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Vitellogenin Transport and Yolk Formation in the Quail Ovary

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ABSTRACT—Morphological and biochemical investigations were made on the yolk formation in ovaries of the quail *Coturnix japonica*. Morphologically, two ways of nutrient uptake were observed in follicles. In small oocytes of white follicles, vitellogenin (VTG) was taken up through fluid-phase endocytosis which was assisted by follicular lining bodies. The lining bodies were produced in follicle cells. They adhered to the lateral cell membrane, moved along the membrane in the direction of the enclosed oocyte and were posted to the tips of the microvilli. These tips, now with lining bodies, were pinched off from the main cell body, engulfed by indented cell membranes of the oocyte, and transported to yolk spheres. In large oocytes of yellow follicles, VTG and very-low-density lipoproteins (VLDL) were taken up through receptor-mediated endocytosis. The VTG and VLDL particles diffused through the huge interspaces between follicle cells, and once in oocytes were transported to yolk spheres via coated vesicles. Immunohistochemistry showed that the VTG resides on or near the surface of the follicle cell membrane at the zona radiata whereas the cathepsin D resides at or near the oocytic cell membranes. Tubular and round vesicles in the cortical cytoplasm of oocytes were also stained with both antisera, suggesting that these vesicles are the sites where the VTG is enzymatically processed by cathepsin D. Upon analysis by SDS-PAGE, a profile similar to that of yolk-granule proteins was produced by incubating VTG with a quail cathepsin D of 40 kD.

Key words: quail, ovarian follicle cells, lining bodies, vitellogenin transport, cathepsin D

INTRODUCTION

In amphibians and birds, the yolk precursor protein vitellogenin (VTG) is synthesized in the liver, transported via the vascular system to the oocytes, taken up by the oocytes and enzymatically processed into yolk proteins that are deposited in yolk platelets or yolk spheres for future use (Wallace, 1985; Burley and Vadehra, 1989; Yoshizaki, 1992; Yoshizaki and Yonezawa, 1994, 1996). In the literature we find that there seems to be a difference between two groups of animals in the way that VTG is transported through the follicle cell layer. In *Xenopus*, the follicle cells are quite separated and VTG appears to be transported by diffusion through wide channels between them (Yoshizaki, 1992). In chickens, the follicle cells lie close together at first, their cell membranes being separated by a gap of about 20 nm (Bellairs, 1965) during the early stages of vitellogenesis when the VTG is being taken up. In the late stages, wide channels between follicle cells are produced for transport of another yolk precursor protein, very low density lipoprotein (VLDL) (Perry and Gilbert, 1979), in addition to VTG. It is impossible to transport avian VTG effectively in such a narrow gap only by diffusion, so the avian follicle cell layer might possess some special mechanism for transport in the early stages of vitellogenesis.

A characteristic feature of avian follicle cells is the presence of certain specializations that are closely associated with the cell membrane (Press, 1964; Bellairs, 1965; Wyburn *et al*., 1965). They appear as structural modifications of the cell membrane such that the membrane is greatly thickened in its inner aspect by small granules (Wyburn *et al*., 1965), which are called lining bodies (Bellairs, 1965). One author reported that they remain permanently attached to the follicle cells (Press, 1964) and another speculated that they may be engulfed by the oocytes (Bellairs, 1965). The fate of these lining bodies, to say nothing of their function, has not been elucidated.

Ultrastructural studies on avian vitellogenesis were actively performed in the 1960's and 1970's, the dawn of

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development of the methodology (Press, 1964; Bellairs, 1965; Wyburn *et al*., 1965; Bakst and Howarth, 1977; Perry and Gilbert, 1979). Because of the inevitably poor fixation owing to methodological immaturity and the large size of eggs, results did not always represent real entities; a reported lack of cell membrane in the vegetative region of ovulating eggs (Bakst and Howarth, 1977) is still influencing illustrations in which the demarcation around yolk sac is drawn ambiguously (Carlson, 1988; Gerhart and Kirschner, 1997). Although biochemical data concerning with VTG- and VLDL-processing have accumulated since then (Retzek *et al*., 1992; Elkin *et al*., 1995), no study has been made of avian vitellogenesis from a morphological viewpoint as far as we know. The present study by electron microscopy is an attempt to totally understand the mechanisms for the transportation of VTG and VLDL into quail oocytes through the follicle cell layer. Results of immunofluorescent staining with antisera against VTG and cathepsin D, and results of in vitro interactions between the two will also be shown as supporting evidence.

MATERIALS AND METHODS

Electron microscopy

Ovaries of Japanese quail *Coturnix japonica* were fixed at 4°C in a mixture of 5% glutaraldehyde and 4% paraformaldehyde in 0.12 M cacodylate buffer (pH 7.4). The ovary of a laying quail contains oocytes varying in size from about 0.05 mm up to about 15 mm in maximum diameter. Developmental stages of the oocyte were determined from the size in diameter or conventional staging which ranges from F1, the largest, to F5, the smallest of yellow follicles. Oocytes with surrounding follicle cells were separated from each other and maintained in fresh mixtures for 2 hr or up to one night, depending on the size of the oocyte. After being washed with the buffer, they were postfixed for 3 hr in similarly buffered 1% OsO_4. The specimens were dehydrated in acetone and embedded in Quetol 812 (Nisshin EM Co., Japan). Ultrathin sections were stained with uranyl acetate and lead citrate, then viewed with the electron microscopes JEM-100SX (JEOL, Japan) and H-8000 (Hitachi, Japan).

Purification of vitellogenin (VTG)

VTG was purified from estrogen-primed male plasma as described by Deeley *et al.* (1975) with some modifications. Briefly, plasma was collected by centrifuging the blood at 800xg for 15 min. The supernatant was re-centrifuged at 100,000xg for 90 min, and the floating layer of lipoprotein was discarded. The remaining plasma (about 2 ml) was diluted with 50 ml of 0.05 M sodium citrate buffer (pH 5.5), and applied to a DEAE52 column (Pharmacia, Sweden). The column was washed with the 0.1 M sodium citrate buffer, and then fractionated with an application of a NaCl gradient, 0–0.25 M in the same buffer. The fractions containing VTG were pooled and dialysed at 4°C against buffer containing 0.15 M NaCl and 1 mM PMSF, pH 7.4. On SDS-polyacrylamide gel electrophoresis (PAGE), the VTG fraction dissociated to give three bands, VTG1, 2 and 3, the molecular weights of which were 230, 210 and 190 kD, respectively (Gibbins *et al*., 1981). To further isolate the VTG2, the 210 kD bands were scraped off the gels and extracted electrophoretically into 2.5 mM Tris-HCl buffer, pH 8.3, in a Max-Yield Protein Concentrator (Atto Co., Japan) at 5 W for 2.5 hr.

