

The development of the region between the preexisting and nascent membranes during the first cleavage of *Rana amurensis* eggs

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ABSTRACT

The region between the preexisting and nascent membranes of a cleaving *Rana* egg is a dense protrusion region of nearly 40 μm wide at 180° stage. Later, this region differentiate, into an upper part, a strip with long, branched and randomly distributed protrusions which are derived largely from the preexisting membrane, and a lower part, a band with microvilli. During 4- and 8-cell stages, the strip is almost vanished and microvilli in the band is shortened. The nascent membrane is smooth at the 180° stage, then a rough area appears below the smooth region and quickly expands. Wheat germ agglutinin which can bind to preexisting membrane interrupts the development of the region between the preexisting and nascent membranes. Detergent, Brij, having the property to increase the area of nascent membrane, does not interrupt the development of the region between the preexisting and nascent membranes.

Key words: *cleavage, region between the preexisting and nascent membranes, surface active agents, wheat germ agglutinin, Rana amurensis eggs.*

INTRODUCTION

The first cleavage furrow of amphibian fertilized eggs begins at animal pole and travels downward with accompanying membrane ingrowth from all sides which deepens into the interior to accomplish cytokinesis in blastomere formation. The

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external surface membrane of blastomeres and the upper parts of furrow walls are the preexisting membrane of the fertilized egg, while the lower parts of furrow walls are of nascent origin. The characteristics of the preexisting and nascent membranes have been extensively described [1—12]. The region between the preexisting and nascent membranes, which plays an essential part in the formation of interblastomeric junction [11, 13], has been variously described as a line [2], a band [7], a ridge [11, 13], in the features of granules [9, 14] or of elongated and branched microvilli [8, 15]. Do these features represent the morphological changes occurred during the development of this region? From which membrane is this region derived? Can its development be influenced by factors which are able to bind with preexisting membrane or to increase the area of nascent membrane [16]? These are questions that remain to be answered.

The present report shows that the region between the preexisting and nascent membranes changes its feature during development, and is derived largely from the preexisting membrane. Factors such as wheat germ agglutinin (WGA) and surface active agents which can influence either the preexisting or nascent membranes are found to affect the feature, development or area of the region between the preexisting and nascent membranes as well.

MATERIAL AND METHODS

Ovulation of *Rana amurensis* eggs was induced by the injection of pituitary glands of the same species. After artificial fertilization, egg jelly was removed by 1% cysteine (pH 7.6) for 3 min. then eggs were thoroughly washed in 1/10 Barth solution (Hepes buffer, pH 7.2), and any remaining jelly should be stripped off by forceps.

All eggs used were enclosed in vitelline membrane. Control eggs were immersed in 1/10 Barth solution. In experiments, eggs were transferred to 1/10 Barth solution containing 500 $\mu\text{g/ml}$ WGA (Sigma) alone or together with 40 mg/ml of its competitive inhibitor, N-acetylglucosamine (NacGlu). About 1h before cleavage. Brij 58 (Sigma), 0.001%, or lysolecithin [Stearoyl, gift of Prof. Yang FY; or from egg yolk (Sigma)], 1—10 $\mu\text{g/ml}$, was added to eggs just at the onset of cleavage.

Fluorescence micrographs of Brij treated eggs were taken after their brief fixation in 10% formalin and then stained with fluorescein labeled WGA (F-WGA).

The areas of nascent membrane and dense protrusion region were measured with mop videoplan (Opton) as follows: when cleavage furrow extending superficially to the equator, i. e. 180° stage [7], eggs untreated or treated with various agents were fixed briefly in 2.5% glutaraldehyde for about 5 min. After the removal of fertilization membrane eggs were pressed under cover glass to make furrow wall exposed, flattened and then photographed. The area of nascent membrane was the unpigmented region. The area of dense protrusion region was the region enclosed by the ends of stress folds of preexisting membrane in furrow walls, which corresponded to the most heavily pigmented line seen under light microscope, with the subtraction of the area of nascent membrane.

For scanning electron microscopy, eggs were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for over 2h. After the removal of vitelline membrane with forceps, eggs were washed for 2h with several changes of cacodylate buffer (pH 7.4), post-treated successively in 1% OsO₄, 2% tannic acid and 1% OsO₄, dehydrated through a graded series of alcohol, rinsed in isoamyl acetate, dried in liquid CO₂ (Hitachi, CPD-II critical point drier), coated with gold, and observed under scanning electron microscope (SEM) (Hitachi, S-450) at 15 kV. Photos were taken at animal poles except otherwise stated.

