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Termination of Vitellogenesis in Follicles of the Moth, *Plodia interpunctella*: Changes in Oocyte and Follicular Epithelial Cell Activities

Grażyna Zimowska, Paul D. Shirk, Donald L. Silhacek, and Eli Shaaya

Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Gainesville, Florida (G.Z., P.D.S., D.L.S.); Department of Invertebrate Physiology, University of Warsaw, Warszawa, Poland (G.Z.); The Volcani Center, Agricultural Research Organization, Bet-Dagan, Israel (E.S.)

The production and uptake of the follicular epithelium yolk protein (FEYP) is terminated coincident with the initiation of vitelline membrane synthesis in follicles of the Indianmeal moth, *Plodia interpunctella* (Hübner). This was determined by visualizing the cytolocalization of the FEYP subunits YP2 and YP4 using antisera to immunolabel ultrathin sections or whole-mounted ovaries. Both subunits of FEYP were detectable in the Golgi apparatus and associated secretory granules of the follicular epithelial cells (FC) in vitellogenic follicles. Before the follicles entered the terminal growth phase, the oocytes began production of specialized organelles, late yolk spheres. Following the appearance of late yolk spheres in the oocyte, the FC initiated the production of vitelline membrane proteins and the rapid clearance of YP2 from their cytoplasm. No YP2 was detected in the Golgi apparatus or in the secretory granules of FC from follicles in terminal growth phase, although YP4 was detected in these organelles. The vitelline membrane of follicles in termal growth phase was a bilayered structure with an electron-dense layer of vitelline membrane proteins that originated in the FC and an electron-translucent layer containing yolk proteins. During this period, late yolk spheres were observed fused with the oolemma exposing and possibly releasing their contents to the electron-translucent layer of the vitelline membrane. From this evidence, we suggest that during termination of vitellogenesis, the oocyte and FC work in concert to end uptake of yolk proteins and begin the synthesis of egg membranes, and that the oocyte contributes to the production of vitelline membrane by the release of previously sequestered yolk proteins.

Key words: yolk proteins, vitelline membrane, immunofluorescent staining, immunogold labeling, Indianmeal moth

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Address reprint requests to Paul D. Shirk, P.O. Box 14565, Gainesville, FL 32604.

INTRODUCTION

As an ovarian follicle completes vitellogenesis in moths, the three cell types within the follicle, i.e., oocyte, nurse cells, and follicular epithelium cells (FC*), must change their activities from those of vitellogenic to post-vitellogenic (compare Telfer and Smith, 1970). During vitellogenesis the tissues of the follicles are involved in processes supporting the accumulation of the maternally contributed nutrient stores, organelles, and positional information within the oocyte that are necessary for directing and supporting early embryogenesis. After vitellogenesis is complete, the follicle enters into the terminal growth phase and begins sealing the oocyte within an impermeable encasement. The nurse cells complete the transfer of cytoplasm to the oocyte, close the cytoplasmic connections, i.e., ring canals, with the oocyte, and become pycnotic, thus ending their role. The oocyte discontinues the endocytotic activities of yolk protein uptake and forms a cortical periplasm. The FC initiate synthesis of the vitelline membrane, form tight junctions between the cells establishing an occlusion zone around the oocyte (Rubenstein, 1979), and secrete the chorion. At the completion of these processes, the mature egg is ready for fertilization.

As described previously for the moth, *Hyalophora cecropia*, the terminal growth phase begins when a follicle finishes vitellogenesis, and it is marked by the follicle becoming impermeable to the stain, Trypan blue (Telfer and Anderson,1968). The impermeability to Trypan blue and other extra-ovarian materials is the result of vitelline membrane synthesis (Telfer and Anderson, 1968) and of the formation of an occlusion zone by the FC (Rubenstein, 1979). It is only after the formation of the occlusion zone that the oocyte becomes isolated from further exposure to the vitellogenins present in the hemolymph of *H. cecropia* (Rubenstein, 1979).

The termination of vitellogenesis in *H. cecropia* has been associated primarily with the initiation of vitelline membrane synthesis (Telfer and Smith, 1970). From studies in the fruit fly, *Drosophila melanogaster*, we know that the proteins that comprise the vitelline membrane are produced by the FC (Fargnoli and Waring, 1982). However, in the moth *Anagasta (Ephestia) kühniella*, there has been conflicting evidence as to the cellular origin of the major components of the vitelline membrane (Cruickshank, 1971, 1972; Cummings, 1972). On the bases of both pulse/chase radioactive amino acid labeling and ultrastructural evidence, Cruickshank (1971, 1972) concluded that the oocyte contributed significant amounts of proteinaceous material to the vitelline membrane. He suggested that the proteins originated from membrane bound organelles in the cortex of the oocyte. On the other hand, using only ultrastructural evidence, Cummings (1972) concluded that the proteins of the vitelline membrane originated in the FC as observed in *Drosophila*. However,

*Abbreviations used: bb = brush border; cm = collapsing membranes; FC = follicular epithelial cells; FEYP = follicular epithelium yolk protein; g = Golgi apparatus; lys = late yolk sphere; m = mitochondrion; mv = microvillus; n = nucleus; nc = nurse cell; oo = oocyte; sp = inter-follicular epithelial cell space; v = vesicle; vm = vitelline membrane; YP = yolk

neither of these works unequivocally resolved the nature and origin of the proteinaceous components of the vitelline membrane, nor did they clarify the origin or function of the sites of protein labeling in the oocyte cortex in *A. kühniella*.

Plodia interpunctella (Hübner) is a pyralid moth that is closely related to *A. kühniella* and that shares similarities in yolk proteins (Shirk, 1987) and reproductive physiology. The availability of synchronously developing pharate adults has provided a temporal framework for the analysis of vitellogenesis in *P. interpunctella*. Previous investigations established the developmental timing of follicle maturation (Zimowska et al., 1991), vitellogenin production and uptake (Shirk et al., 1992), initiation of vitellogenesis in terminal follicles (Zimowska et al., 1994), and their correlation with ecdysteroid titers (Shaaya et al., 1993) during metamorphosis.

The terminal follicles within the ovarioles of *P. interpunctella* become vitellogenic at 105 h after pupation (Zimowska et al., 1994) and complete vitellogenesis and initiate choriogenesis within 2 to 6 h after adult eclosion (eclosion is 136 h after pupation) (Zimowska et al., 1991). During vitellogenesis of *P. interpunctella*, two major yolk proteins, vitellin and follicular epithelium yolk protein (FEYP), are accumulated in the yolk spheres of oocytes (Shirk et al., 1984; Bean et al., 1988). As is typical of Lepidoptera, the fat body secretes vitellogenin, which consists of two subunits, YP1 (M_r = 153,000) and YP3 (M_r = 43,000), into the hemolymph. Vitellogenin is accumulated, without major change, in the yolk spheres of the oocyte as vitellin (M_r = 475,000). The follicular epithelial cells secrete FEYP (M_r = 235,000) which consists of subunits YP2 (M_r = 69,000) and YP4 (M_r = 33,000) (Shirk et al., 1984; Bean et al., 1988; Zimowska et al., 1994).

In this investigation, the changes in the follicles during the termination of vitellogenesis and initiation of vitelline membrane synthesis in *P. interpunctella* were examined. To identify events during this period, we examined the subcellular distribution of the individual subunits of the FEYP with antisera for each of the subunits (Bean et al., 1988) using immunofluorescent and immunogold labeling of tissues. We determined the sites and timing of synthesis and release of the two FEYP subunits by the FC during the termination of vitellogenesis. In addition, we describe evidence that suggests that the oocyte actively participates in the termination of vitellogenesis and the initiation of vitelline membrane synthesis.

MATERIALS AND METHODS

Insect Preparations

The *Plodia interpunctella* colony was reared according to Silhacek and Miller (1972) in a 16 h light:8 h dark cycle at 30°C and 70% relative humidity. White pupae (± 1/2 h) were collected just prior to the beginning of the scotophase to obtain synchronous cohorts. The cohorts were determined to be of a specific developmental stage during metamorphosis as identified on the basis of time from pupation and on external morphological characters as described in Zimowska et al. (1991). Insect age is expressed in hours from the time of pupation or adult eclosion.

Immunohistochemistry

The polyclonal rabbit antiserum for YP2 used in immunoreactions was described in Bean et al. (1988). The polyclonal rabbit antiserum for YP4 was prepared as described in Bean et al. (1988) and the specificity is shown in Figure 10. Non-reactive serum for each antiserum was collected from preimmune rabbits. Preabsorbed antisera for YP2 and YP4 were prepared by serially exposing each of the antisera to the corresponding antigen electroblotted to nitrocellulose (Shirk et al., unpublished data). The absence of antigen recognition by a preabsorbed antiserum was determined by exposing the blot to a goat-antirabbit-horseradish peroxidase conjugate (BioRad, Hercules, CA) and then developing a color reaction using an Immuno-Blot assay (BioRad). Background levels of immunoreactivity for each of the preabsorbed and non-reactive sera were determined by labeling whole-mounts and paraffin sections of ovaries from 120 h pharate adults (Zimowska et al., 1994).