Purification of cathepsin D

Yolks were obtained from spawned eggs by removing the egg white and vitelline membrane manually. Isolated yolks or the liver homogenates were mixed with two volumes of acetone, chilled to –20°C, and the supernatant was removed following centrifugation at 10,000xg for 10 min. Further purification of cathepsin D was performed with affinity chromatography on QA52 (Whatman, England) and pepstatin-Sepharose columns according to the method for the purification of *Xenopus* (Nakamura *et al*., 1996) or rat (Yonezawa *et al*., 1987) cathepsin D.

Preparation of antisera

Antisera against quail VTG2 and rat liver cathepsin D were obtained by injecting each purified protein into rabbits. Monospecific anti-rat cathepsin D antibody was purified by using porcine liver cathepsin D-Sepharose 4B as an immunoadsorbent, as described previously (Yonezawa *et al.,* 1987).

Immunohistochemistry

Oocytes were fixed in Bouin's solution and embedded in paraffin. Sections of oocytes 5 μm thick were treated with rabbit antiserum against rat cathepsin D or quail vitellogenin for 1 hr at 37°C. The sections were washed thoroughly with phosphate-buffered saline and treated with fluorescein isothiocyanate-conjugated goat antiserum against rabbit IgG (Seikagaku Kogyo Co., Japan) for 1 hr at 37°C. Control sections were treated with normal rabbit serum instead of antiserum.

Immunoblotting

After SDS-PAGE, the proteins were electroblotted onto polyvinyliden difluoride membranes. The membranes were treated with the antiserum and then with horseradish peroxidase-conjugated goat antiserum (E-Y Lab. San Mateo, CA). The peroxidase was detected using a saturated concentration of 4-chloro-1-naphthol and 0.03% H_2O_2.

Enzyme and protein assays

Purified cathepsin D was mixed with VTG in 0.1 M citrate-Na_2HPO_4 buffer (pH 3.0–5.0). After incubation for 1 day at 37°C, the mixtures were analyzed by SDS-PAGE on a 7.5% gel. Gels were stained with Coomassie brilliant blue.

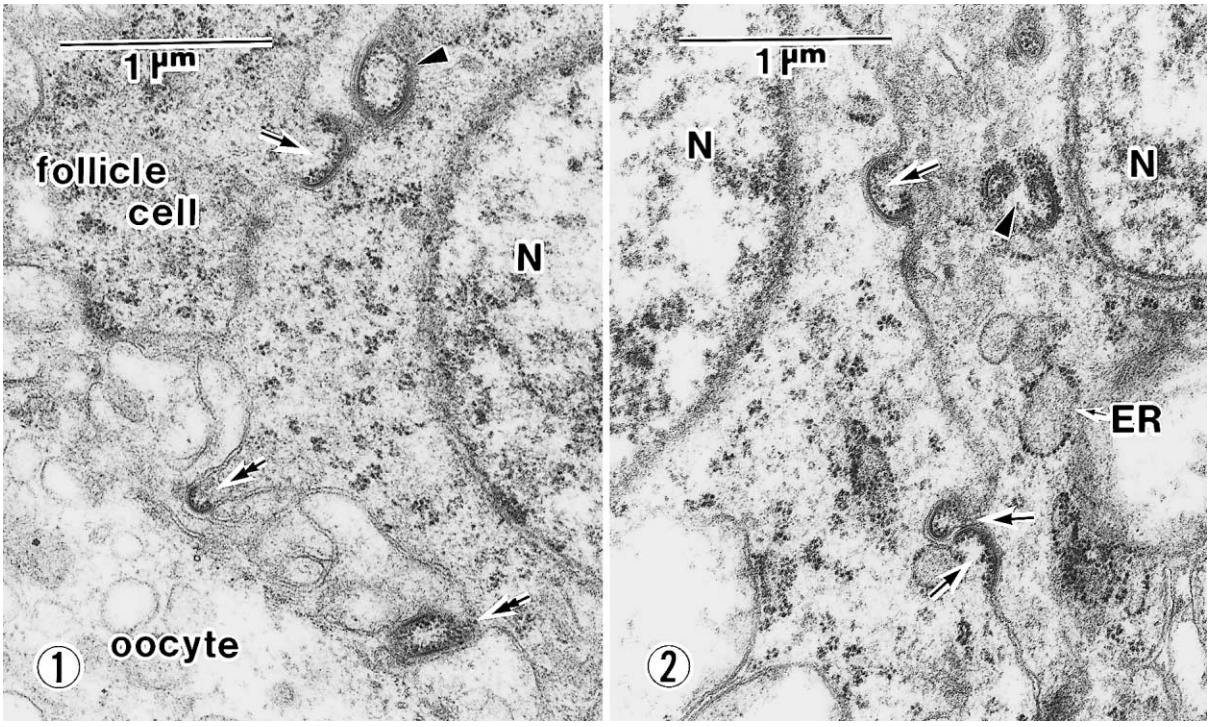
Protein was determined according to the manufacturer's protocol for the Pierce BCA protein assay kit (Pierce, USA).