For transmission electron microscopy, eggs were fixed in 2.5% glutaraldehyde, washed in cacodylate buffer, and postfixed in 1% OsO₄. A small piece of cleavage furrow was chipped from animal pole and coated with agar, dehydrated in graded ethanol solutions and acetone, embedded in Epon 812, sectioned, stained in lead citrate-uranyl acetate and observed under JEM-100B transmission electron microscope.

RESULTS

The development of the region between the preexisting and nascent membranes

In order to observe the developmental changes of the feature of the region between the preexisting and nascent membranes during first cleavage, cleaving eggs were broken manually along cleavage furrow after fixation. Under lower magnification of SEM at 360° stage a ridge stretched in the form of arc was situated between preexisting and nascent membranes (Plate 1, Fig. 1). The ridge was more conspicuous near the animal pole than near its tips, indicating that the ridge might have started to develop when the preexisting membrane began to invaginate.

Under higher magnification of SEM, a dense protrusion region of about 40 μ m width located between the preexisting and nascent membranes could be seen at the 180° stage (Plate 1, Fig. 2A). The origin of dense protrusion region could be traced from animal pole to equatorial region where a string of patches of different sizes with denser protrusions was present near the border of invaginated preexisting membrane. Toward animal pole the patches gradually increased in size and number and connected with each other to form dense protrusion region. When furrow reached 210°—240° stage, this dense protrusion region differentiated into an upper part, a strip, with less protrusions and a lower part, a band, full of long protrusions and with a sharp boundary to nascent membrane (Plate 1, Fig. 2B). During 270°—360° stage, the strip was full of branched protrusions (Plate 1, Fig. 2C). In some eggs or in some areas of egg, a line with much less protrusions might be seen between the preexisting membrane and the strip. The band was composed of microvilli. During 4- and 8-cell stages at animal pole the width of strip was decreased and microvilli in the band became shortened (Plate 1, Fig. 2D), while at equatorial region, however, the feature of the strip and band were nearly the same as that at animal pole of 270°—360° stage. Under TEM, close contacts could be found between the protrusions of the opposite furrow walls of 2-cell stage (Plate 1, Fig. 3).

The origin of the region between the preexisting and nascent membranes

There were evidences indicating that the region between the preexisting and nascent membranes was derived largely from the preexisting membrane: (1) The protrusions in the region between the preexisting and nascent membranes had WGA-binding sites which were markers of the preexisting membrane (Plate 2, Fig. 4). (2) Pigment was another marker of the preexisting membrane and the strip was also proved to have pigment. By pricking the pigmented membrane nearest to the unpigmented region with a glass needle in fixed eggs under dissecting microscope, it was found that the pricked holes were located in the strip in scanning electron micrographs. (3) The protrusions of the preexisting

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membrane could be elongated (Plate 2, Fig. 5).

The development of the nascent membrane

During the development of the region between the preexisting and nascent membranes, nascent membrane itself also developed. At 180° stage, nascent membrane with less protrusions was often narrower than, or nearly equal to the dense protrusion region, and folded in some cases (Plate 1, Fig. 2A). At 210°—240° stage, nascent membrane developed into an outer zone with less protrusions and more "openings" and an inner zone with more protrusions and less "openings". The area of the inner zone increased rapidly (Plate 1, Fig. 2B). At 360° stage, the outer zone became narrower with more conspicuous "openings", while the inner zone greatly expanded and studded with irregular protrusions (Plate 1, Fig. 2C). Similar features could be seen during 4- and 8-cell stage (Plate 1, Fig. 2D).

