig. 16). The electron-dense coat continues along the inner surface of the cisternae. Serial sections show that the open cisternae are in communication with cisternae lying deeper in the cytoplasm. The RR has penetrated to some extent into the system, and the cisternae lying deeper show a decreasing amount of RR bound to their inner surface. The communication of the cisternae with the external milieu is via a narrow opening (Figs. 8 and 16 d).

In the cytoplasm the interconnecting necks between cisternae are also very narrow (Fig. 16 a and f).

Apart from the dense surface coat, three other dense cellular structures become manifest as a result of RR treatment; they are designated as plaques, droplets, and spheroids. Along the furrow surface small groups of electron-dense cytoplasmic inclusions are present in the cytoplasm (Figs. 18 and 20). Some of them, the so-called droplets, are round and measure 0.1–0.2 µm across (Figs. 18 and 19). Others, the so-called plaques, are larger and more irregular in outline (Figs. 17 and 20).

Unlike the plaques, which invariably occur along the cell surface only (Fig. 20), the droplets are seen scattered throughout the cytoplasm beneath the furrow, down to a depth of approximately 30 µm. It is not possible to distinguish an investing membrane around the droplets or the plaques. Infrequently plaques are seen closely associated with lipid bodies. Some plaques are composed of evenly spaced dense lamellae (Fig. 17), but most plaques and droplets are inhomogeneously dense, showing a typical substructure (Fig. 19).

Complex masses, the so-called spheroids, partly consisting of dense material, are attached to the unpigmented outer furrow surface (Fig. 20). Almost invariably the material making up a spheroid is directly adjacent to plaques in the cytoplasm. The electron-dense coat of the furrow surface continues along the surface of the spheroid. In the spheroid evenly spaced dense lamellae are typical constituents (Fig. 21). Apart from this material in myelinlike configuration, no recognizable substructure is seen in other constituents which are either amorphously dense or electron translucent. SEM micrographs show that the spheroids occur exclusively along the smooth surface in the furrow (Fig. 22). The smaller ones are almost perfect spheres, whereas the larger ones show large indentations (Figs. 23). The diameter of the spheroids varies between 1 and 30 µm.

Eggs without vitelline membrane, exposed to 10 µg/ml CCB, were fixed 8 min after the SS stage in a RR-containing fixing fluid. TEM analysis shows that the typical morphological changes seen in normally cleaving eggs concomitantly with the appearance of new membrane surface also occur in CCB-treated eggs. SEM analysis reveals that numerous spheroids are present in the region of the regressed furrow (Fig. 24). The surface to which the spheroids are attached (= unpigmented surface) is very smooth when compared with the rough surface (= pigmented surface) outside the regressed furrow region.

**DISCUSSION**

The surface-marking experiments have provided evidence that the smooth, unpigmented surface is formed de novo and not at the expense of pigmented pre-existing surface. Surface-marking experiments in the sea urchin egg have shown that constriction is followed by de novo surface formation in a localized region around the cleavage stalk (Dan...
et al., 1938; Dan and Dan, 1940; Dan 1954 a, 1954 b). 5-7 min of constriction followed by new membrane formation (as seen in *Xenopus* eggs) also occurs in the sea urchin egg (compare Schroeder, 1972, with Dan, 1954 b). The timing and sequence of events seem surprisingly alike. The fate of the rough early furrow surface in *Xenopus* is unknown. It may be dispersed when new membrane material is inserted randomly or it may be “pushed aside.” Accepting the latter possibility, it may contribute to the formation of the folded ridge (Fig. 24) which arises at the perimeter of the smooth furrow surface (Selman and Perry, 1970; Bluemink, 1971 a).

Exposure of *Xenopus* eggs without vitelline membrane to CCB results in an enhanced increase in new membrane area (Table II). The following interpretation seems plausible. Without the support of an investing membrane the egg flattens under gravity. The tendency to increase the surface/volume ratio is counter-balanced by the mechanical rigidity of the surface. It is likely that a cortical filament network controls rigidity (Selman and Perry, 1970; Bluemink, 1972). Evidence exists that CCB penetrates into the egg only when the cell membrane permeability increases, i.e., when new membrane is formed (de Laat et al., 1973). CCB may penetrate directly via the new membrane and/or via the open cisternae. From then on CCB may interfere with the cortical filament system as well as with the process of new membrane growth. The rate of enhanced membrane growth is highly variable (see Table II). In our view the rate of growth is greatly influenced by the speed and extent of egg flattening under the effect of gravity. In *Xenopus* eggs devoid of their vitelline membrane but not exposed to CCB (see de Laat et al., 1973) new membrane area is also exposed to the external medium. Such eggs, which manage to complete division, show a reduced rate of membrane growth. Up until the onset of second cleavage an intact filament layer ca. 0.07-μm thick is present along the advancing end of the first cleavage furrow (own unpublished observation) whereas in eggs cleaving within the vitelline membrane the filaments fade away in the course of cleavage (Kalt, 1971; Bluemink, 1971 b). Probably the contractile system along the leading end of the furrow is capable of controlling the rate as well as the direction of growth of new membrane. In CCB-treated eggs this mechanism of control apparently is not functioning. The primary site of CCB action leading to this effect is still open to discussion.