Immunofluorescent detection of yolk polypeptides YP2 in whole-mounts of ovaries was performed as described previously (Zimowska et al., 1991). Immunogold-localization of YP2 and YP4 in ultrathin sections was performed as described previously (Zimowska et al., 1994).

Transmission Electron Microscopy

Ovaries were prefixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate, pH 7.5, 0.15 mM $CaCl_2$) at 4°C for 2 h and fixed in 1% OsO_4 in cacodylate buffer plus 2.5% sucrose at 4°C overnight. The tissues were dehydrated in a graded ethanol-acetone series and embedded in Epon-Araldite (Mollenhauer, 1964). Ultrathin sections were poststained with 2% uranyl acetate followed by 0.2% lead citrate (Reynolds, 1963). Ultrastructural examination was performed on a Hitachi H-600 transmission electron microscope operating at 75 kV.

Vitelline Membrane/Chorion Purification

The procedure for purifying the vitelline membrane/chorion is a modification of that used in *Drosophila melanogaster* (Fargnoli and Waring, 1982). One-hour-old embryos were washed twice in 0°C distilled water and once in 0°C homogenization buffer (40 mM Tricine, pH 6.8, 20% sucrose, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM PMSF, 1% Triton X-100). The embryos were disrupted in homogenization buffer by 1 min of sonication at 0°C with a cup-horn sonicator (Heat Systems Ultrasonics, Inc., Farmingdale, NY). The homogenate was centrifuged at 50*g* for 5 min at 4°C. The pellet was resuspended in homogenization buffer, sonicated, and pelleted by centrifugation six times. The pellet was then resuspended in homogenization buffer, the large pieces of chorion were allowed to settle by gravity for 30 min at 0°C, and the supernatant was removed. The washing and settling of the chorion pieces was repeated five times until no yolk spheres were present by visual inspection with a microscope (data not shown). The preparation was placed in SDS-sample buffer (Shirk et al., 1984) and boiled for 5 min. The proteins were resolved by SDS-PAGE and electroblotted to Trans-Blot membrane (BioRad) as described previously (Bean et al., 1988). The immunoblots were blocked and reacted as described previously (Shirk et al., 1992). The indi-

vidual blots were reacted with antisera for YP2 and YP4. The immunoreactive bands were visualized with an Immun-Blot color assay (BioRad) using horseradish peroxidase-linked goat anti-rabbit IgG as the second antibody.

RESULTS

Cytological Distribution and Secretory Pathways for YPs From Follicular Epithelial Cells

YP2 was observed in the FC and oocytes of vitellogenic follicles. In whole-mounted ovaries that contained vitellogenic follicles, immunofluorescent staining of YP2 was restricted to the columnar FC surrounding the oocytes of vitellogenic follicles (Fig. 1A; Table 1). The columnar FC surrounding the oocyte contained similar levels of immunofluorescently staining YP2 and, therefore, uniformly accumulated YP2 without apparent polarization around the oocyte. However, YP2 was not detected in the squamous FC surrounding the nurse cell cap. Staining of whole-mounted ovaries with either non-reactive serum (Fig. 1B) or preabsorbed sera for YP2 (Zimowska et al., 1994) produced minimal, non-specific fluorescence in the preparations.

YP2 was detected within the columnar FC that surround the oocytes of all ages of vitellogenic follicles (Table 1). The presence of YP2 was examined in follicles of the same whole-mounted ovariole of a vitellogenic 124 h pharate adult, and YP2 was detected in the columnar FC of the terminal follicle through the 29th follicle in this ovariole (Fig. 2A,B,C). However, YP2 was not detected in any of the follicles that were younger than the 29th follicle in this preparation (data not shown). Because the columnar FC surrounding the oocytes were clearly stained with the YP2 antiserum, structural features of the columnar FC could be observed. The columnar FC were generally arranged in rosettes, and as the follicles became more mature, the planar surface of the FC increased approximately four-fold from the 29th follicle to the most mature terminal follicle (Fig. 2A–C).

Within the columnar FC of the vitellogenic follicles, YP2 and YP4 were localized in Golgi apparatus and the secretory granules. Immunogold labeling of ultrathin sections of vitellogenic follicles showed that YP2 was present in the forming and mature secretory granules associated with the Golgi apparatus (Fig. 3A). In addition, YP2 labeling was observed in secretory granules near the plasma membrane. Labeling was also observed in secretory granules fused with the plasma membrane of the columnar FC (Fig. 3B). Similar labeling patterns in the Golgi apparatus and secretory granules were observed for YP4 in vitellogenic and terminal growth phase follicles (see Figs. 6A and 7A).

Transformation of Follicles From the Vitellogenic Stage to the Terminal Growth Phase

After follicles of moths complete vitellogenesis, they initiate a terminal growth phase (Telfer and Anderson, 1968). During the terminal growth phase, the follicles of *P. interpunctella* lose the property of staining with the vital dye, Trypan blue (Table 1; Zimowska et al., 1991). The presence of YP2 in the

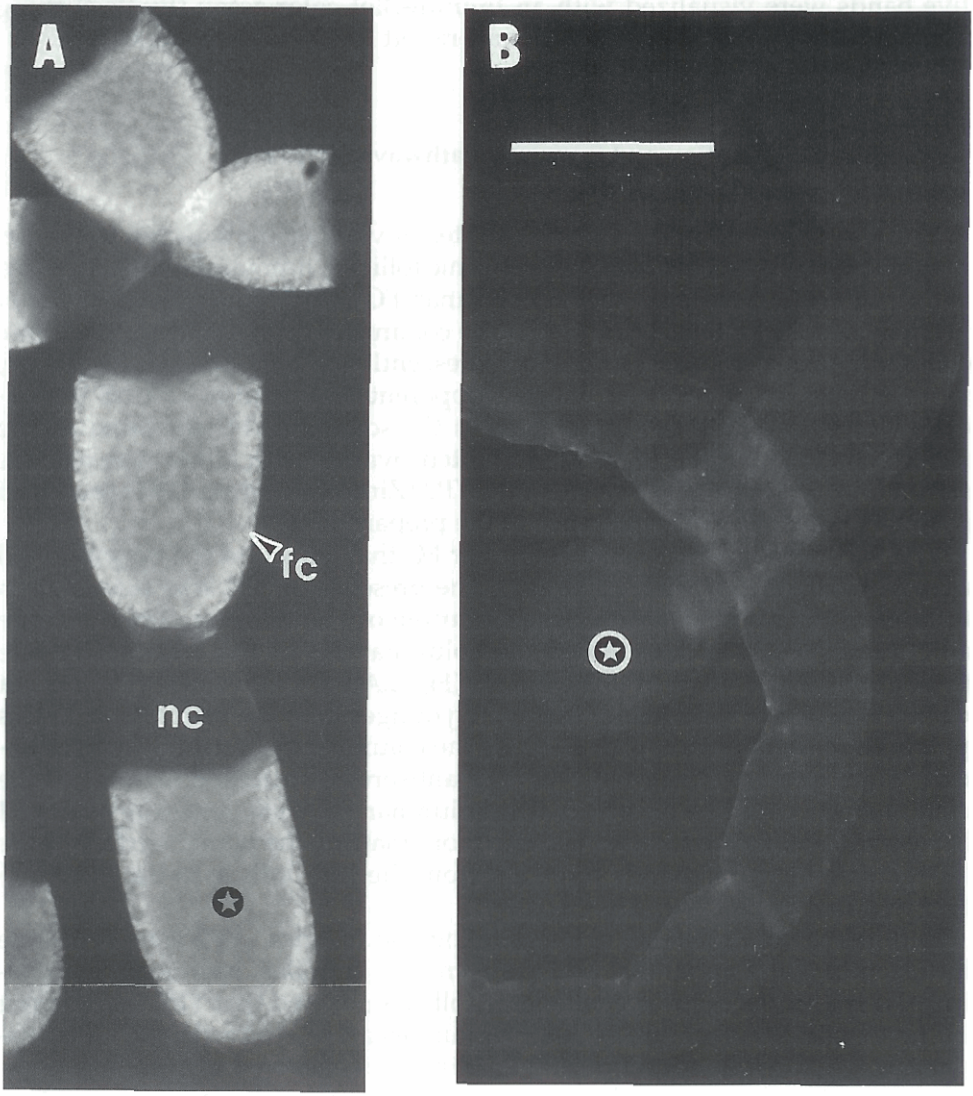

Fig. 1. YP2 *is localized within the follicular epithelial cells of vitellogenic follicles from 124 h* pharate adult female. Whole-mounted ovaries were immunofluorescently stained by reacting with YP2 antiserum **(A)** as primary antisera. As a control, ovaries were incubated with a non-reactive serum **(B)** as the primary serum. Preabsorbed antisera for YP2 produced fluorescent staining levels equivalent to the non-reactive serum (data not shown). The terminal follicles are marked with an asterisk. Abbreviations: fc, follicular epithelial cell; nc, nurse cell cap. Scale bar = 100 μm.

