AN ULTRASTRUCTURAL STUDY OF OVARIAN DEVELOPMENT IN THE otu\textsuperscript{7} MUTANT OF DROSOPHILA MELANOGASTER

DONNA L. BISHOP AND ROBERT C. KING

Department of Ecology and Evolutionary Biology, Northwestern University, Evanston, Illinois 60201, U.S.A.

SUMMARY

Females homozygous for the otu\textsuperscript{7} allele produce ovarian tumours, as well as egg chambers that reach a relatively late stage of development. Mutant ovarian nurse cells contain giant polytene chromosomes. These are transcriptionally active, and RNA is transported to the oocyte through ring canals, although at a reduced rate. Vitellogenic oocytes are endocytotically active. Protein (α yolk) spheres are formed, but glycogen (β yolk) spheres were never seen in the ooplasm. Follicle cells migrate normally and secrete more vitelline membrane and chorion than is required to cover the slowly growing oocyte. Specialized follicle cells also secrete relatively normal dorsal appendages. The micropylar cone is secreted by another cluster of specialized follicle cells called border cells. These are out of phase with the oocyte, and the forming micropylar cone prevents the nurse cells from passing the remainder of their cytoplasm to the oocyte. The result is a morphologically abnormal chamber blocked at the p-12 stage. Sections through the micropylar cone of a p-12 chamber demonstrated that one of the border cells formed a projection containing a bundle of microtubules. Secretions of the border cells and oocyte were deposited against this tube, which later degenerates or is withdrawn. Normally this results in a canal, the micropyle, through which the sperm enters the egg. The slowed growth of the mutant oocyte presumably results from a defect in the transport of fluids or charged molecules to it, and the otu\textsuperscript{+} gene is therefore believed to play a vital role in this process.

INTRODUCTION

An interesting gene named otu (for ovarian tumour) has been found in Drosophila. It resides at 22-7 on the genetic map and within subdivision 7F of the cytological map of the X chromosome (see King, Cassidy & Rousset, 1982, table 1). Mutations at the otu locus produce a number of different types of ovarian pathology, and hence, the alleles have been grouped into three different classes. In class 1, the ovarioles lack germ cells; in class 2, most ovarioles contain only tumorous chambers; and in class 3, cystocyte clusters are produced, some of which contain oocytes that fail to complete development. The alleles otu\textsuperscript{1}, otu\textsuperscript{2} and otu\textsuperscript{3} belong to class 3, and otu\textsuperscript{4} and otu\textsuperscript{5} belong to class 2 and class 1, respectively (King & Riley, 1982). During mutagenesis experiments carried on in the laboratory of Dr Anthony Mahowald three new female sterile mutations were induced, using ethylmethane sulphonate. The mutations were isolated by Dr Lee Engstrom and were given the designations fs209, 1304 and 1396. Since they all proved to be alleles of otu\textsuperscript{7}, stocks were kindly provided for the Northwestern collection. We renamed the alleles otu\textsuperscript{6}, otu\textsuperscript{7} and otu\textsuperscript{8}, respectively, and made
cytological studies of the ovaries of homozygotes. \textit{otu}^6 and \textit{otu}^8 belonged to class 1 and \textit{otu}^7 to class 3.

Heretofore cytological studies of ovaries from flies carrying class 3 \textit{otu} mutations have been restricted to Feulgen-stained, ovarian whole mounts. The investigations to be described concern analyses of sections from plastic-embedded ovaries viewed with the light and electron microscopes. We hoped, from studying the pathological changes in mutant ovaries, to understand why oocytes carrying class 3 alleles failed to grow to maturity and how the enveloping follicle cells behaved in the presence of the defective oocyte.