RESULTS

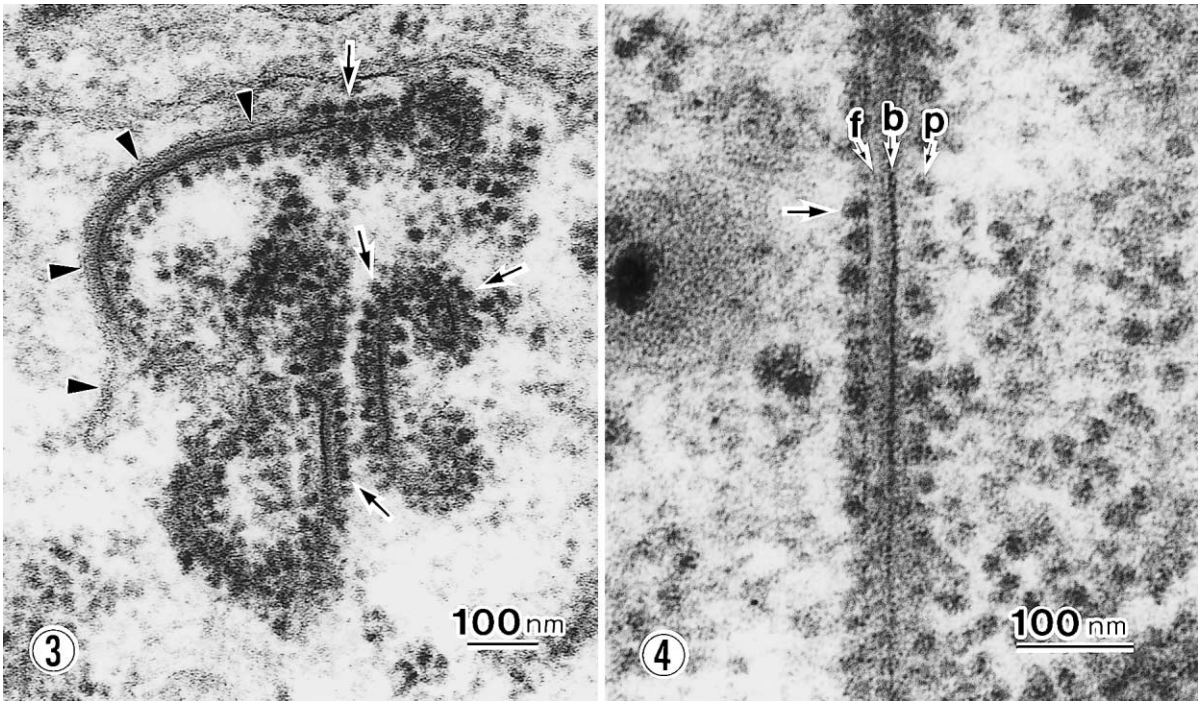
Ultrastructural observations of the relationship between vitellogenic oocytes and follicle cells

At the border between an oocyte 0.8 mm in diameter and the surrounding follicle cells, the two types of cells are in contact by means of short microvilli in the early stages of oogenesis (Fig. 1). The microvilli of follicle cells have special structures, called lining bodies (see below), at their tips. The lining bodies were most often observed at the lateral follicular cell membranes (Fig. 2). Follicle cells lie close together along their lateral cell membranes, the gap between them being about 20 nm. The lining bodies have a tendency to be concave, pushing the follicular cell membrane outwards from the inside. Thus the distance between the membranes of two apposing cells decreased to less than 10 nm at these points. Frequently observed was a mutual bulginess at approximately the same site on neighboring cells.

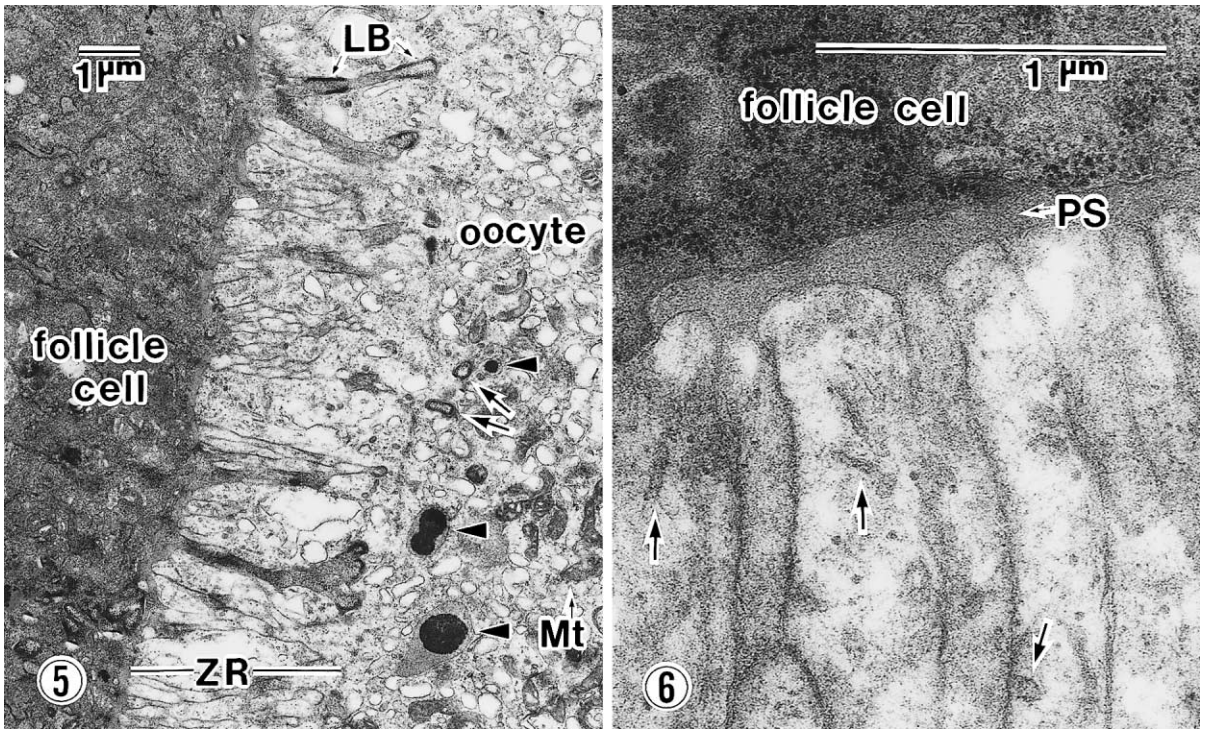
A stack of lining bodies possibly decorated with ribo-