able with Trypan blue (Fig. 4A; Table 1). In whole-mounted ovarioles that contained both vitellogenic and choriogenic follicles, only follicles that retained a nurse cell cap and were vitellogenic had immunofluorescently detectable YP2 in the columnar FC (Fig. 4A, follicles "a" and "b"). Once the

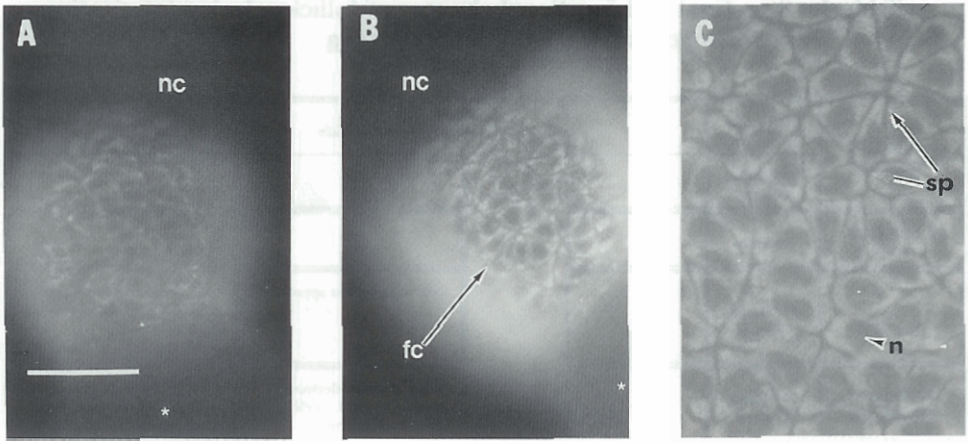

Fig. 2. YP2 is localized in follicular epithelial cells of different stages of follicles from 124 h vitellogenic pharate adult females. Whole-mounted ovaries were reacted with YP2 antiserum as primary antisera. **A:** The follicular epithelial cells of the youngest follicle, the 29th follicle from the terminal follicle, that contains YP2 antigen. **B:** YP2 antigen in follicular epithelial cells of the 23rd follicle from the terminal oocyte, which was an active vitellogenic follicle, from the same preparation as shown in A. **C:** YP2 antigen in the follicular epithelial cells of the vitellogenic terminal follicle from the same preparation as shown in A. The nurse cell cap of the next distal (posterior) follicle is marked with an asterisk in A and B. Abbreviations: fc, follicular epithelial cell; n, nucleus; nc, nurse cell cap; sp, inter-follicular epithelial cell space. Scale bar = 25 μm.

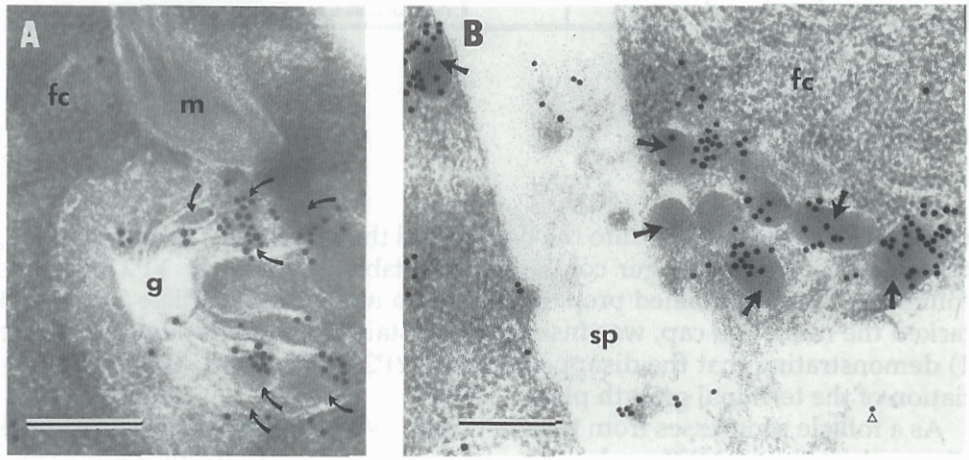

Fig. 3. YP2 is localized within the Golgi apparatus and secretory granules of follicular epithelial cells of vitellogenic follicles. Ultrathin sections were reacted with YP2 antiserum as the primary antiserum. **A:** A Golgi apparatus producing secretory granules. The arrows point to forming or mature secretory granules. **B:** Secretory granules juxtaposed to the portion of the plasma membrane of a follicular epithelial cell that is part of the brush border with an oocyte. The arrow points to a mature secretory granule. The △ in B points to a 20 nm gold particle linked IgG. Abbreviations: fc, follicular epithelial cell; g, Golgi apparatus; m, mitochondrion; sp, inter-follicular epithelial cell space. Scale bar = 0.25 μm.

Table 1. Structural and functional changes of follicles during termination of vitellogenesis in Plodia interpunctella

WHOLE FOLLICLE	Position in Ovariole		(Anterior)	Vitellogenic Follicle	(a) Penultimate vitellogenic Follicle
	Trypan Blue Staining			+	+
NURSE CELL CAP	Presence			+	+
OOCYTE	Structural Changes			Late yolk spheres appear.	Late Yolk spheres become more irregular with less dense contents.
VITELLINE MEMBRANE	Structural Changes			Broad granular electron-translucent band. YPS present throughout.	Electron dense granules fuse to form electron dense layer. YPs present throughout.
FOLLICULAR EPITHELIAL CELLS	Functional Changes	VM[1] Synthesis		--	Apical surface releases electron dense droplets to VM
		Cytoplasm	YP2	Max[3]	Max
			YP4	Max	Max
		IFS[2]	YP2	ND[4]	ND
			YP4	Max	Max
	Structural Changes			Large inter-follicular cell spaces	
				FC[7] surround oocyte except for cytoplasmic bridges	
				FC columnar (long axis of nuclei oriented perpendicular to oocyte)	

[1]VM = Vitelline membrane
[2]IFS = inter-follicular cell spaces
[3]Max = Maximum immunogold labeling
[4]ND = No detectable immunogold labeling
[5]Min = Minimum immunogold labeling
[6]Mod = Moderate immunogold labeling
[7]FC = Follicular epithelial cells

nurse cells were absorbed into the oocyte and the nurse cell cap disappeared, the columnar FC no longer contained detectable amounts of YP2 (Fig. 4A, follicle "c"). In live stained preparations (data not shown), follicle "c," which lacked the nurse cell cap, was insensitive to staining with Trypan blue (Table 1) demonstrating that the disappearance of YP2 was coincident with the initiation of the terminal growth phase.

As a follicle progresses from the vitellogenic stage into the terminal growth phase, the columnar FC undergo a rapid change in activity by terminating the synthesis of yolk protein and initiating the production and deposition of the vitelline membrane proteins (Fig. 5; Table 1). In the penultimate vitellogenic follicle, the vitelline membrane was identified as a broad electron-translucent region outside of the brush border of the oocyte and along the apical surface of the columnar FC (Figs. 5B; 6A,B). Along the apical surface of the columnar FC, electron-dense granules were deposited and incorporated into the vitelline membrane region (Fig. 5B). The columnar FC

Table 1. (continued)

(b) Most vitellogenic follicle		(c) First post-vitellogenic follicle (lacking nurse cells)	(Posterior)
+ Vitellogenic		-- Terminal Growth Phase	
+		--	
Late yolk spheres release contents (YPS) through the brush border. Transfer of nurse cell cytoplasm		Late yolk spheres release contents (YPs) through the brush border. Cytoplasmic bridges close after transfer of nurse cell cytoplasm. Hydration increased volume.	
Oocyte surrounded by electron-dense layer. YPs restricted to translucent layer	Transformation of Follicular Epithelium	Bilayered structure: Electron-translucent VM (YPs present) Electron-dense VM (YPs not present)	
		+	
Min[5]		ND	
Max		Min	
ND		ND	
Mod[6]		Min	
FC establish apical inter- cellular contacts		Occlusion zone between FC complete	
		FC completely surround oocyte	
		FC flat & elongated (long axis of nuclei oriented parallel to oocyte surface)	

the apical surfaces (Fig. 5A,B). Within the oocyte, a few late yolk spheres structural variants of yolk spheres as described below, could be observed that were positioned close to the oolemma and associated with collapsed mem branes (Fig. 5B).