Factors affecting the development of the band and the areas of dense protrusion region and nascent membrane

(1) WGA

If the region between the preexisting and nascent membranes is largely derived from the preexisting membrane, factor such as WGA which can bind to the preexisting membrane may obstruct the development of the region between the preexisting and nascent membranes. In eggs treated with WGA the dense protrusion region did not differentiate and remained the same from 180° to 4-cell stage. The area of dense protrusion region was significantly smaller than those of control group (Table 1). In this area a characteristic zipper-like structure composed of apposing ridges of about 40μm long appeared on both sides of the

Table 1. Areas of dense protrusion region and nascent membrane at 180° stage*
Area of dense protrusion region

	$\bar{x} \pm S.D. ** ()$	C.I.***	r****
Control	0.183 ± 0.056(19)	0.159—0.208	1
WGA	0.118 ± 0.067(20)	0.089—0.148	0.645
WGA+NacGlu	0.176 ± 0.040(15)	0.156—0.196	0.962
Brij	0.228 ± 0.057(23)	0.205—0.251	1.24
WGA+Brij	0.142 ± 0.095(15)	0.094—0.189	0.776
Area of nascent membrane			
	$\bar{x} \pm S.D. ()$	C.I.	r
Control	0.135 ± 0.097(19)	0.091—0.197	1
WGA	0.025 ± 0.035(20)	0.009—0.004	0.185
WGA+NacGlu	0.122 ± 0.063(15)	0.090—0.154	0.904
Brij	0.227 ± 0.107(23)	0.234—0.321	2.05
WGA+Brij	0.203 ± 0.133(15)	0.136—0.271	1.504

* eggs from one frog.

** mean area (mm²) ± S.D.

() number of eggs measured.

*** 0.95-confidence interval for the mean

**** area ratio to control.

furrow tips(Plate 2, Fig. 6A)and became more characteristic at later stages (Plate 2, Fig. 6B). This structure may be resulted from the cross-linking of WGA to its binding sites on the egg surface as evidenced from the presence of numerous thread-like links among protrusions. Furthermore, in WGA treated eggs, many precipitates adhered to the egg surface and heavy floccules in the perivitelline space could be often seen after fixation, which indicated that much WGA was present in perivitelline cavity after its penetration through fertilization membrane.

The other effects of WGA were: (1) the area of nascent membrane became much smaller (Table 1) and (2) many wrinkles randomly distributed all over the egg surface before cleavage. During cleavage wrinkles persisted and stress folds on both sides of cleavage furrow were higher and longer than those in control. Apparently, the formation of wrinkles was also due to the cross-linking effect of WGA but not due to the egg shrinkage since the equatorial area, of WGA treated egg was the same as that of control (2.07 mm^2 , an average value from 10 eggs in each group from the one and the same frog).

None of the aforementioned phenomena appeared when NacGlu, the competitive inhibitor of WGA, was added with WGA.

(2) Brij

If the region between the preexisting and nascent membranes derives largely from preexisting membrane, factor such as Brij which can increase the area of nascent membrane [16] will not influence noticeably the development of the region between the preexisting and nascent membranes. In eggs treated with 0.001% Brij solution the development of the region between the preexisting and nascent membranes was nearly the same as in control(Plate 2, Fig. 7). Protrusions on nascent membrane were usually elongated. The areas of dense protrusion region and nascent membrane (Table 1), egg height [16] and the equatorial area ($2.12 + 0.02 \text{ mm}^2$) were significantly larger than those in control ($2.08 + 0.03 \text{ mm}^2$). Those suggest that Brij can incorporate into plasma membrane and change its permeability.

(3) Lysolecithin

Lysolecithin, either stearyl or from egg yolk, could also enlarge the areas of dense protrusion region and nascent membrane as Brij did, but to a lesser extent. The microvilli in the band assumed spoon-shaped in some cases (Plate 2, Fig. 8). It could also temporarily expose nascent membrane as in the treatment of *Xenopus* zygote [2].

(4) Brij and WGA

The areas of dense protrusion region and nascent membrane of this group lay between those of the groups of WGA and Brij (table 1). The development, of dense protrusion was retarded, but less markedly than that in WGA. The nascent membrane was also less exposed than in Brij alone. These results show that the combined effects of WGA and Brij are additive.

DISCUSSION

The increase of the length and density of protrusions at a narrow and long area of amphibian egg surface is the first external sign where cleavage furrow will be formed. Later, this area differentiates into 3 parts. The middle one is the presumptive contractile ring, while the two bilateral parts are dense protrusion region of 40 μm width, which differentiate further into a strip and a band. During these continuous differentiation a common feature is the elongation of protrusions. This may be caused by actin-actin-binding protein interactions induced by the change of intracellular ionic environment [17]. The elongation and erection of protrusions can be prevented by WGA either through its transmembrane effect which interferes the interaction between actin-actin-binding protein, or simply by X-linking effect of WGA with its binding sites on egg surface to increase the rigidity of egg surface. Thus WGA may be used as a tool to investigate the development of the region between the preexisting and nascent membranes.