In CCB-treated eggs inside the vitelline membrane (Table II, series 4) the vegetal half remains hemispherical whereas the animal half flattens when new membrane forms. Because of the constraint exerted by the vitelline membrane the area of new membrane can grow to a limited extent only, i.e., until the animal surface has completely flattened.

It has been shown that after initial contraction the cell surface in the region of the furrow under-
FIGURE 16  Furrow bottom of an egg (HAF stage) fixed (− VM) in the presence of RR. This series of thin sections shows that the cisternae have narrow interconnecting necks (see arrow, a and f) and a narrow opening to the outside. (See arrow d). Arrow in f = interconnecting neck in cross section. The penetration of RR into the system is restricted (compare e, f, and g). Unstained section. × 48,500.
goes a marked alteration. Until ca. 4 min after the onset of cleavage (SG stage) the furrow is rough and folded. Just like the rest of the egg surface it has no specific affinity for RR. Apparently the surface of the early furrow still consists of preexisting cell membrane. This conclusion is in accordance with the observation that during this phase of cleavage the electrical membrane properties remain unchanged (de Laat et al., 1973).

7–8 min after the onset of cleavage (HAF stage) the surface along the furrow has changed. Now the furrow surface is smooth and clearly distinguishable from the pigmented rough surface outside the furrow (see Fig. 22). After exposure to RR only the unpigmented, smooth furrow surface stains intensely red as seen under the dissecting microscope. Gingell (1970), using a 0.1% nile blue sulfate solution, observed a similar precisely delimited staining pattern in cleavage furrows of early blastulae of Xenopus. None of the other dyes he tried caused staining. It is interesting to know that nile blue sulfate is used in cytochemistry to stain acidic lipids and phospholipids. This information fits in with the concept that RR stabilizes pooled membrane precursors in the cytoplasm, probably phospholipids (see below).

The electron-dense droplets, plaques, and spheroids were found exclusively along the furrow surface. Similar droplets and plaques have been observed at the wound surface in damaged amebas (Szubinska, 1971) and amphibian eggs (Bluemink, 1972). They have been identified provisionally as phospholipid droplets and are present also in other cell types (see Szubinska, 1971, for references). Near the membrane the droplets seem to accumulate and to fuse into plaques. That the droplets and the plaques were invariably observed only in the furrow region calls for comment. Apparently these structures only occur here because otherwise it must be assumed that they would have been seen along the rest of the egg surface after 21 h fixation in the presence of RR. It is appropriate to mention here three papers which have reported the occurrence of osmiophilic dense bodies in close association with the forming furrow (Arnold, 1969, 1971; Robbins and Gonatas, 1964). However, these authors did not relate them to new membrane formation.

Fusion of dense droplets with the cell membrane has been interpreted as a possible way in which material is inserted to expand the cell membrane (Szubinska, 1971). Apparently in the amphibian egg the biogenesis of new membrane during cytokinesis occurs in the same way. The newly formed membrane along the unpigmented surface is different from the preexisting cell membrane in having a less well-defined trilamellar appearance. This may reflect the still labile state of organization of new membrane. The material contributed to the membrane presumably is rich in phospholipids. Electron microscopy so far has shown that only polar phospholipids emulsified in water orient themselves into layers which can be visualized as periodic dense lamellae. In the hydrated state typical membrane phospholipids generate myelin forms in the presence of RR (Luft, 1971a). We therefore conclude that the structures seen as droplets, plaques, and spheroids are the precipitation forms of phospholipids, i.e., membrane precursors.

The spheroids external to the egg surface are frequently close to plaques inside the egg. Probably

![Figure 17](image-url) Egg (HAF stage) fixed (−VM) in the presence of RR. Plaque in the subcortical cytoplasm showing a substructure of evenly spaced dense lamellae. Unstained section. × 85,000.

![Figure 18](image-url) Egg (HAF stage) fixed (−VM) in the presence of RR. Small dense droplets and a plaque beneath the furrow bottom. RR containing ER cisterna (arrow). p = pigment granule. Unstained section. × 14,400.

![Figure 19](image-url) Electron-dense droplet from the same egg in Fig. 20 showing its typical substructure. Unstained section. × 108,000.