Immunogold labeling of ultrathin sections from vitellogenic follicles showed that both YP2 and YP4 were associated with the Golgi apparatus and secre tory granules of the columnar FC adjacent to the vitellogenic oocyte (Fig 6A,B). The immunogold labeling also showed that YP4, as well as YP2, wa detectable within the late yolk spheres of vitellogenic oocytes (Fig. 6A) and that YP4 (Fig. 6A) and YP2 (Fig. 6B) were present in the broad, diffuse vi telline membrane.

In the terminal vitellogenic follicle, identified by the presence of the nurse cell cap, the vitelline membrane formed a condensed, electron-dense regio between the oocyte and the columnar FC (Fig. 5C,D; Table 1). In addition t the condensed vitelline membrane, the columnar FC developed cytoplasmi contacts between cells at the apical surface so that the vitelline membran was continuously in contact with the FC (Fig. 5C).

During the terminal growth phase, the condensed vitelline membrane be

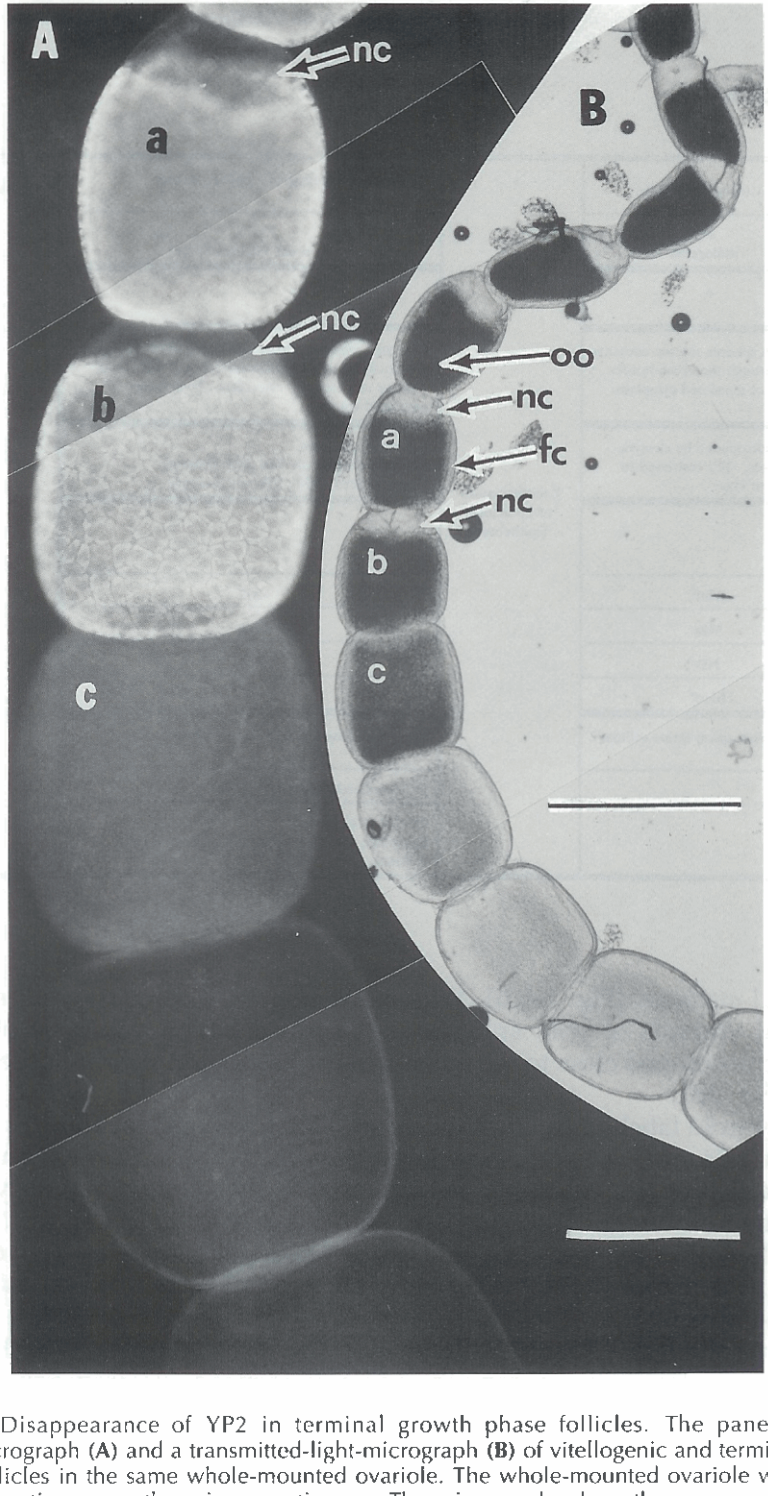

Fig. 4. Disappearance of YP2 in terminal growth phase follicles. The panels show a fluoromicrograph **(A)** and a transmitted-light-micrograph **(B)** of vitellogenic and terminal growth phase follicles in the same whole-mounted ovariole. The whole-mounted ovariole was reacted with YP2 antiserum as the primary antiserum. The micrographs show the presence of a nurse cell cap and YP2 in the FC of the penultimate vitellogenic follicle "a" and the "most" vitellogenic follicle "b." The nurse cell cap is absent and YP2 is not detectable in the adjacent terminal growth phase follicle "c." Abbreviations: fc, follicular epithelial cell; nc, nurse cell cap; oo, [illegible] to. Scale bars = 100 μm (A) and 250 μm (B).

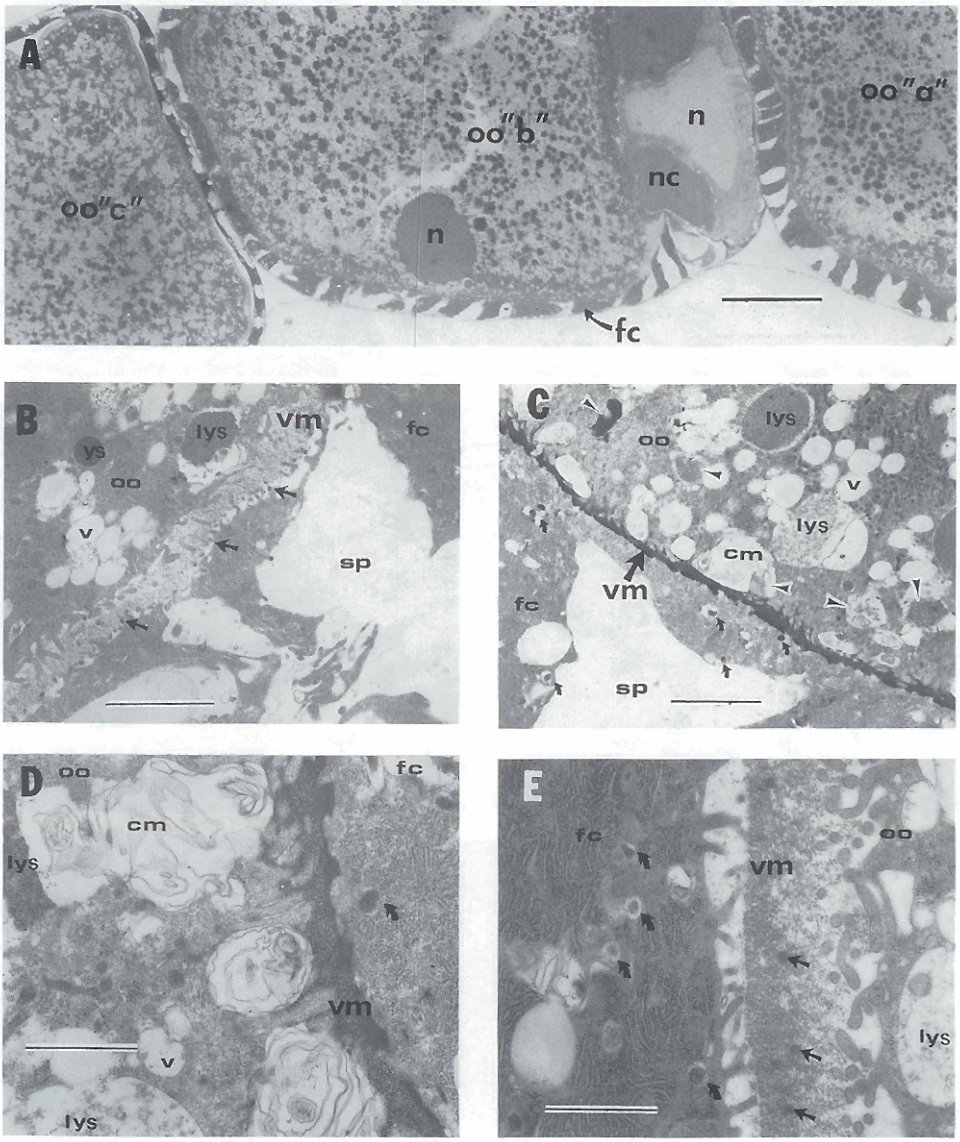

Fig. 5. Ultrastructural changes in vitelline membrane during the termination of vitellogenesis and the initiation of vitelline membrane synthesis. Tissues were osmicated before embedment in epon. **A:** The penultimate vitellogenic follicle "a," the "most" vitellogenic follicle "b," and the adjacent terminal growth phase follicle "c" in an ovariole as designated in Figure 4. **B:** The vitelline membrane and an adjacent follicular epithelial cell from follicle "a" in A. **C** and **D** show the vitelline membrane and adjacent follicular epithelial cells from follicle "b" in A. **E:** The vitelline membrane and an adjacent follicular epithelial cell from follicle "c" in A. Arrows in B–E point to electron-dense droplets in vesicles from the apical portion of a follicular epithelial cell and in thickening vitelline membranes. Arrowheads in C point to late yolk spheres associated with collapsed membranes. Abbreviations: cm, collapsing membranes; fc, follicular epithelial cell; lys, late yolk sphere; n, nucleus; nc, nurse cell; oo, oocyte; sp, inter-follicular epithelial cell space; v, vesicle; vm, vitelline membrane. Scale bars = 25 μm (A), 2.5 μm (B,C), and 1 μm (D,E).