The formation of nascent membrane is a main process to meet the requirement of the increment of surface area during cleavage. The growth of nascent membrane, according to Bluemink and de Laat [18], may be performed by the insertion of cytoplasmic vesicles [2—4, 7, 13] and incorporation of molecules. Lipid is one of the main components of membrane. Membrane destabilizing agents such as Brij and lysolecithin may incorporate into nascent membrane at places where the trilamellar structures are less well defined, as seen in *Xenopus* [4] and *Rana amurensis* (unpublished data), reflecting the labile state of nascent membrane organization at these places. In our previous work [16], we have provided evidence that Brij can interact with proteins in perivitelline space, and membrane structure may be formed through such interaction and inserted into nascent membrane. The insertion of cytoplasmic vesicles in localized area of nascent membrane has been mentioned in *Xenopus* [7]. From our present SEM studies of the structure and development of nascent membrane, we are of opinion that the probable sites of insertion of cytoplasmic vesicles are the openings in the outer zone of nascent membrane. The inserted profuse membrane, pushed inward and formed the part of the studded irregular protrusions in the inner zone of nascent membrane (Plate 1, Fig. 2C).

In measuring the area of nascent membrane during first cleavage of *Xenopus* eggs, Bluemink and deLaat [4] showed that nascent membrane area was largest in eggs devoid of vitelline membrane and treated with cytochalasin, next in egg within vitelline membrane and untreated, and smallest in eggs within vitelline membrane and treated with cytochalasin. They interpreted their results by the difference in surface mechanical rigidity in different groups. In the present experiment, the degree of depth of cleavage furrow in eggs treated with WGA is less than that in controls, suggesting that surface rigidity is increased or the surface tension in the area of cleavage furrow is decreased through the binding of WGA to preexisting membrane, including cleavage furrow bottom. The decrease of surface tension in

cleavage furrow may make the insertion of cytoplasmic vesicles into nascent membrane to a much lesser extent. Thus the area of nascent membrane formed will be smaller than that in control. So the decrease of the amount of nascent membrane by WGA is through its change on surface rigidity, while the increase of the amount of nascent membrane by brij is through its direct insertion or the insertion of membrane structures into nascent membrane.