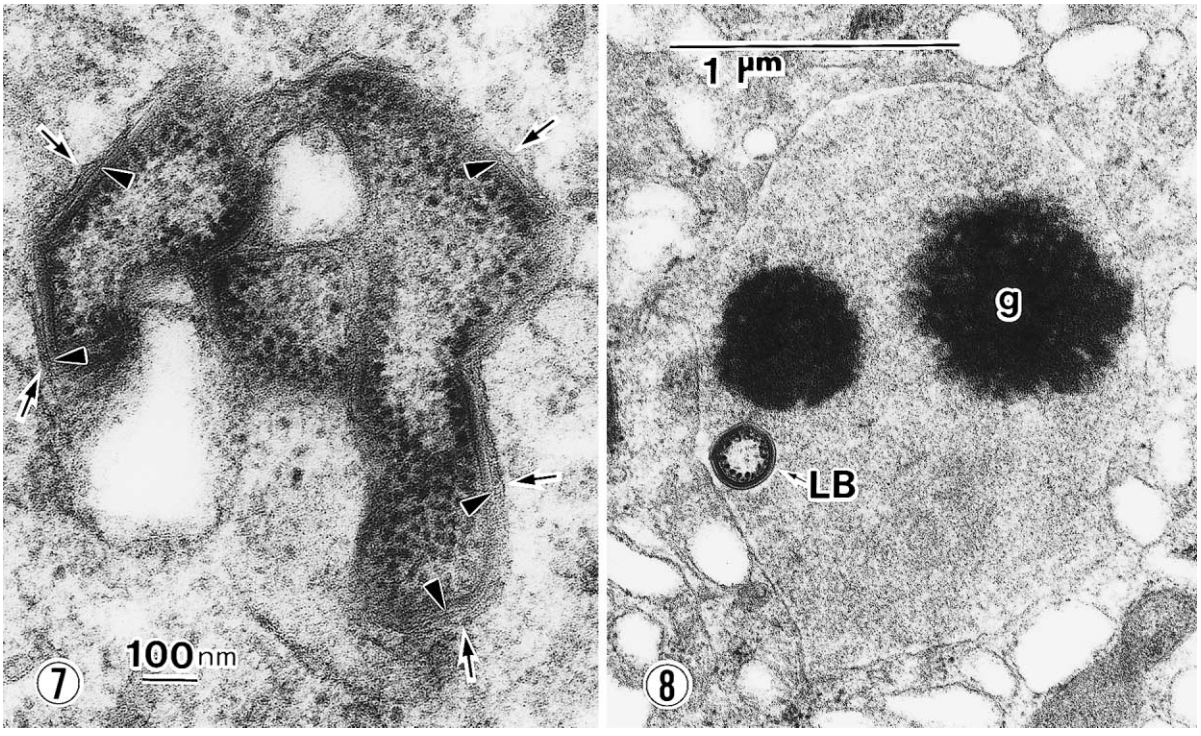
Figs. 1 and 2. Electron micrographs of follicle cells surrounding a 0.8 mm oocyte, showing lining bodies adhering to the inner face of follicle cell membranes. Double arrows indicate lining bodies which are pushing against the cell membrane of their follicle cells at the microvilli towards the oocyte (Fig. 1), and single arrows indicate those on the lateral cell membrane of the follicle cells (Figs. 1 and 2). The arrowhead in Fig. 1 indicates the cross-section of a bulge from an adjacent follicle cell. The arrowhead in Fig. 2 indicates lining bodies just forming on free poly-ribosomes. ER, rough-surfaced endoplasmic reticulum; N, nucleus.



Figs. 3 and 4. Lining bodies in follicle cells surrounding a 0.8 mm oocyte. Arrows indicate ribosomes. The lining body consists of three components (Fig. 4): a fuzzy, electron-lucent layer (f), a chain of electron-dense beads (b) and a line of 20-nm particles (p). Occasionally a very thin sheet remains on the fuzzy layer (arrowheads in Fig. 3) after the ribosomes detach.



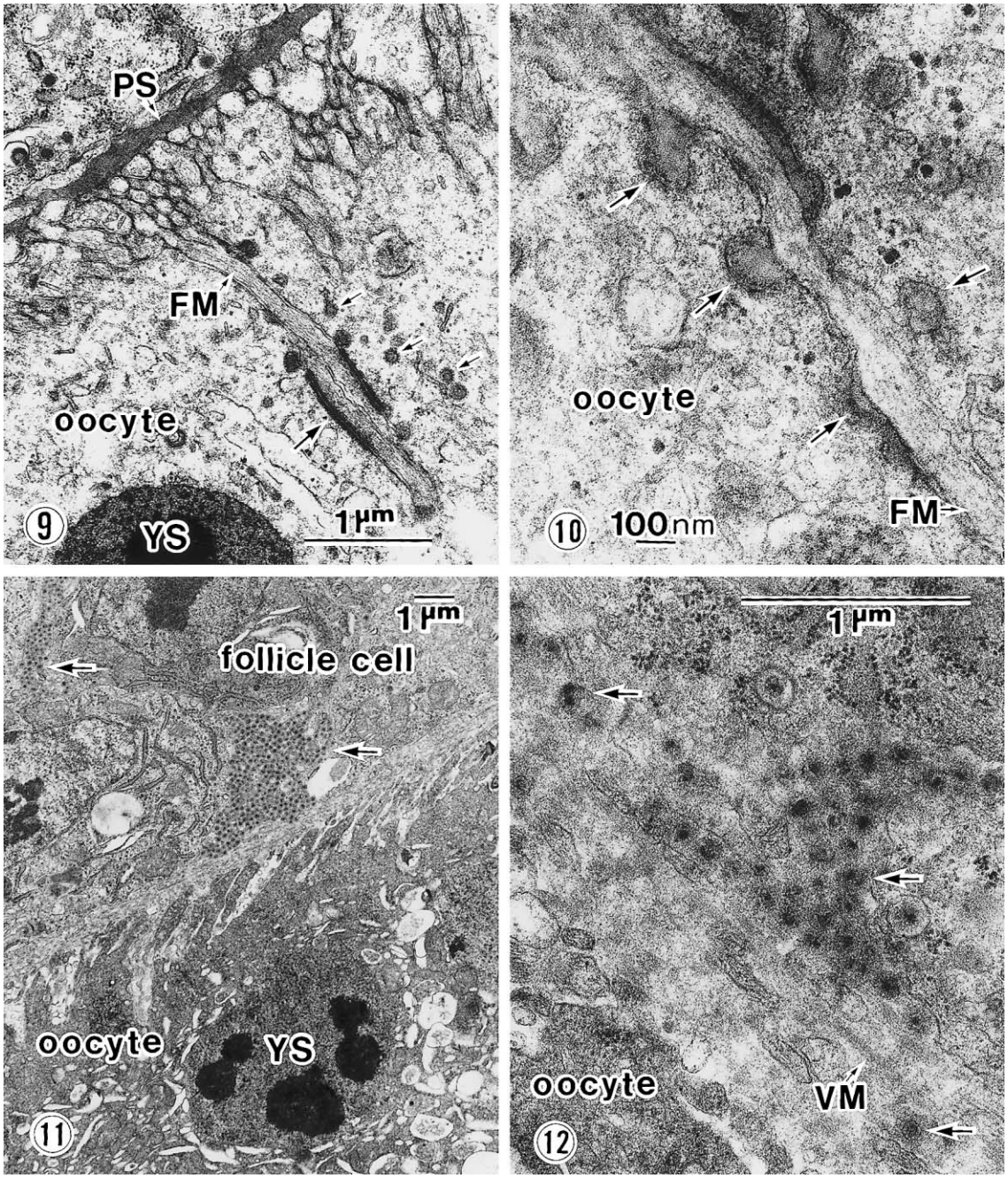
Figs. 5 and 6. Electron micrographs of follicle cells surrounding a 1.3 mm oocyte. Lining bodies (LB) are present at the tips of follicular microvilli or occasionally at their necks. Vesicles containing LB (arrows) and the nascent yolk spheres containing yolk granules (arrowheads) are present in the cortical region of the oocyte (Fig. 5). Presumably what accumulates in the perivitelline space (PS) between follicle cells and a stack of oocytic microvilli is VTG (Fig. 6). Very small vesicles are present in the oocytic microvilli (arrows in Fig. 6). Mt, mitochondrion; ZR, zona radiata.