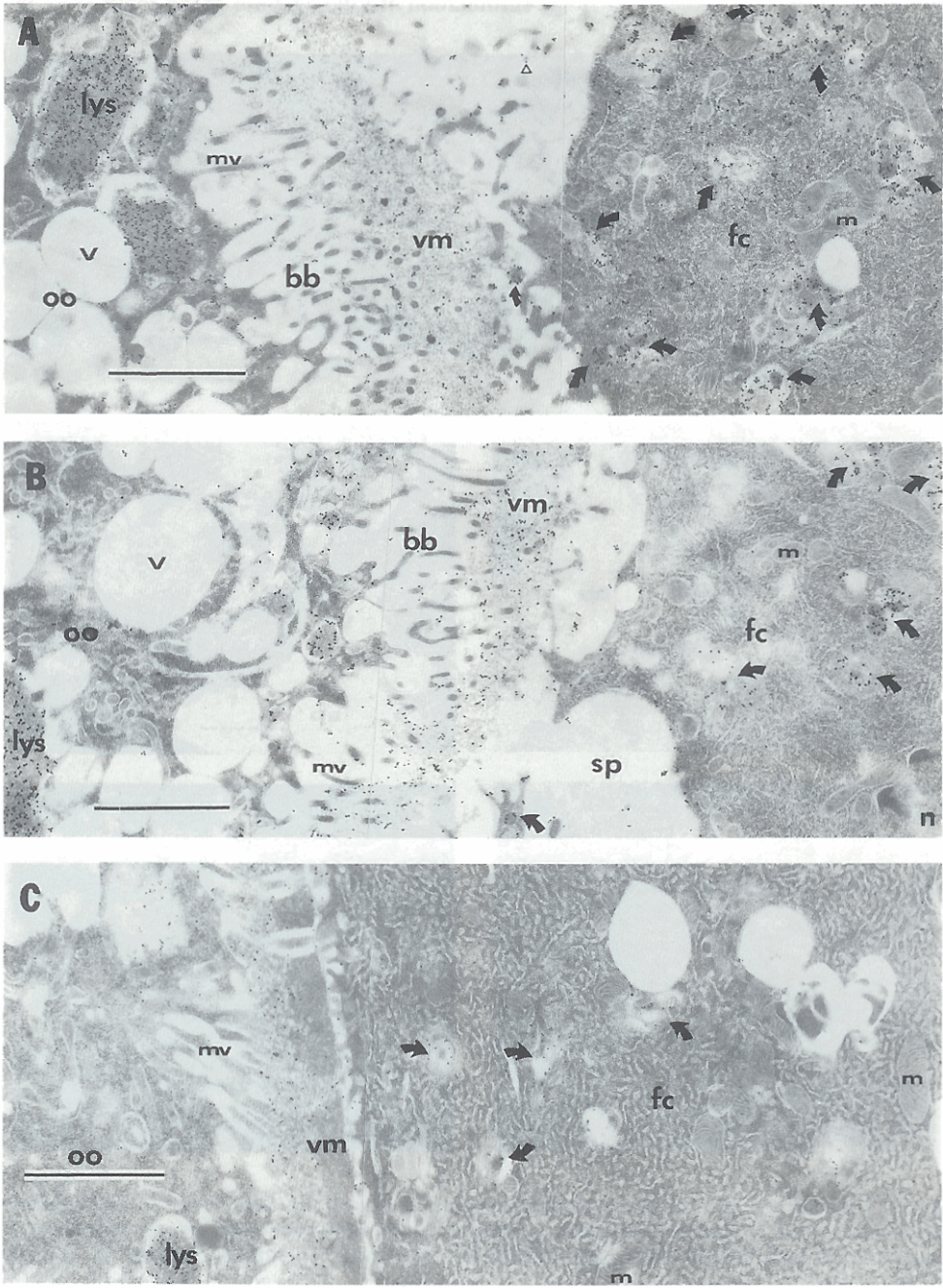

Fig. 6. YP2 and YP4 are localized in follicular epithelial cells and oocytes of vitellogenic (A and B) and terminal growth phase follicles (C). Ultrathin sections were reacted with a YP4 antiserum (A) and YP2 antiserum (B and C) as primary antisera. In the follicular epithelial cells, the Golgi apparatus (arrows) and associated secretory granules (A and B) and dissociating Golgi apparatus (C) contain immunogold-labeled YP4 and YP2. Immunogold-labeled YP2 and YP4 were detected in the broad, diffuse vitelline membrane (A and B). In the oocyte, yolk spheres contained immunogold-labeled YP2 (B and C) and YP4 (A). The △ in A points to a 20 nm gold particle linked IgG. Abbreviations: bb, brush border; fc, follicular epithelial cell; lys, late yolk sphere; m, mitochondrion; mv, microvillus; oo, oocyte; sp, inter-follicular epithelial cell space;

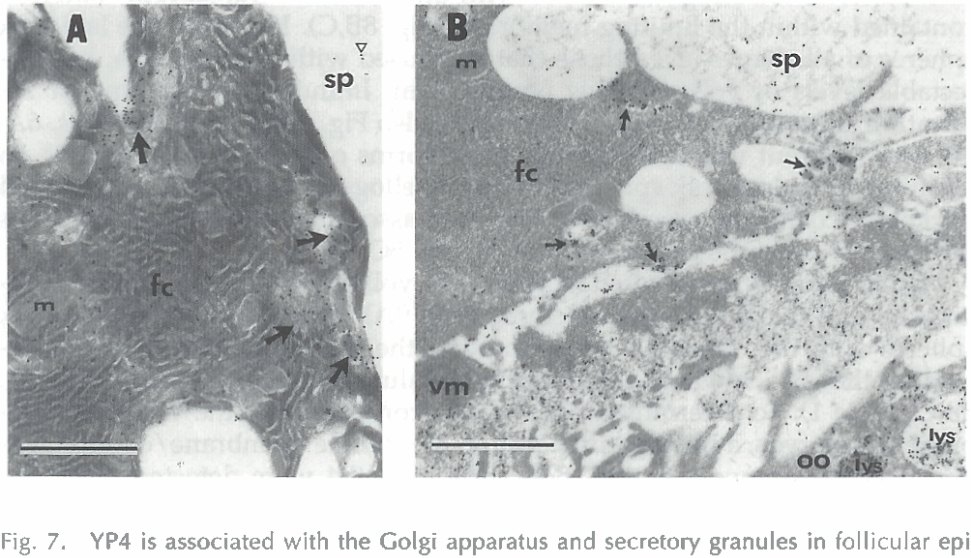

Fig. 7. YP4 is associated with the Golgi apparatus and secretory granules in follicular epithelial cells of follicles synthesizing vitelline membrane (terminal growth phase). Ultrathin sections were reacted with YP4 antiserum as the primary antiserum. Electron micrographs show sections of a follicular epithelial cell close to the tunica propria (A), and adjacent to the vitelline membrane (B). Arrows point to Golgi apparatus and associated secretory granules. The △ in A points to a 20 nm gold particle linked IgG. Abbreviations: fc, follicular epithelial cell; lys, late yolk sphere; m, mitochondrion; oo, oocyte; sp, inter-follicular epithelial cell space; vm, vitelline membrane. Scale bar = 1 μm.

cent component (Figs. 5E, 6C; Table 1). The electron-dense region of the vitelline membrane was juxtaposed to the apical surface of the columnar FC and the electron-translucent region was oriented to the surface of the oocyte.