\textbf{MATERIALS AND METHODS}

All the mutant alleles of \textit{otu} that have been studied have no effect upon spermatogenesis, and hemizygous males are fertile. In laboratory cultures the mutation is transmitted from generation to generation by matings between \textit{otu} males and \textit{otu}+/ females. The \textit{otu}+ allele is carried by FM3, a balancing chromosome marked by the dominant gene \textit{Bar}. FM3 also contains a series of paracentric inversions and two recessive lethals. The monograph of Lindsley & Grell (1968) contains a detailed description of the FM3 chromosome and the marker genes referred to above. Flies were reared on David's (1962) medium at room temperature (22 ± 1 deg. C) in a normal dark–light cycle. Ovaries from females homozygous for each of these four alleles were studied at the light-microscopic level, and so were ovaries from flies of genotype \textit{otu}^7/\textit{otu}^4 and \textit{otu}^4/\textit{otu}^7. Oregon R wild-type females and heterozygotes (\textit{otu}^s/FM3 and \textit{otu}^7/FM3) served as controls.

Ovaries to be observed at the light-microscopic level were fixed in 4% glutaraldehyde in 0.117 M-sodium phosphate buffer, pH 7.1, and embedded in JB-4 resin according to the instructions of the manufacturer (Polysciences, Inc., 400 Valley Rd, Warrington, PA). Sections (1–2 μm thick) were stained in an aqueous solution containing 0.5% each of methylene blue, azur II, and sodium tetraborate. Polysaccharides were stained by the periodic acid/Schiff (PAS) procedure.

Electron-microscopic studies were restricted to ovaries from \textit{otu}^7 homozygotes and heterozygotes. Ovaries were fixed according to a procedure suggested by M. Locke. The fixation was accomplished in several steps. The ovaries were dissected from females immersed in a solution containing 5% (w/v) glutaraldehyde, 0.1 M-phosphate buffer (pH 7.7), and 2% (w/v) sucrose. After 1 h in the first fixative, they were transferred to a solution containing 5% gallic acid in a sample of the initial fixative. After 1 h in the second fixative, the ovaries were washed in phosphate buffer containing sucrose, and then placed in the phosphate buffer/sucrose solution containing 1% osmium tetroxide. They were left in the third fixative for 1 h. Fixation was done at 0–4°C. After four washes in distilled water, the tissues were stained in 50% saturated aqueous uranyl acetate solution for 3 h. The ovaries then were rapidly dehydrated through a graded series of ethanol solutions, followed by propylene oxide, and embedded in Polybed/Araldite resin (Polysciences; Warrington, PA). Polymerization took 8 h at 65°C. Blocks were oriented so that ovarioles would be sectioned longitudinally. A series of serial sections were picked up on Formvar-coated, one-hole copper grids. The grids were stained with uranyl acetate and lead citrate. Formulations for the buffer and staining solutions are given by Locke & Huie (1980). A JEOL 100 CX electron microscope was used, operating at 80 kV.

\textbf{RESULTS}

\textit{Ovarian tumours}

Tumorous chambers do occur in \textit{otu}^7/\textit{otu}^7 ovaries, generally in the anterior portion of ovarioles. One tumorous chamber (Fig. 1A) contained anterior and posterior populations of small, undifferentiated cells. Overlapping electron micrographs were
Fig. 1. A. Transmission electron micrograph (TEM) of tumour from 9 to 10-day-old *otu* homozygote. The lower third of the section contains a group of about 80 tumour cells enclosed by an abnormally thick vitelline membrane (vm). The lateral and posterior portions of this membrane are surrounded by material that resembles disorganized chorion (ch). Between this and the tunica propria (tp) is a monolayer of squamous follicle cells (sfc). Above this area lies a group of about 100 tumour cells, and above this a diagonal layer of follicle cells (fc), outlined in black. Above the follicle cell layer is another mass of tumour cells, and about 30 of these are included in the section. The positions of canals joining 14 pairs of tumour cells are shown by small circles. Bar, 10 μm. B. An enlarged view of a ring canal (rc) connecting two tumour cells. The inner surface of the plasmalemma surrounding the canal contains a coating of dense material. G, Golgi material; n, nucleus. Bar, 1 μm.
taken of sections from five levels in this tumour. From the assembled montages, we
plotted the location of ring canals connecting 14 pairs of tumour cells within a segment
of the tumour about 3 μm thick. Since these cells had undergone incomplete
cytokinesis, they were behaving like germarial cystocytes. King (1979), in a more
detailed analysis, has shown this to also be true for tumour cells in ovaries of females
homoygous for *otu*. A typical canal is illustrated in Fig. 1B.