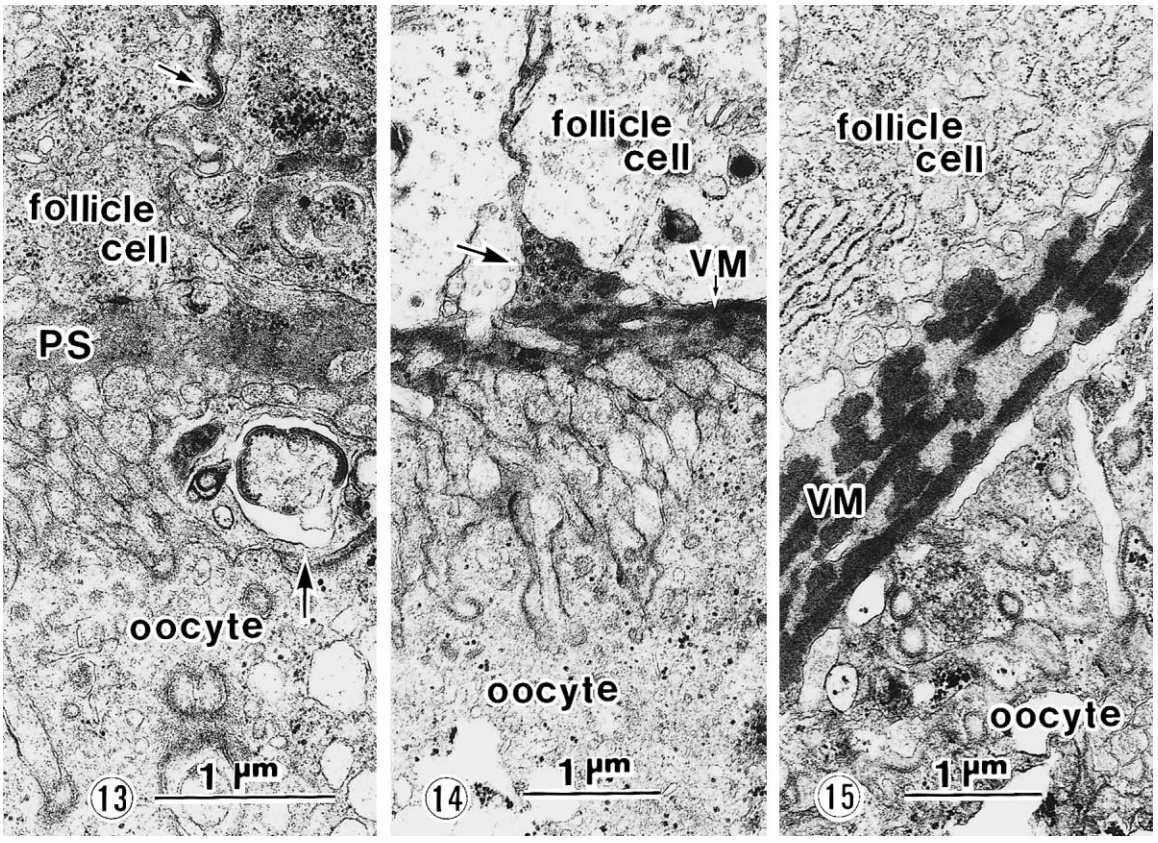
Figs. 7 and 8. Electron micrographs of the cortical region of a 1.3 mm oocyte, showing the vesicles containing lining bodies (Fig. 7) and the nascent yolk sphere (Fig. 8). In Fig. 7, arrows indicate limiting membranes originating from the oocytic cell membrane and arrowheads indicate those from the follicle cell membrane. Electron dense materials fill the space between the two membranes. In Fig. 8, a lining body (LB) surrounded by a single membrane appears with fine particles and yolk granules (g) in a yolk sphere.

somes was frequently observed in the cytoplasm of follicle cells (Figs. 2 and 3). High power view showed that the lining body consists of three components: (a) a fuzzy, electron-lucent layer 13 nm in width, (b) a chain of electron-dense beads 6 nm in size, and (c) a line of particles 20 nm in size (Fig. 4). Occasionally a very thin sheet was observed on the fuzzy layer. It is not clear at present whether the sheet is a normal constituent of a lining body or an unrelated substance accidentally adhering. The lining bodies after being produced on the ribosomes migrate to the cell membrane and bind with it at their fuzzy layer.

In the case of an oocyte 1.3 mm in diameter, the microvilli of both the oocyte and the surrounding follicle cells elongate to 3 μm and form the so-called zona radiata (Fig. 5). The microvilli of follicle cells could be easily distinguished from oocytic microvilli by their high electron-density and the



Figs. 9–12. Electron micrographs of F5 follicles possessing a 4.5 mm oocyte. Two types of follicles are present. In the first type, follicular microvilli (FM) are bound to the oocytic cell membrane with desmosome-like junctions (large arrow in Fig. 9). Coated vesicles (small arrows in Fig. 9) or coated pits (arrows in Fig. 10) are present on or near the oocytic cell membrane. In the second type of follicle (Figs. 11 and 12), the interstitial space between follicle cells widens and is filled with VLDL particles (arrows). The zona radiata is obscure in the perivitelline space (PS), but the fibers of vitelline membrane (VM) appear (Fig. 12). Yolk spheres (YS) contain yolk granules but no lining bodies.