Although the numbers of secretory granules diminished in the columnar FC of follicles in terminal growth phase, YP4 (Fig. 7A,B) labeling associated with Golgi apparatus was still evident. In contrast to YP4, YP2 labeling in the Golgi apparatus and secretory vesicles of these follicles was at background level (Fig. 6C). The Golgi apparatus and secretory granules were distributed throughout the columnar FC and were observed in the basal regions of the cells (Fig. 7A) as well as the apical regions (Fig. 7B). Secretory granules were observed juxtaposed and fused with the plasma membrane of the columnar FC which apparently released YP4 into the inter-FC spaces (Fig. 7).

Proteinaceous Yolk in the Vitelline Membrane

Within the oocyte during late vitellogenesis, a specialized form of yolk sphere, designated here as late yolk spheres, appeared in the cortical region of the oocyte (Fig. 5C,D; Table 1). The morphology of the late yolk spheres progressively changed from the time that they appeared during late vitellogenesis. The first detectable late yolk spheres were nearly spheroid and similar to normal yolk spheres except that the electron-dense matrix contained a few internal vesiculations and occasional non-immunolabeled electron-dense "cores" (Fig. 8A,C). As the termination of vitellogenesis progressed, the late yolk spheres became more numerous and less uniform in shape. Most late

yolk spheres had irregular shapes with only electron-translucent materials contained within the limiting membrane (Fig. 8B,C). However, the late yolk spheres of all shapes, even those that had fused with the oolemma, had detectable levels of yolk proteins within them. Immunogold labeling of the ultrathin sections showed that YP2 (Fig. 8; also Fig. 6B,C) and YP4 (Figs. 6A, 7B) were present within the matrix of all forms of the late yolk spheres. In addition to the late yolk spheres, the late vitellogenic oocytes also developed pockets of collapsing membranes that were associated with late yolk spheres that were close to the oolemma ("cm" in Fig. 5C,D).

During termination of vitellogenesis, late yolk spheres were observed juxtaposed and fused with the oolemma (Fig. 9A). The fusion of the late yolk spheres with the oolemma would suggest the release of their contents, including the YPs, towards the electron-translucent vitelline membrane (Fig. 8B,C; Table 1). To determine if YPs were a component of the vitelline membrane in embryos, a purified preparation of vitelline membrane/chorion was examined by immunoblotting. Both YP2 and YP4 were detected in the vitelline membrane/chorion preparation (Fig. 10).

Immunogold labeling of ultrathin sections of a follicle producing vitelline membrane proteins showed that YP2 was present in the electron-translucent vitelline membrane but not in the electron-dense region of the vitelline membrane (Fig. 9B; also Fig. 6C). Immunogold labeling of YP4 was also observed in the electron-translucent vitelline membrane (Fig. 7B). In addition, both subunits of vitellin (YP1 and YP3) were present in the late yolk spheres and the electron-translucent vitelline membrane (data not shown).

DISCUSSION

As a follicle completes vitellogenesis in the Indianmeal moth, *P. interpunctella*, we observe five integrated changes in cellular activities delineating the end of vitellogenesis and the beginning of the terminal growth phase (Table 1): (1) the nurse cells transfer their cytoplasm to the oocyte and become pycnotic; (2) the oocyte produces late yolk spheres; (3) the columnar FC terminate the production of FEYP; (4) the columnar FC begin synthesis and release of the vitelline membrane proteins; and (5) the columnar FC establish intercellular junctions to form occlusion zones around the oocyte. This investigation focused primarily on the changes in the activities of the follicular epithelium and the oocyte that lead to the termination of vitellogenesis and to the initiation of the production of the egg membranes that surround the oocyte.

Transformation of the Follicular Epithelium

During vitellogenesis in *P. interpunctella*, the columnar FC produce FEYP that forms part of the proteinaceous yolk of the oocytes. The subunits of FEYP were localized in the Golgi apparatus and in the secretory granules in the columnar FC. Both YP2 and YP4 were observed in all of the secretory granules located from the apical to the basal surfaces within the columnar FC suggesting that the synthesis, packaging, and secretory pathway of the two subunits is uniform throughout the cells and identical for the two subunits

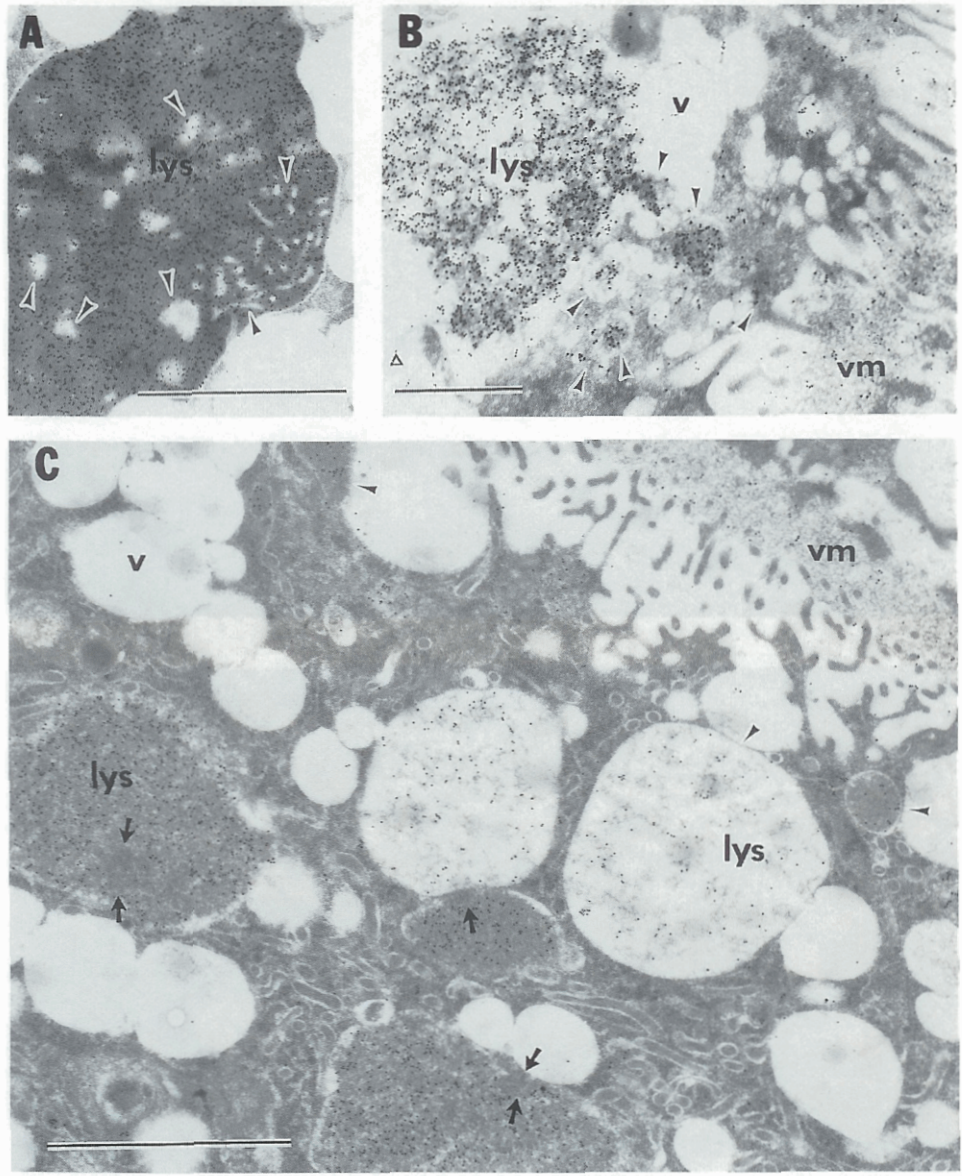

Fig. 8. Electron-translucent late yolk spheres appear as structural variants of yolk spheres from the cortex of oocytes during the termination of vitellogenesis. Ultrathin sections were reacted with YP4 antiserum as the primary serum. Late yolk spheres appearing in an oocyte during late vitellogenesis (A) and follicles during the initiation of vitelline membrane synthesis (B and C) (see follicle "a" in Fig. 4). Arrowheads in A indicate pieces of membranes and electron-translucent vesicles within a yolk sphere. Arrowheads in B indicate vesicles containing yolk proteins dissociating from late yolk spheres. Arrowheads in C indicate yolk proteins in late yolk spheres that are opening to the brush border, and the arrows point to non-labeled electron-dense cores in the late yolk spheres. The △ in B indicates a 20 nm gold particle. Abbreviations: lys, late yolk