The posterior population of tumour cells was surrounded by a thick vitelline mem-
brane. There was no membrane secreted around the anterior population of tumour
cells, although follicle cells were present. Thus it appears that cells in the posterior
region of the tumour had developed surface properties that stimulated the adjacent
follicle cells to secrete vitelline membrane, whereas the anterior cells had not. In this
limited sense, the posterior cells displayed a characteristic property of oocytes.

**Chambers containing pseudonurse cells**

The ovaries of females possessing *otu* alleles of class 3 contain cells, which because
of their large nuclei, resemble nurse cells. They are called pseudonurse cells (PNC)
because their chromosomes are morphologically different from those of wild-type
nurse cells. In normal nurse cells the endomitotic cycles produce dispersed
chromosomes that are of little cytological value (King, 1970, fig. VI-1). In PNCs,
however, the replicating chromatids remain together to form giant polytene
chromosomes (Dabbs & King, 1980; King et al. 1981). Recent studies have shown
that the largest chromosomes of *otu* PNCs have undergone 12 cycles of endomitotic
DNA replication (Rasch, King & Rasch, 1984).

When sections of the giant chromosomes of *otu* PNCs were viewed under the
electron microscope, they were found to contain numerous masses of dense material.
Since in adjacent thick sections this material stained with basic dyes, it is assumed to
contain ribonucleoproteins (RNP). The RNP deposits were present as a series of
vertical bands each 3–5 μm thick; meandering tube-shaped masses of RNP sometimes
extended up to 20 μm to either side of the chromosome (see nucleus 2 in Figs 2, 3).
Ribosomes were abundant in the cytoplasm. The nuages seen in Fig. 3 presumably
represent places where transport of nuclear RNA to the cytoplasm had occurred
recently.

Females homozygous for *otu* occasionally produce chambers containing
pseudonurse cells, but no oocyte (Fig. 2). The pseudonurse cells in a given chamber
presumably represent a clone of interconnected cells, and some sections included ring
canals. Other sections demonstrated binucleate cells (Fig. 3).

**Chambers containing abnormally placed oocytes**

Mutant ovarioles also contained chambers that possessed both pseudonurse cells
and a single abnormal oocyte. Such 'pseudo-oocytes' contained cytoplasmic yolk
spheres, but they were not enclosed by a normal vitelline membrane, and they
sometimes occupied an abnormal position in the chamber (Fig. 4). The PNCs and the
pseudo-oocyte were surrounded by a small number of flattened follicle cells. Such
chambers often contained a cluster of follicle cells at the posterior pole, and these
Fig. 2. Low-power TEM of a longitudinally sectioned PNC chamber from an *otu*<sup>7</sup> homozygote. The PNCs are surrounded by an envelope of flattened follicle cells. The section passes through six PNCs, and their nuclei are numbered. The perinuclear cytoplasm contains mitochondria (m), lipid droplets (l) and lysosomes (ly). Each PNC nucleus contains banded polytene chromosomes (*pc*) to which are attached dense deposits of ribonucleoproteins (*RNP*). Bar, 50 μm.

sometimes secreted a small deposit of vitelline membrane that protruded into a corner of the oocyte.

Fig. 5 shows a region of the perinuclear cytoplasm of one of the pseudonurse cells. Note that some of the nuclear pores contain a central particle – evidence for transport
of RNA from the nucleus to the cytoplasm. Annulate lamellae occurred in stacks adjacent to the nucleus of the pseudonurse cell. At their edges the lamellae were often continuous with tubular, rough-surfaced endoplasmic reticulum. Typical mitochondria, lipid droplets and clusters of Golgi material lay in the neighbouring cytoplasm. The annulate lamellae presumably are formed by delamination from the nuclear envelopes of the nurse cells, and at least some of the annulate lamellae in the oocyte may arise from this source.