Figs. 13–15. Electron micrographs of F4, F3 and F1 follicles. In an F4 follicle possessing a 5.6 mm oocyte, a lining body is present in the follicle cell (small arrow in Fig. 13), but in the oocyte it is in a vesicle which locates near the cell surface (large arrow). In an F3 follicle possessing a 8.5 mm oocyte, the perivitelline space (PS) is filled with the vitelline membrane (VM), the VLDL particles (arrow) and an electron-dense matrix of presumably VTG (Fig. 14). The fibers of VM increase in their width. In an F1 follicle possessing a 15 mm oocyte, the perivitelline space is occupied by the fibers of VM and an electron-lucent matrix (Fig. 15). The zona radiata is obscure in this follicle.

presence of lining bodies, located mostly at the microvilli's tips and occasionally at their necks. Oocytic microvilli contain small vesicles (Fig. 6). A single follicular microvillus often branches at its front into several tips, each of which possesses a lining body. Lining bodies continued to be produced in the follicle cells and thus there are many bulges in the lateral cell membranes where the lining bodies are present (not shown). There is an accumulation of electron-dense substances in the space between the follicle cells and the tips of oocytic microvilli (Fig. 6); these substances might include vitellogenin (VTG), as shown later with immunohistochemistry.

The most conspicuous feature in the cortical cytoplasm of a 1.3 mm oocyte is the presence of yolk spheres and vesicles, in both of which there are lining bodies (Fig. 5). The vesicles are apparently delimited by two membranes (Fig. 7). The structure of such vesicles can be understood if the tips of the follicular microvilli with the lining bodies inside had been pinched off and engulfed by indentations of an oocytic cell membrane. Then the outer membrane of the vesicles might originate from the oocytic cell membrane and the inner membrane from the follicular cell membrane. The yolk spheres contain yolk granules and lining bodies (Fig. 8). Yolk spheres grow in size by repeated mutual fusions, form-

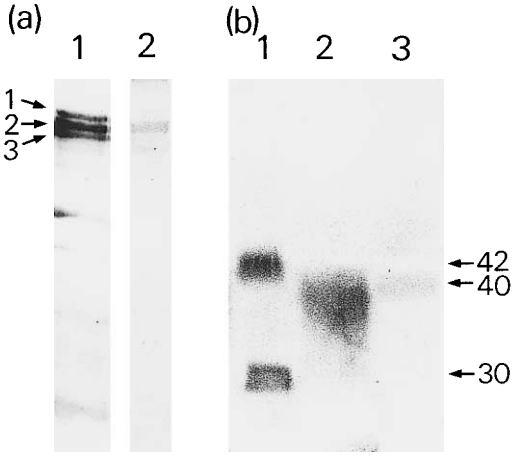


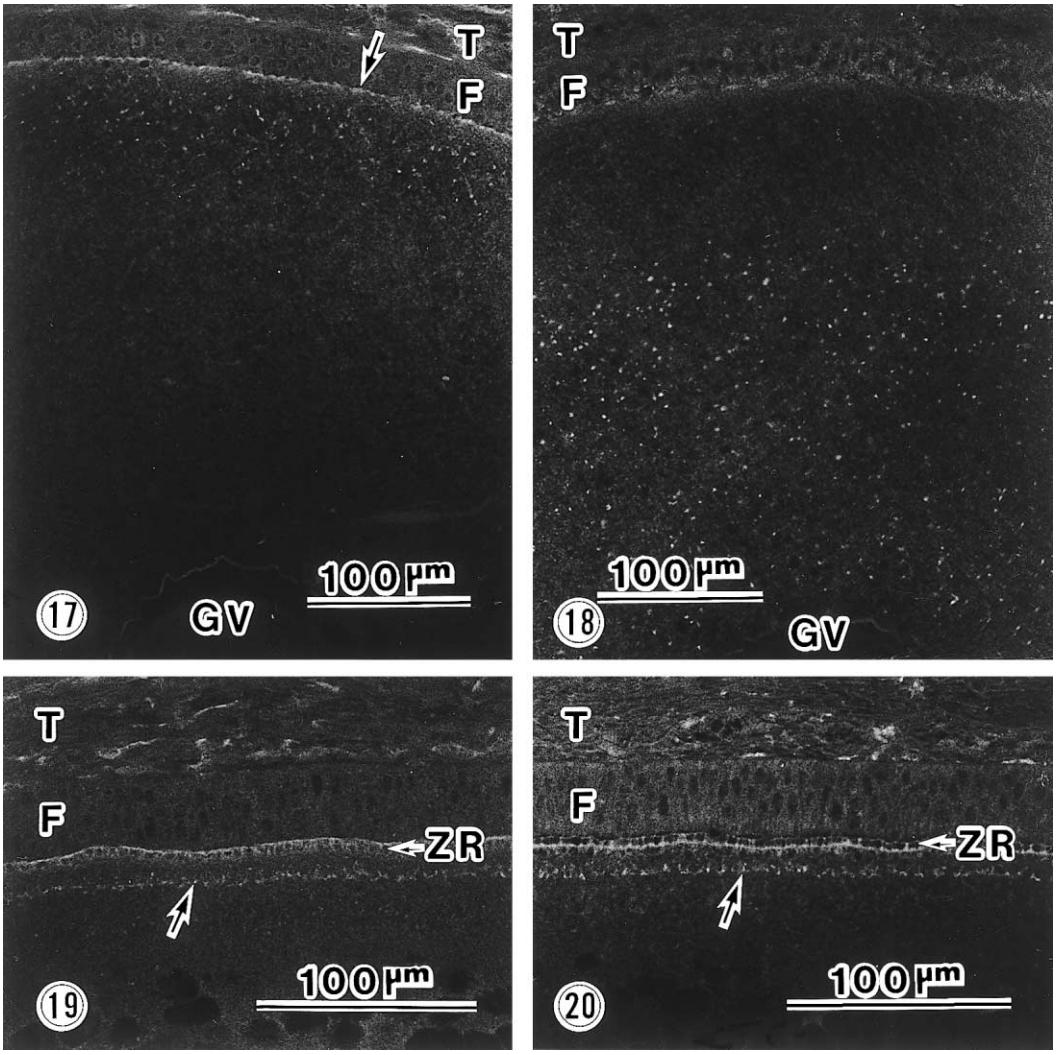
Fig. 16. SDS-PAGE showing cross-reactivity of used antisera. (a) Blood plasma (4 μg protein) from estradiol-primed males was electrophoresed and stained with Coomassie brilliant blue (lane 1). Numbers at the left indicate the bands of VTG1, 2 and 3, respectively. On immunoblotting, an antiserum against the quail VTG2 specifically stains the band (lane 2). (b) An antiserum against the rat cathepsin D stains the 40 kD band of cathepsin D from quail liver (500 ng protein; lane 2) or yolk (lane 3). Lane 1 shows the rat liver cathepsin D. Molecular sizes are shown to the right in kD.

ing a large yolk sphere in which many yolk granules and lining bodies have accumulated in a 3 mm oocyte (not shown).