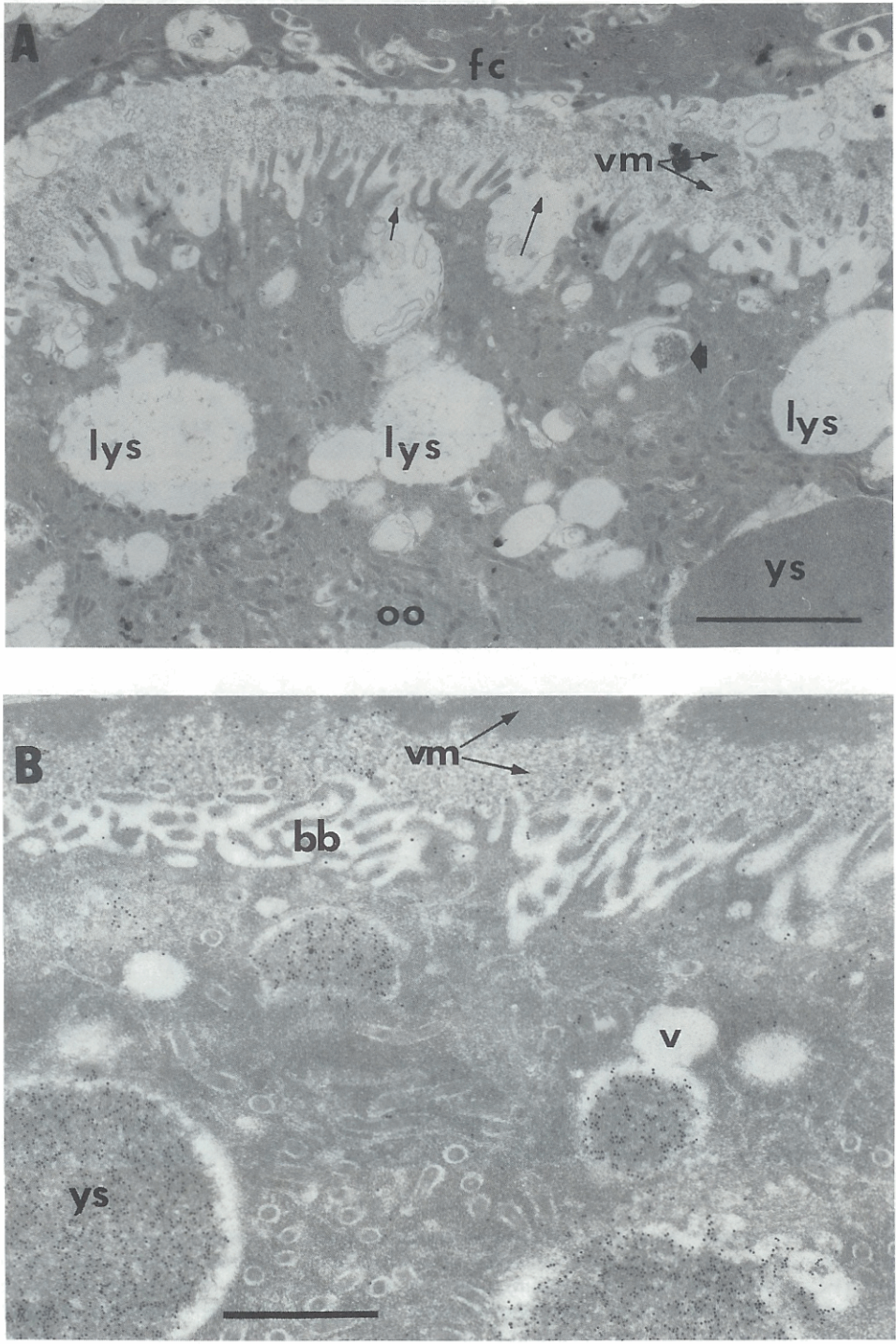

Fig. 9. Late yolk spheres fuse with the oolemma and yolk protein is present in the electron translucent vitelline membrane of a terminal growth phase oocyte. Late yolk spheres were ob

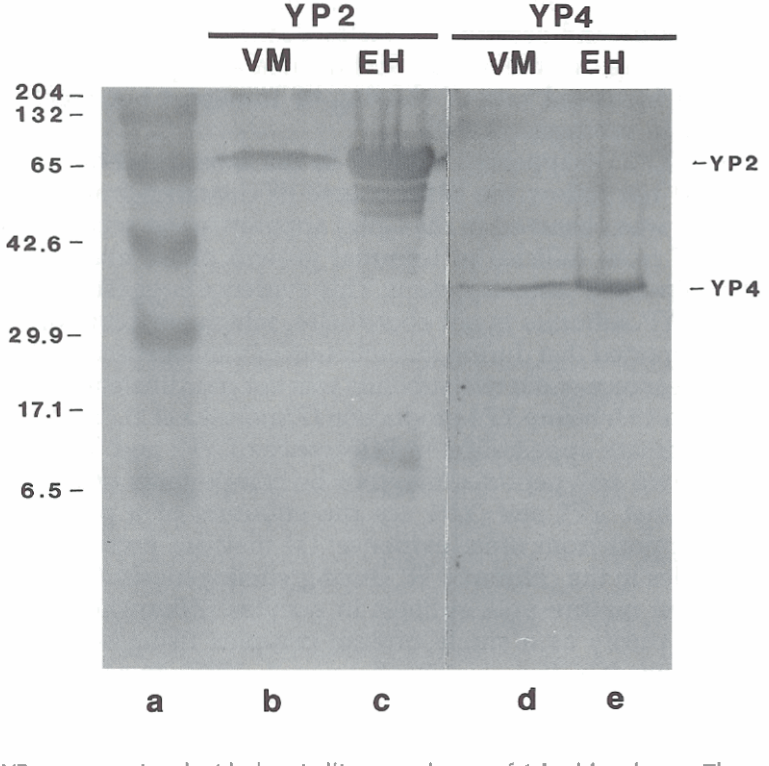

Fig. 10. YPs are associated with the vitelline membrane of 1-h-old embryos. The proteins of purified vitelline membrane/chorion preparation were resolved by SDS-PAGE and electroblotted Reaction of the electroblots with antisera to YP2 (lanes b and c) or YP4 (lanes d and e) showe that both YPs were present in vitelline membrane extracts. The cross-reactive material in lane has been shown to be due to the presence of YP2 degradative products (Bean et al., 1988). Lan designations: **(a)** molecular weight standards; **(b, d)** purified vitelline membrane/chorion prepa ration; **(c, e)** homogenate of 1-h-old eggs. Molecular weight standards (X 10^{-3}) are marked o the left, and the position of the YPs are marked on the right.

Because there was no apparent pooling of the secretory granules within th FC or regulated release of the granules from the FC, the pattern of FEYI secretion from the columnar FC is consistent with the "fast pathway" as de scribed for other tissues (Arvan and Castle, 1992).

Synthesis of YP2 by the columnar FC appears to terminate rapidly during the transition from the vitellogenic to the terminal growth stage. This wa evidenced by significant differences in the amount of YP2 labeling in the co lumnar FC, generally, and in the Golgi apparatus and secretory vesicles, spe cifically, between the most mature vitellogenic follicle and the first follicle i

points to electron-dense yolk in a late yolk sphere. Immunogold labeling of ultrathin section with YP2 antiserum as the primary serum showed the presence of YP2 material in the yol spheres and in the electron-translucent vitelline membrane **(B).** Abbreviations: bb, brush border fc, follicular epithelial cell; lys, late yolk sphere; oo, oocyte; v, vesicle; vm, vitelline membrane

terminal growth phase. The amount of YP2 labeled material associated with the Golgi apparatus and secretory vesicles declined rapidly between these two stages, indicating that the overall decline in labeled protein was not solely the result of an increased rate of clearing or transport but most likely involves a decline in synthesis as well.

Even though YP2 disappeared rapidly in the most posterior vitellogenic follicle, YP4 did not follow the same course of clearance from the FC. YP4 labeled material was observed in the Golgi apparatus and secretory granules of columnar FC from follicles in terminal growth phase even though YP2 labeled material was no longer present. This evidence suggests that the regulation of the FEYP subunits is not coordinate, which supports previous observations. We reported that during the initiation of vitellogenesis in the terminal follicles, the appearance of the two subunits was not coordinate in the FC as YP2 production began 13 h before YP4 production (Zimowska et al., 1994).

The non-coordinate appearance and clearance of YP2 and YP4 might suggest that these two polypeptides may not be components of the same protein. However, that YP2 and YP4 are the subunits of a protein receives considerable support from other evidence: (1) they are present in the same secretory granules in the columnar FC during vitellogenesis, (2) they are uniformly present in mature yolk spheres in oocytes (Zimowska et al., 1994), and (3) they co-purify as a single protein complex using several different separatory methods (Shirk et al., 1984; Bean et al., 1988). In addition, the non-coordinate regulation of subunits of other proteins has been reported, e.g., globins in vertebrates (compare Evans et al., 1990) and the yolk polypeptides, YP1, YP2, and YP3, in the FC of *Drosophila* ovaries (Brennan et al., 1982). The significance of the non-coordinate production of YP2 and YP4 to oocyte development in *P. interpunctella* has not been determined, and it may impact on the proliferation and growth of yolk spheres. Alternatively, the increased production of YP4 that is coincident with the period of FC patency (Zimowska et al., 1994) and its propensity to reside in the inter-FC matrix suggests that YP4 may have a role in the stabilization and/or transport of materials through the inter-FC spaces.