Fig. 5 shows an organelle never observed in normal nurse cells. This 'condensing vacuole' is surrounded by an aggregation of short, dense tubules. Many of these are forked, and some are joined to the condensing vacuole. Similar aggregations of

Fig. 3. A portion of a binucleate PNC. This section is from an area 10 μm deeper into the block and is located near the vicinity marked 2 in Fig. 2. Both nuclei contain segments of sectioned, banded polytene chromosomes (pc), and an attached deposit of ribonucleoprotein (RNP) appears in the upper nucleus. m, mitochondria; fcn, follicle cell nucleus. Arrows point to nuages at the nuclear membrane. Bar, 5 μm.
Fig. 4. Low-power TEM of a longitudinally sectioned otu^7 chamber. Six PNCs are included in the section, and their nuclei are numbered. An area (see rectangle) adjacent to nucleus 6 is shown at higher magnification in Fig. 5. The nuclei contain banded polytene chromosomes and attached, dense RNP deposits. The chamber would be classified as stage 9 because of the size of the nurse cells. The lower cell contains numerous dense yolk spheres, and on this basis, can be classified as an oocyte. A deposit of material resembling vitelline membrane (vm) resides in the ooplasm. Possibly this was secreted by columnar follicle cells lying above or below the section. fc, squamous follicle cell. Bar, 50 μm.
Fig. 5
Ovarian pathology of *otu* mutant

Tubules also occurred in the cytoplasm of pseudonurse cells from chambers that lacked an oocyte.

'Normal' chambers

The majority of the ovarioles from *otu* mutants belonging to class 3 produce chambers that are normal in the sense that each contains a posterior oocyte and 15 nurse cells. However, in the case of *otu*<sup>3</sup>, *otu*<sup>4</sup> and *otu*<sup>7</sup> homozygotes, nurse cells contain banded polytene chromosomes. These PNCs grow as large as normal nurse cells, but the accompanying oocyte is always abnormally small.

Comparative studies were made of Feulgen-stained whole mounts of ovaries from *otu*<sup>7</sup>/*otu*<sup>7</sup> and *otu*<sup>7</sup>/+ females 1, 1-5, 2 and 2-5 days after eclosion. From measurements of the oocytes in the most posterior chambers, estimates could be made of the ooplasmic volumes of representative mutant and normal cells. In day-old females mutant oocytes were one-sixth of the volume of normal cells, and they reached their maximum size one day later than normal cells. Furthermore, their maximum volume was usually one-third to one-half of that of normal, stage 14 oocytes. So it is clear that mutant oocytes grow more slowly than normal cells, at least during vitellogenic stages.

Part of the growth of the oocyte is due to the transport of cytoplasm from nurse cells to it. During this process ribosomes synthesized by the nurse cells pour into the oocyte through ring canals that connect it to four adjacent nurse cells. The result is the formation of basophilic deltas that can be seen in sections. Fig. 6 shows a normal and a mutant chamber that are about the same size, and the nurse nuclei have equivalent volumes. Both chambers would be classified as stage 8, since alpha yolk spheres are present, but the follicle cells have not yet begun to secrete vitelline bodies against the oolemma. These first form at stage 9. The mutant oocyte (lower) is smaller than the normal oocyte (upper), there are fewer alpha spheres in the mutant, and it lacks deltas.

Serial sections were made of ovaries from flies of five genotypes: *otu*<sup>+</sup>/*otu*<sup>+</sup> (Oregon R), *otu*<sup>5</sup>/otu<sup>+</sup>, *otu*<sup>5</sup>/otu<sup>5</sup>, *otu*<sup>+</sup>/otu<sup>+</sup> and *otu*<sup>5</sup>/otu<sup>5</sup>. The sections were stained with methylene blue, and subsequently scored for the number of ring canals that occurred in sections of chambers in stages 8-10. Ring canals were detected in sections from normal chambers about three times more often than from *otu* chambers. In cases where sections were found that contained ring canals joining oocytes and adjacent nurse cells (as in Fig. 6), we noted the presence or absence of deltas. The number of different sectioned canals observed was 40 in *otu*<sup>+</sup>/+ and 16 in *otu*/otu. Deltas were