In yellow follicles of F5, drastic changes occur in the relationship between oocytes and follicle cells. Microvilli of a 4.5 mm oocyte and those of follicle cells maintain the configuration of the zona radiata by binding each other with a desmosome-like junction in most follicles (Fig. 9). Many coated pits or vesicles were observed on or near the cell membrane of oocytic microvilli (Figs. 9 and 10). In some other follicles of F5, the microvilli of the oocyte and those of follicle cells lose their intimate association (Fig. 11). Thus the structure of the zona radiata becomes obscure and then the perivitelline space widens. The most prominent feature of the perivitelline space in this follicle is the appearance of fibers of vitelline membrane and many VLDL particles (Fig. 12). An accumulation of VLDL particles was observed here and there in the widened spaces between follicle cells (Fig. 11), but not in the oocyte.

Lining bodies were not prominent in yellow follicles. Although they were still observed in an F4 follicle, those dislodged from follicular microvilli remained near the surface in a 5.6 mm oocyte but were not incorporated into yolk spheres (Fig. 13).

In an F3 follicle, the VLDL particles, VTG and apparent fibers of the vitelline membrane filled the perivitelline space. The zona radiata, now 2 μm in length, and coated vesicles were again observed in an 8.5 mm oocyte (Fig. 14). The ultrastructural features of an F2 follicle were similar to those of an F3 follicle. In an F1 follicle, just before spawning, the zona radiata was not observed in a 15 mm oocyte and the perivitelline space was filled with the fibers of vitelline membrane and electron-lucent matrix (Fig. 15).



Figs. 17–20. Immunofluorescent micrographs of a 0.8 mm oocyte (Figs. 17 and 18) and 1.6 mm oocyte (Figs. 19 and 20). Sections from the same oocyte were stained with the antibodies raised against VTG2 (Figs. 17 and 19) or cathepsin D (Figs. 18 and 20). In the 0.8 mm oocyte, VTG2 is present in small vesicles in the cortical region and in the perivitelline space (arrow in Fig. 17), whereas cathepsin D is present in the small vesicles around a germinal vesicle (GV; Fig. 18). In the 1.6 mm oocyte, both substances are present in round or tubular vesicles in the cortical region (arrows in Figs. 19 and 20). In addition, VTG2 is present in the perivitelline space (Fig. 19), and cathepsin D is present at or near the oocytic cell membrane (Fig. 20), including its microvilli. F, follicle cell; T, theca; ZR, zona radiata.

Cross-reactivity of antisera

Immunoblotting analyses were performed to show the cross-reactivity of the antisera used. An antiserum raised against quail VTG2 specifically stained the VTG2 in blood plasma from estradiol-primed males (Fig. 16a). Fig. 16b shows that the partially purified, 40 kD cathepsin D from quail liver or yolk cross-reacts with an anti-rat liver cathepsin D antiserum.

Immunohistochemical observations on vitellogenic oocytes and follicle cells

To detect the location of VTG and cathepsin D, which participates in cleaving the VTG molecules, immunofluorescent staining of oocytes and follicle cells was carried out using the antisera mentioned above. Anti-VTG antiserum stained the perivitelline space and vesicles in the cortical cytoplasm of a 0.8 mm oocyte (Fig. 17). On the other hand, anti-cathepsin D antiserum stained the small vesicles in the deep cytoplasm around the germinal vesicle of the oocyte (Fig. 18). Thus the two proteins distributed separately, confirming the absence of yolk spheres in such an oocyte.

The zona radiata of an oocyte 1.6 mm in diameter appears to be stained with antisera for both VTG (Fig. 19) and cathepsin D (Fig. 20). However, closer observation revealed that the VTG resides on or near the surface of the follicular cell membrane whereas the cathepsin D resides at or near the oocytic cell membrane. Tubular or round vesicles in the cortical cytoplasm of oocytes were also stained with both antisera.

Large yolk spheres located in the deep cytoplasm of a 1.6 mm oocyte were not stained by either antiserum. A cluster of small vesicles which were stained by the antiserum against cathepsin D were still present in the center of the oocyte after the germinal vesicle had moved toward the cell surface (not shown).

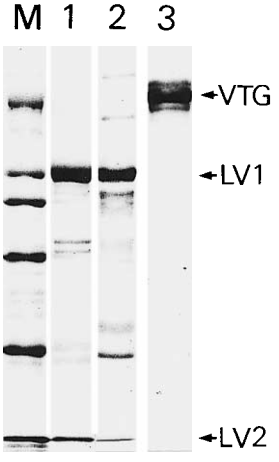


Fig. 21. SDS-PAGE showing the cleavage of the VTG by cathepsin D. Purified VTG (4 μg protein) was incubated with quail liver cathepsin D (50 ng protein) for 1 day at 37°C, electrophoresed and stained with Coomassie brilliant blue (lane 2). Lane 1, yolk proteins; lane 3, VTG. M, marker proteins of 200, 116, 97, 66, 45 and 31 kD from top to bottom; LV1 and 2, lipovitellin 1 and 2.

The staining pattern of both antisera just described for the 1.6 mm oocyte was maintained in an oocyte of an F5 follicle. We could not test oocytes larger than 7 mm in diameter of an F4 follicle because of a poor fixation.

Cleavage of VTG by cathepsin D

Proteins of yolk granules consist of lipovitellins 1 and 2 and phosvitin, the molecular weights of which are 110kD, 30 kD and 42 kD, respectively (Elkin *et al.*, 1995; lane 1 in Fig. 21). The band of phosvitin is not shown in Fig. 21, because it is not stainable with Coomassie brilliant blue. Both lipovitellins were apparently produced when the purified VTG was incubated with quail liver cathepsin D at pHs of 3 to 5 (lane 2). The same cleavage pattern of VTG was obtained by incubation with yolk cathepsin D (not shown).