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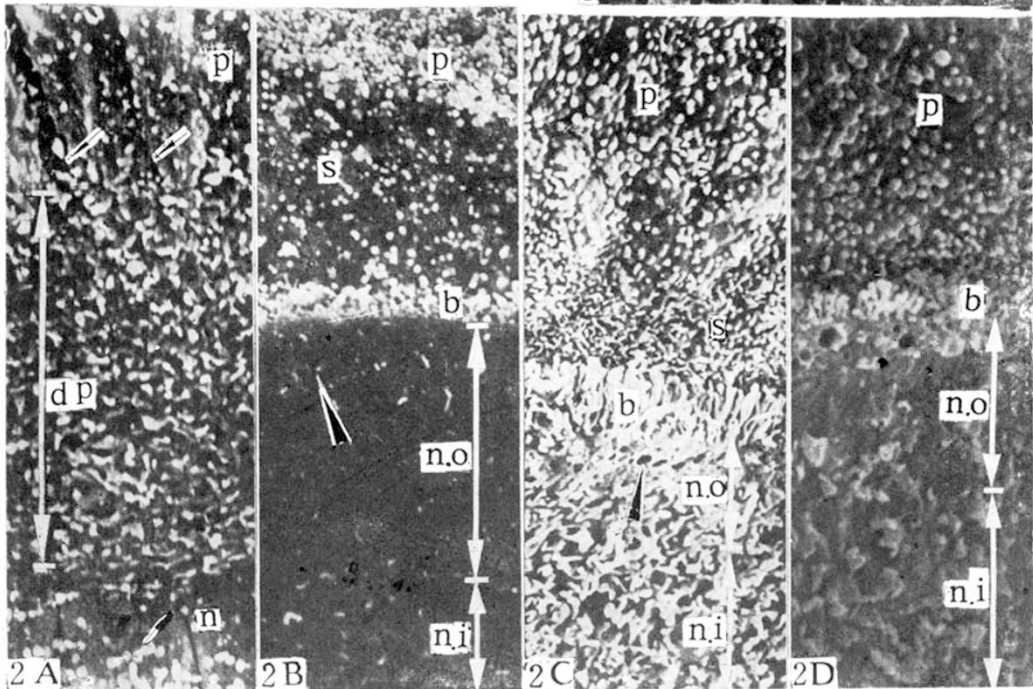
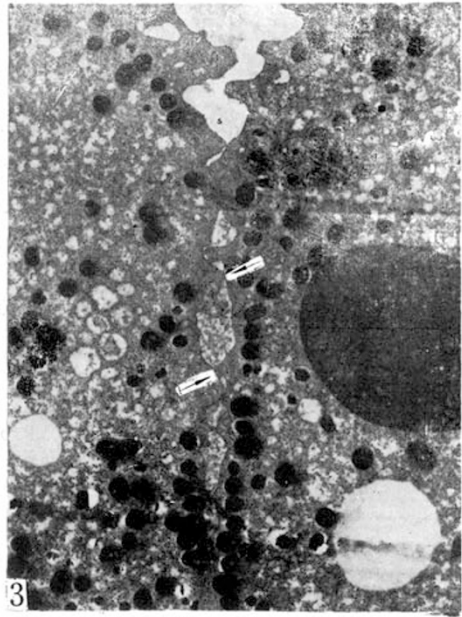
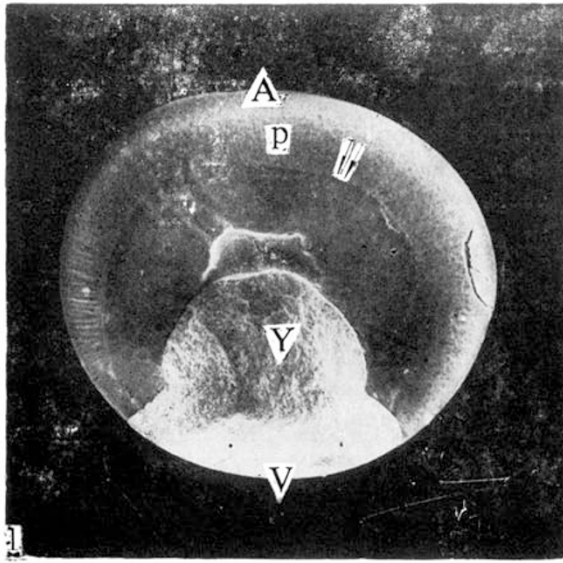


Plate I

Fig. 1 A scanning electron microscopic photograph of cleavage furrow wall showing a ridge (double arrow) stretched in the form of arc situating between the preexisting (p) and nascent membrane (n). A, animal pole, V, vegetal pole. Y, yolk 360° stage. $\times 40$

Fig. 2 A-D The development of the region between the preexisting and nascent membrane during cleavage.

A. The dense protrusion region (d.p) between the ends of stress folds (arrow) of the preexisting (p) and nascent membrane (n). 180° stage. SEM. $\times 1500$

B. The dense protrusion region differentiating into strip (s) and band (b), and nascent membrane differentiating into outer zone (n. o) with more "opening" (arrowhead) and inner zone (n. i) with more protrusions. 210° stage SEM. $\times 1000$.

C. Strip (s) full of branched protrusions, band (b) full of microvilli, outer zone (n. o) with openings (arrowhead) and inner zone (n. i) with irregular protrusions 270° stage SEM $\times 1500$.

D. The strip almost vanishing, the microvilli in the band being short. 4-cell stage. SEM. $\times 2000$.

Fig. 3 Close contacts (arrow) between the protrusions of opposite wall of the animal pole. TEM. $\times 9000$.