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Fig. 5. Perinuclear cytoplasm from the mutant PNC chamber in the rectangle drawn in Fig. 4. The region shown is from a section 5 μm deeper into the block. Arrows point to an area where the convoluted nuclear envelope has been tangentially sectioned. Many of the nuclear pores contain a central dense particle. Tubules of rough-surfaced, endoplasmic reticulum (er) lie alongside the nucleus, and mitochondria (m) and lipid droplets (l) are abundant. The largest organelle is a dense collecting vacuole (cv), and below it lies a system of branching canaliculi. Each tubular element is about 50 nm in diameter and contains material of density similar to the contents of the collecting vacuole. ↔, Region in which tubules are attached to the collection vesicle. Bar, 1 μm.
Fig. 6. A. Drawing of a longitudinally sectioned, stage 8 egg chamber from a fertile (otu$^3$/otu$^+$) D. melanogaster. The nurse cell cytoplasm is more basophilic than the ooplasm, and deltas of basophilic material extend into the oocyte at the mouths of the canals. B. Drawing of a section of a stage 8 chamber from an otu$^3$/otu$^5$ female showing a ring canal between the egg and an adjacent nurse cell. There is no delta. The follicle cells surrounding the oocyte are becoming columnar. Note that the alpha spheres in the egg of the mutant are less numerous and irregular in shape. Bar, 50 μm.
Fig. 7. A longitudinally sectioned egg chamber belonging to a p-12 stage from a 9 to 10-day-old \textit{otu$^2$/otu$^2$} female. The ring marks the position of a ring canal joining adjacent nurse cells. \textit{bc}, border cell; \textit{en}, endochorion; \textit{fcn}, follicle cell nucleus; \textit{ncn}, nurse cell nucleus; \textit{on}, oocyte nucleus; \textit{v}, vacuole; \textit{vm}, vitelline membrane; \textit{ys}, alpha yolk sphere. Bar, 50 \textmu m.
Fig. 8A and B
Ovarian pathology of otu$^7$ mutant

Fig. 9. The distribution of ribosomes in four cytoplasmic areas, all photographed at the same magnification. A, B, and C are from a p-12 chamber from an otu$^7$ homozygote. D is from a stage 12 oocyte from an otu$^7$/otu$^+$ female. A. The cytoplasm of a nurse cell directly connected to an oocyte; c, the ooplasm; and b, the cytoplasm of a border cell. In b most ribosomes are attached to the membranes of the endoplasmic reticulum (er), whereas in the other three cells the ribosomes are suspended in the cytoplasm. ays, alpha yolk sphere; m, mitochondrion. The square in A represents 0.5 μm on each side.

observed in heterozygotes, but not in homozygotes. We concluded that in mutant chambers basophilic material passed abnormally slowly from nurse cells to the oocyte.

The terminal chamber in each otu$^7$ ovariole generally develops until it reaches the pseudo-12 (p-12) stage. Fig. 7 shows a typical p-12 egg chamber. Its component cells appear to be out of developmental register. An endochorion and a micropylar cone have formed, so the follicle cells behaved as they would in a normal stage 12 chamber. However, the nurse cells are the size normally seen late in stage 10, and the oocyte has only the volume of an early stage 10. The PNCs have banded polytene chromosomes. The condensing vacuoles and associated tubular aggregates previously described were not seen in the cytoplasm of these PNCs.

Fig. 8. TEMs showing the location of tubular elements (arrows) in the cortical ooplasm of vitellogenic oocytes from otu$^7$/otu$^+$ (A) and wild-type females (B). Note that some of the tubes are attached to membrane-enclosed vesicles, which contain deposits of material similar in density to that of alpha yolk spheres (ays). vm, vitelline membrane; mv, microvilli. Bars, 1 μm.
The oolemma facing the vitelline membrane as well as that bordering the vacuoles was thrown into microvilli. Pinocytotic pits were abundant, and these showed dense coatings about 20 nm thick. The oolemma of the p-12 was similar in appearance to that of a wild-type oocyte that is micropinocytosing vitellogenins. Receptor-mediated endocytosis is the normal method of internalizing these components of alpha yolk spheres (Telfer, Huebner & Smith, 1982). The cortex of vitellogenic oocytes contains large numbers of tubular elements. In both mutant and wild-type oocytes (Fig. 8A, B) these appeared as branching canaliculi that were sometimes attached to membrane-enclosed spheres with diameters between 0.1 and 0.4 μm. Dense material similar to that seen in alpha spheres was deposited in these vesicles. The tubules had similar diameters, about 50 nm in both mutant and normal chambers.