DISCUSSION

The present study revealed that lining bodies are produced in the follicle cells of quail ovaries, adhere to the inside of the follicular cell membranes, move along the membranes in the direction of the enclosed oocyte and are posted at the tip of the follicular microvilli. They could be a marker for tracing the movement and fate of the membranes of follicle cells. The microvilli of follicle cells elongate to 3 μm, forming the so-called zona radiata with similarly elongated microvilli of oocytes. The fact that at all the tips of such follicular microvilli there are lining bodies as well as the fact that what appear to be vesicles in the cortical region of the oocyte also possess lining bodies suggest that the follicular microvilli are engulfed by the oocyte. The presence of lining bodies in small nascent yolk spheres in this region would strengthen such a view. Although how the tips of the follicular microvilli are pinched off from the main body of the follicle cell is not yet known, such a removal of cell membranes may promote the movement of lining bodies from the lateral cell membrane to the microvilli in follicle cells.

Ovarian follicle cells are known to assist growing oocytes by accumulating sufficient amounts of nutrients. However, the major nutrient components, VTG and VLDL, are synthesized in the liver (Perry *et al.*, 1984; Wallace, 1985). These lipoglycoproteins traverse the interstitial space of the follicle cell layer and are taken in by the oocyte. It was shown in *Xenopus* that the process of taking the VTG into the oocyte does not require any direct help from the follicle cells (Opresko and Karpf, 1987). The substance which is really produced by the follicle cells during the period of vitellogenesis is E2, a hormone stimulating the liver to produce lipoglycoproteins (Sretarugsa and Wallace, 1997). The presence of lining bodies in yolk spheres is the first evidence that the follicle cells are actually involved in the accumulation of nutrients by the growing oocytes.

A lining body is a sheet which may be a complex of three components, that is, fuzzy, particle and granular entities, or a polymer consisting of a single molecule with three different domains. The lining body is not bound by a mem-

brane. Thus, it might reasonably be concluded that they are produced on free ribosomes but not on the rough-surfaced endoplasmic reticula. Figs. 3 and 4 may represent such poly-ribosomes with the lining body just forming. However, further study is needed to verify this proposal, since no comparable photograph has been published that shows housekeeping proteins in the process of formation on free polyribosomes.

The biological function of the lining body is a matter of speculation at present. However, the following facts indicate that its role may be to transport VTG into the oocyte through the follicle cell layer. (1) Follicle cells surrounding a small oocyte in a quail bind very closely with each other along their whole lateral cell membrane, the distance between the membranes being about 20 nm. It is difficult for the VTG to pass smoothly through this space only by the force of diffusion. This contrasts with VTG transport in *Xenopus* (Yoshizaki, 1992) and VLDL and VTG transport in large quail oocytes of yellow follicles (Perry and Gilbert, 1979; the present study), in which a huge space appears between the follicle cells that enables transportation only by diffusion through the follicle cell layer. (2) The lining body adhering to the inside of the follicular cell membrane has a tendency to become concave, thus pushing its own cell membrane outwards toward the adjacent cell's membrane. This outward movement occurs in neighboring cells at approximately the same place and narrows the intercellular space to less than 10 nm at that point. When this mutually bulging site moves in the direction of the oocyte, as mentioned earlier, the VTG entrapped in the interstitial space of the follicle cell layer will be transported in the same direction. (3) Immunofluorescent observations show that VTG accumulates in the perivitelline space and is also present in the vesicles and small yolk spheres in the cortical region of the oocyte, all locations where there are also lining bodies.

The phenomenon of extracellular substances being taken up occurs in a wide variety of cells. This process, when occurring at the cell membrane, is called receptor-mediated endocytosis; in the process, specific macromolecules are endocytosed in the cell by being bound to complementary cell-surface receptors lined with a clathrin coat (Alberts *et al*., 1989). *Xenopus* VTG (Yoshizaki, 1992) and chicken VLDL (Perry and Gilbert, 1979) apparently enter the cell as receptor-macromolecule complexes in endocytic coated vesicles, and those very receptors have been identified (George *et al*., 1987; Stifani *et al*., 1990). In the present study, the same endocytic coated vesicles were found in the oocytes, but only of yellow follicles. On the other hand, Stifani *et al.* (1988) have succeeded in identifying the VTG receptor in chicken oocytes of yellow follicles. Then there might be two different mechanisms for the uptake of nutrients in avian oocytes. In small oocytes of white follicles, the VTG is taken up through so-called fluid-phase endocytosis which is assisted by follicular lining bodies, and in large oocytes of yellow follicles, the VTG and VLDL are taken up through receptor-mediated endocytosis.

In the follicles of which the perivitelline space is filled with VLDL particles, microvilli of an oocyte and those of follicle cells tend to lose their intimate association. They may recover the association, forming a zona radiata at subsequent stages. We could not obtain results to explain such a change of the zona radiata. There might be some rhythm in the uptake of VTG and VLDL, coordinating with the change of the follicle cell layer, since it is well known that concentric bands having different shades of yellow are often discernible in boiled yolk (Romanoff and Romanoff, 1949).

The engulfment of follicular microvilli by the oocyte is apparently achieved by phagocytosis, the generic term signifying the endocytosis of large structures via large vesicles. However, as far as we know, phagocytosis is performed mostly by those cells which clear away dead cells and their debris or foreign organisms. The engulfment of follicular microvilli by the avian oocyte, then is a unique example of phagocytosis by which certain portions of cells that are healthy are taken up by cells belonging to the same individual.

After being taken into the oocytes, VTG is cleaved by cathepsin D and processed into yolk proteins, lipovitellins and phosvitin (Retzek *et al*., 1992; Elkin *et al*., 1995; the present study). The VLDL is also cleaved to several fragments by cathepsin D (Retzek *et al*., 1992; Elkin *et al*., 1995). Retzek *et al.* (1992) isolated cathepsin D from chicken oocytic yolk and liver. Upon analysis by SDS-PAGE, both enzyme preparations were found to consist of three bands of 43, 30 and 14 kD. This seemed to be consistent with the known existence of single-chain (42 kD) and two-chain (30 and 12 kD) forms of mammalian cathepsin D (Yonezawa *et al*., 1987). However, the present study showed that the cathepsin D from quail liver and yolk consists only of a single-chain form of 40 kD. It is also known that the cathepsin D from *Xenopus* ovary is a single-chain form of 43 kD (Nakamura *et al*., 1996). The absence of a two-chain form of avian and amphibian cathepsin D may be explained by a missing proteolytic processing region in the cDNA sequence (Mukai *et al*., 1995). How the VTG is encountered by the cathepsin D in quail oocytes is not yet known. But, tubular or round vesicles in the cortical cytoplasm of oocytes, which were stained with both antisera against VTG2 and cathepsin D, might be the sites where yolk proteins are enzymatically produced from the VTG.

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