The initiation and termination of YP2 production during follicular development may provide a model for examining the nature of the changing regulatory milieu in the FC as the follicles develop. Once choriogenesis is initiated in *Antherea polyphemus* (Paul and Kafatos, 1975), *Bombyx mori* (Swevers and Iatrou, 1992), and *D. melanogaster* (compare Kafatos et al., 1986), the process continues as a developmental cascade and does not require further extrafollicular regulators to complete chorion production. In *B. mori*, ovarioles containing choriogenic terminal follicles were cultured in vitro (Swevers and Iatrou, 1992). Those vitellogenic follicles that were 34 h or less before the start of choriogenesis were committed to further development and autonomously regulated the completion of follicular development in vitro. The developmental program, which included vitelline membrane synthesis and choriogenesis, in follicles beyond the 34 h stage was not influenced by addition of 20-hydroxyecydsone (20E) nor was the chorionic potential of follicles that were within the 34 h stage enhanced. Previously, we reported that YP2

licles of *P. interpunctella* (Shirk et al., 1990). Because YP2 production terminates at the beginning of the terminal growth phase, which is most likely controlled by a developmental cascade, we may be able to use the changes in YP2 production to examine the regulatory mechanisms.

Terminal Growth Phase and Formation of Vitelline Membrane

At the end of the vitellogenic stage, the follicles shift from the accumulation of maternally produced components to the enclosure of the oocyte within the egg membranes. The activities and structures of both the oocyte and the FC change from vitellogenic to choriogenic within a 2 h period.

In the penultimate vitellogenic follicles of *P. interpunctella*, large electron-translucent vesicles that contain yolk proteins form in the cortical region of the oocyte. Similar organelles appear in the cortical region of the late vitellogenic oocytes of other Lepidoptera but the presence of yolk proteins within them has not been confirmed for the other species. Cortical "refractile bodies" have been described in *B. mori* (Akutsu and Yoshitake, 1977; Yamauchi and Yoshitake, 1984) and *H. cecropia* (Rubenstein, 1979); "accessory yolk nuclei" and "electron-lucent coated vesicles" were reported in the butterfly, *Calpodes ethlius* (Griffith and Lai-Fook, 1986); and "accessory yolk nuclei" and "membrane-bound vesicles" were described in *A. kühniella* (Cruickshank, 1971, 1972). Because the homologous organelles in *P. interpunctella* contain yolk proteins, we suggest that "late yolk spheres" is a more descriptive and appropriate designation for these organelles.

The vitelline membrane of the most mature vitellogenic follicle was a single-layered condensed structure, and electron-dense granules were being secreted from the FC into this structure. In *D. melanogaster*, electron-dense granules that contain vitelline membrane proteins are released from the apical surfaces of the FC (Mahowald and Kambysellis, 1980; Fargnoli and Waring, 1982). We observed similar structures in *P. interpunctella* and conclude that the role of the FC in the production of vitelline membrane protein is similar to that in *Drosophila*. This is consistent with the conclusions of Cummings (1972) who suggested that FC have a role in vitelline membrane production that is similar in insects from Odonata to Diptera.

Subsequently, the vitelline membrane of the first follicle in terminal growth phase was a bilayered structure. The electron-dense layer, being produced by the FC, had become much more uniform in thickness and density than in the most mature vitellogenic follicle. In addition to the electron-dense layer, an electron-translucent layer became apparent that was juxtaposed to the oocyte surface. A bilayered vitelline membrane structure has also been observed in the follicles of the moths *B. mori* (Akutsu and Yoshitake, 1977; Fig. 6), *H. cecropia* (Rubenstein, 1979; Fig. 1C), and the butterfly, *C. ethlius* (Griffith and Lai-Fook, 1986). However, a bilayered vitelline membrane was not observed in *Drosophila* (Cummings et al., 1971; Giorgi, 1977; Giorgi and Postlethwait, 1985) or the stick insect, *Carausius morosus* (Giorgi et al., 1993).

Are Yolk Proteins Components of Vitelline Membrane?

Yolk proteins were localized immunocytologically in the vitelline membrane of various stages of vitellogenic and terminal growth phase follicles from

P. interpunctella. We present two non-mutually exclusive hypotheses that could account for the presence of the yolk proteins in the electron-translucent layer of the terminal growth phase follicles: (1) the yolk proteins are trapped material that does not enter the oocyte during uptake; and/or (2) the yolk protein in the vitelline membrane is due to the release of previously sequestered yolk proteins from the oocytes.

The first alternative has merit from the following observations. In the penultimate vitellogenic follicle, the vitelline membrane is broad, and the presence of the yolk proteins is most likely due to the passage of proteins through the vitelline membrane and brush border for entry into the oocyte. In the first terminal growth phase follicles, the FC have stopped producing YP2 and YP4 production is considerably reduced. The FC have also formed an occlusion zone around the oocyte and the electron-dense vitelline membrane originating from the FC has become thicker and more uniform, thus blocking the entry of vitellogenin into the oocyte. In addition, the structure of the brush border has declined, and the formation of nascent yolk spheres has ceased. These observations suggest that any material remaining in the vitelline membrane may be trapped there and be incorporated as a structural component of the vitelline membrane.

The second hypothesis, which has been suggested previously (Cruickshank, 1971, 1972), gains credence from the following observations. Beginning in the penultimate vitellogenic and becoming more frequent in the more developmentally advanced follicles of *P. interpunctella*, some late yolk spheres were observed fused with the oolemma and appeared to be releasing their contents into the broad electron-translucent layer of the vitelline membrane. Fusion of late yolk spheres with the oolemma was especially evident in the first follicles in terminal growth phase when FEYP production had stopped and vitellogenin entry was blocked because of the presence of the oclussion zone and the formation of the electron-dense vitelline membrane. Strong labeling for the yolk proteins was present throughout the whole region of the electron-translucent vitelline membrane during this period of the terminal growth phase.

This evidence is consistent with the previous reports that suggested the release of material from the oocyte at the termination of vitellogenesis. On the bases of pulse chase autoradiographic evidence, Cruickshank (1971) showed that labeled material accumulating in vesicles localized in the cortex of late vitellogenic oocytes was released into the vitelline membrane of *A. kühniella*. Ultrastructural evidence also indicated that the vesicles in the cortex of the oocyte discharged their contents into the vitelline membrane (Cruickshank, 1972). The vesicles that release their contents into the vitelline membrane in *A. kühniella* are most likely to be identical to the late yolk spheres in *P. interpunctella* described here. The release of materials from vesicles within oocytes into the vitelline membrane was also reported for the butterfly, *C. ethlius* (Griffith and Lai-Fook, 1986). Electron-lucent granular material was reported to be exocytosed from "coated vesicles" into an electron-lucent layer of the vitelline membrane in late vitellogenic follicles of this butterfly in a process similar to that described for the late yolk spheres in *P. interpunctella*.

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release of yolk proteins from the oocyte into the vitelline membrane is likely and may be operative in many other Lepidoptera.

The release of yolk proteins from the oocyte into the vitelline membrane was not apparent in either *Drosophila* (Cummings et al., 1971; Giorgi, 1977; Giorgi and Postlethwait, 1985; Butterworth et al., 1992) or in *C. morosus* (Giorgi et al., 1993). The cortical regions of the oocytes of these two insects do not contain organelles similar in structure to late yolk spheres and all vitelline membrane proteins apparently originate in the FC. In fact, the presence of yolk proteins in the vitelline membrane of *Drosophila* appears to be detrimental to normal formation and function of vitelline membrane. The production of mutant yolk polypeptides in *Drosophila* resulted in the formation of YP precipitate plaques in the vitelline membrane that disrupted formation of eggshell membranes and led to sterile phenotypes (Giorgi and Postlethwait, 1985; Butterworth et al., 1922).

From our observations, we propose that both the follicular epithelial cells and the oocyte of *P. interpunctella* contribute proteins to the vitelline membrane for the encasement of the oocyte in the egg membranes. This perspective places the oocyte in an active role in the termination of vitellogenesis rather than a passive one, i.e., once the oocyte has accumulated sufficient supplies for embryogenesis, it also participates in its own closure. Because the yolk proteins can form condensed aggregates, it is most likely that they provide structural components for the formation of a water tight component of the vitelline membrane. If the oocyte is actively participating in the termination of vitellogenesis, what then would be the nature of the signal that tells the oocyte (and/or the follicular epithelium) that vitellogenesis is complete, that the formation of late yolk spheres be initiated, and that their fusion with the oolemma for release of the yolk proteins into the vitelline membrane begin? These questions remain to be addressed.

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