To compare ribosomal densities, we photographed at a higher magnification areas containing nurse cell cytoplasm (Fig. 9A), border cell cytoplasm (Fig. 9B), and ooplasm from the p-12 chamber (Fig. 9C). At the same magnification we also photographed similar areas of normal stage 12 ooplasm (Fig. 9D). Representative squares of 0.5 μm x 0.5 μm were marked on each micrograph (see Fig. 9A), and the numbers of ribosomes within were counted. From the measurements we calculated an average of 167 ± 17 (n = 13) ribosomes per 0.25 μm² in the nurse cell cytoplasm, 34 ± 19 (n = 8) free ribosomes per 0.25 μm² in the border cell cytoplasm, and 129 ± 26 (n = 11) ribosomes per 0.25 μm² in the mutant ooplasm. The number of ribosomes counted in comparable areas of normal stage 12 ooplasm averaged 181 ± 38 (n = 10).

The vast majority of oocyte ribosomes are synthesized by the nurse cells, and since ribosomes were abundant in the p-12 ooplasm, it was clear that transport of nurse cell ribosomes to the oocyte had occurred in the mutant chamber. However, since normal stage 14 oocytes are double the size of the largest p-12 oocytes, the normal nurse cells have transferred at least twice as many ribosomes to the oocyte as the PNCs.

**Follicle cell–oocyte interactions**

One of the events characterizing stage 9 during normal oogenesis is the migration of a cluster of follicle cells called border cells from the anterior pole of the chamber to the surface of the oocyte. In a normal chamber the oocyte is located more posteriorly than any of the other cystocytes in the clone. However, it is quite common to find chambers with their oocytes pointing anteriorly in ovaries from *otu³*, *otu⁴* or *otu⁵* females (King & Riley, 1982). We also observed such chambers in sections of ovaries...
Ovarian pathology of otu\(^7\) mutant

from otu\(^7\) homozygotes. Since the border cells were in the normal position in chambers of reversed polarity, it was clear that the border cells migrated toward the oocyte, irrespective of the polarity of the chamber in the ovariole.
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During stages 9 and 10 of normal oogenesis, a monolayer of columnar follicle cells secretes a vitelline membrane about the posterior and lateral surfaces of the oocyte. In the case of mutant chambers carrying alleles of class 3 the vitelline membrane was sometimes secreted against anterior nurse cells, and it was abnormally thick. During stages 11 and 12 of normal oogenesis, the columnar follicle cells secrete a compartmented endochorion on top of the vitelline membrane. In the otu\textsuperscript{7} mutant the compartmented endochorion was thrown into folds, which generally coincided with the cell junctions. This observation suggests that endochorion is secreted for a short distance into the clefts between neighbouring cells, which never occurs in the normal epithelium. The follicle cells secrete an exochorion on top of the endochorion during...
stage 13. In some terminal chambers in mutant ovaries, an endochorion was secreted, and it stained normally (i.e. magenta with the PAS procedure).

During normal oogenesis the dorsal appendages begin to form at stage 11, and between stages 12 and 13 they grow to three-quarters of their maximum length. Mutant follicle cells also secreted dorsal appendages. They were thicker than those of normals and usually reached a length equivalent to that of a stage 13. However, appendages of nearly normal length were sometimes formed. The micropylar apparatus is normally secreted during stage 13, and most terminal chambers in mutant ovaries initiated the formation of a micropylar cone.