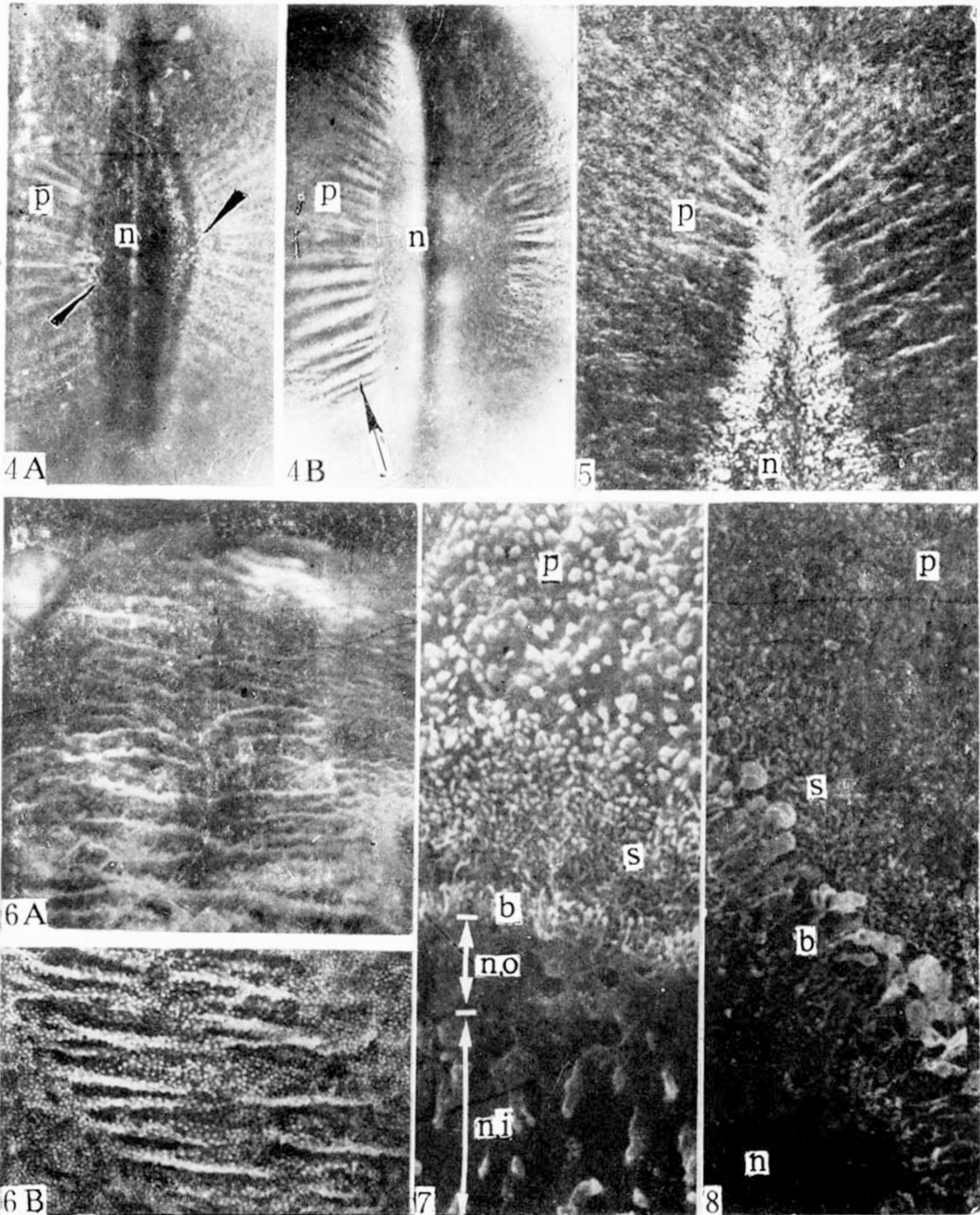


Plate II

- Fig. 4** F-WGA stained granules (arrowheads) in the region between the preexisting membrane (p) with stress folds (arrow) and nascent membrane (n). Brij treated egg, 180° stage. ×50.
 A. Fluorescence micrograph. B. Ordinary light micrograph.
- Fig. 5** Cleavage furrow tip, showing the elongation of protrusions in the preexisting membrane (p). n nascent membranes. SEM. ×250.
- Fig. 6** Zipper-shaped structure at cleavage furrow region of WGA treated eggs.
 A. at cleavage furrow tip, 270° stage SEM. ×500.
 B. at first cleavage furrow, 4-cell stage. SEM. ×500.
- Fig. 7** The band (b) composed of short microvilli. Brij treated eggs. p, s, n.o, n.i, as in Fig. 2. 4-cell stage. SEM. ×1500.
- Fig. 8** The band (b) composed of spoon-shaped microvilli Lysolecithin treated egg, p, s, n, as in Fig 2. 360° stage. SEM. ×1500.