![Figure 20](image-url) Egg (HAF stage) fixed (−VM) in the presence of RR after 8-min exposure to 10 µg CCB per ml. Spheroid at the furrow surface and a droplet and dense plaques in the cytoplasm. The shell of the spheroid consists of evenly spaced dense lamellae, like the plaque shown in Fig. 17, whereas the dense material of the inner mass is more like that seen in the droplet shown in the inset. Unstained section. × 42,500.
the spheroids form upon fusion of the plaques with the cell membrane. The fact that a large amount of presumed membrane precursor is found outside the membrane as spheroids is difficult to fit in with the concept that these substances are to be inserted into the membrane. Although closely related, the fusion of the dense plaques with the membrane and the formation of spheroids should be considered separately. We suggest the following explanation. The formation of spheroids can be seen as a concurrent effect of fixation during the process of insertion. The initial contact with the fixing fluid may have a perturbing effect on the labile, newly formed membrane. Upon interaction, liquid lipid droplets may sprout from the pools of membrane precursor at the cell surface. In fact the spheroids closely resemble liposomes which arise by swelling upon mixing phospholipids with aqueous salt solutions (Papahadjopoulos and Miller, 1967). Apparently the composition of RR- and osmium-containing fixing fluids is adequate to stabilize this artificially released liquid lipid (see Luft, 1971 a) into myelin forms which would then take the form of spheroids. Routine fixations apparently fail to stabilize the lipid (see Fig. 7). This interpretation fits in with the observation that the largest spheroids are found in eggs without vitelline membrane which form less new membrane surface area than normally (see Table II). In these eggs excess amounts of noninserted precursor material will be available.

The local variation in cell membrane structure in CCB-treated eggs described previously (Bluemink, 1971 b) cannot be distinguished very well from the biological variation in membrane thickness in eggs involved in new membrane formation during cytokinesis. Therefore, the previous assumption that CCB might change the dimensions of the cell membrane remains unproven.

In TEM micrographs the RR-stained unpigmented furrow surface has an electron-dense surface coat. The RR staining may result from the binding of RR to sites in the newly formed membrane, to an exudate which coats new membrane surface secondarily, or to both. In the context of this paper we used the term "coat" without making a distinction between these possibilities. The time of appearance and the localization of the coat correspond with those of the lanthanum-binding exudate in the furrow of Xenopus eggs described earlier (Bluemink, 1971 b). The observed RR staining of unpigmented surface is at variance with the observation that in Triturus eggs the new membrane in the furrow has a thinner fuzzy layer than the preexisting surface (Selman and Perry, 1970).

The RR-coated cisternae are very probably identical with the endoplasmic reticulum (ER) cisternae described earlier (Bluemink, 1971 b). The latter were brought into relation with the secretion of lanthanum-binding exudate, probably a carbohydrate complex (see Motomura, 1960). The regularly occurring, locally narrowed passages in the cisternal system (Fig. 16) may be functional. They may reflect the existence of a peristaltic-type transport mechanism, or of a sluice system protecting internal ionic conditions against external influences. As regards the continuity between ER cisternae and the cell membrane the following interpretations are possible: it may be that the ER cisternae are involved in the transport of exudate only, but alternatively it may
FIGURE 22 SEM micrograph of the animal pole surface. Egg fixed (-VM) 6 min after the onset of cleavage in the presence of RR. Spheroid masses are attached to the smooth furrow surface only. × 479.

FIGURE 23 SEM micrograph of the furrow surface. Egg fixed (-VM) 8 min after the onset of cleavage in the presence of RR. The larger spheroid masses show indentations. Dirt particles (asterisk) and folds at the bottom of the furrow (arrow). × 908.
well be that the ER cisternae themselves represent newly synthesized membrane. The latter view, viz. that ER cisternae unfold and add new membrane to the surface, is represented by Ap Gwynn and Jones (1972), Agrell (1966), Buck and Tisdale (1962), Buck and Krishan (1964), and Fullilove and Jacobson (1971).

The CCB experiments suggest that the amount of new membrane formed is easily adapted to needs created by extrinsic influences and is not restricted by intrinsic activity. Rappaport (1971) has proposed self-assembly from units in the underlying cytoplasm as a method of new membrane formation independent of metabolic activity. The findings described in this paper provide evidence for two modes of membrane formation, but they do not allow one to discriminate between them. The two possibilities are: (a) new membrane formation occurs by self-assembly of pooled precursors using the preexisting cell membrane as a matrix; cisternae form a canalicul system which transport surface exudate, or (b) cisternae represent newly formed membrane and additional areal growth takes place by direct insertion of pooled precursors into the cell surface. These alternative possibilities deserve further experimental analysis.

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