Fig. 13. The anterior portion of a stage 12 chamber from a 9 to 10-day-old, phenotypically normal, otu/otu Drosophila. The arrow points to a partially formed micropylar apparatus surrounded by border cells. Cellular debris, the remains of the nurse cells that have been digested by lysosomes, lie in the upper portion of the figure. Follicle cells shown on the lateral portions of the egg, have deposited the vitelline membrane (vm) and have begun to form the chorion (ch). bcn, border cell nucleus; fcn, follicle cell nucleus; ys, yolk sphere; l, lipid droplet. Bar, 25 μm.
Fig. 14. The forming micropylar apparatus in the oocyte of the otu$^2$ heterozygote shown at low magnification in Fig. 13. A projection from a border cell (bcp) containing a bundle of microtubules extends into the cavity of the micropylar cone (cm). A space (s) separates the process from the lamellate material (lm) of the vitelline membrane. Microvilli (mv) from adjacent border cells touch the newly secreted endochorion (en). Bar, 1 μm.
The micropylar apparatus of p-12 chambers

Electron micrographs were taken of a series of longitudinal sections through the p-12 chamber shown in Fig. 7. These sections cut through the border cells and the tube of cytoplasm they surrounded. Three ring canals could be seen in this tube, and they contained ribosomes and mitochondria (Fig. 10). We concluded that the ring canals were transporting cytoplasmic materials to the oocyte when the tubular connections between the oocyte and the four adjacent nurse cells were withdrawn into the nurse cell sub-chamber. Where the tube joined the oocyte, a plug of lamellate material was secreted (Figs 10, 11). This plug presumably prevented the further transport of organelles between nurse cells and the oocyte. Simultaneously, the border cells secreted a projection destined to form the endochorionic portion of the nipple-shaped micropylar apparatus.

The cytoplasm of a typical border cell is shown in Fig. 9b. Note that the majority of the ribosomes are attached to the membranes of the tubular, endoplasmic

Fig. 15. TEM of a section 5 μm deeper in the micropylar apparatus than the one shown in Fig. 14. The border cell process (bcp) is embedded in lamellate material (lm). Each dense lamellar layer is about 30 nm thick and is separated from the next layer by a space about 15 nm thick. The cavity of the micropylar cone contains flocculent material. The surrounding border cells are joined by desmosomes (d), and their cytoplasm contains numerous droplets, which are secreted against the surface of the endochorion (en) at the microvillar border. Bar, 1 μm.
Fig. 16. The border between the oocyte and its adjacent nurse cells in a longitudinally sectioned, early stage-10 egg chamber from a 4 to 5-day-old otu°/otu° female. The border cells are secreting lamellar material (lm) against the oolemma. ays, alpha yolk sphere; bcn, border cell nucleus; f, lipid droplet; on, oocyte nucleus. Bar, 5 µm.
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reticulum. The border cells secrete the layer of endochorion that forms the nipple-shaped projection that surrounds the micropyle. The secretory surface is lined by short microvilli. Dense secretory droplets pass from the border cell cytoplasm by exocytosis and fuse with the endochorionic plaques already present (Figs 10, 12). The droplets are enclosed in membranes derived from Golgi vesicles. A projection from one of the border cells extended into the neck of cytoplasm (Fig. 12). It contained a population of microtubules oriented parallel to its long axis, and its tip was embedded in the deposit of lamellate material.

Later stages in the development of the normal micropylar apparatus

To compare a p-12 micropylar apparatus with the normal one, we examined sections of a normal stage 12 that contained a partially formed micropylar apparatus (Fig. 13). Although it was sectioned tangentially, a border cell projection containing the bundle of microtubules was located in the central portion of the forming micropylar cone (Fig. 14). A dense deposit of endochorionic material, similar to that seen in the mutant, was forming around the micropylar cone, and the border cell projection rested against dense lamellar material. Therefore, the morphogenesis of the micropylar cone is similar in the mutant p-12 and normal stage 12 chambers, although the normal chamber was developmentally more advanced. A section deeper in the block (Fig. 15) showed that the border cell process was flanked on both sides by lamellate material. The lamellae consist of folded, parallel deposits of dense fibrils. Each dense layer was thicker than those seen in the p-12 chamber (Fig. 11).

Next we analysed sections of an early stage 10 chamber from a normal female. In this chamber (Fig. 16), the border cells had reached the surface of the oocyte, and a dense layer of lamellar material was forming at the interface between the border cells
Fig. 18. For legend see p. 109.
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Fig. 19. For legend see p. 109.
Ovarian pathology of otu\(^7\) mutant and the oocyte. It follows that the first event in the formation of the micropylar apparatus is the synthesis and deposition of lamellar material against the oolemma by the border cells. Presumably, the lamellate material is chemically different from the vitelline membrane secreted by the other follicle cells that surround the oocyte.

We next examined sections through the micropylar apparatus of a normal stage 14 oocyte from an otu\(^7\)/otu\(^+\) female. Fig. 17 demonstrates that the tip of the micropylar apparatus was formed by a cone of dense endochorion. This was composed of bundles of fibres that showed a pattern of transverse bands reminiscent of collagen (Fig. 18). The dense endochorion was covered by a layer of pale exochorion (Fig. 17).

The outer micropyle, a canal about 1 \(\mu\)m in diameter, passes through the chorionic cone. Presumably, this canal marks the position once occupied by the cytoplasmic extension from one of the border cells. If at an earlier stage lamellate and chorionic material were deposited about the projection, then the subsequent withdrawal of the process would result in a canal that penetrated the cone.

**Later stages of the development of mutant chambers**

In otu\(^7\) ovarioles adjacent PNCs often fuse with the oocytes of the terminal chambers. Sections have shown an entire nurse cell and a few follicle cells lying in the anterior ooplasm. The nucleus of the extruded pseudonurse cell contained typical banded, polytene chromosomes.

Some terminal chambers have characteristics of stage 13, i.e. the follicle cells can secrete exochorion and dorsal appendages (Fig. 19). The compartmented endochorions possess the convolutions typical of the mutant (Fig. 20). It is common to see oocytes containing autophagic vacuoles of various sizes. Giant cytosegresomes (Fig. 20), containing secondary lysosomes, yolk spheres, mitochondria and lipid droplets, may occupy as much as one-third of the volume of an egg.

Normal stage 13 oocytes contain beta yolk spheres, which first appear during this stage. The terminal otu\(^7\) oocytes observed under the electron microscope showed no organelles resembling beta spheres. These observations support the results of the light-microscope studies on the ovaries of females carrying various otu alleles. Ovaries from otu\(^5\)/otu\(^5\), otu\(^+\)/otu\(^5\) and otu\(^1\)/otu\(^1\) females were sectioned and stained by the PAS procedure. A total of 46 p-12 oocytes were identified, but none contained PAS-positive, beta spheres. In the case of otu\(^5\)/otu\(^+\) females, 14 oocytes belonging to stages 13 or 14 all contained beta spheres. It follows that, while mutant follicle cells complete their normal synthetic activities, the oocyte is unable to synthesize beta spheres.

Late in stage 13, the nuclear envelope must break down, since in stage 14 oocytes the chromosomes are aggregated in the centre of an island of cytoplasm free of yolk spheres (King, 1970, fig. 11-17). An oocyte nucleus was present in each of the mutant terminal chambers sectioned. However, nuclei were often abnormally large and surrounded by

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*Fig. 20. TEM showing the cytosegresome at higher magnification. It contains a population of autophagic secondary lysosomes (ly) and alpha yolk spheres (ays). The endochorion (en) is thrown into complex folds. ex, exochorion; fcn, follicle cell nucleus; il, inner lamella of endochorion; vm, vitelline membrane. Bar, 5 \(\mu\)m.*
vesicles, which presumably formed by blebbing of the nuclear envelope. Possibly these oocytes represent a stage in nuclear breakdown that normally happens rapidly and has heretofore been undetected.

**DISCUSSION**

*Comparison of nurse cells and PNCs*

One of the principal roles of the nurse cells is to provide ribosomal RNA for the growing oocyte. The rRNA genes are clustered together to form nucleolus organizers that are located in the X and Y chromosomes of *Drosophila melanogaster* (see review by Lefevre, 1976). In the PNCs examined in this study, RNP deposits occur at many sites along the sectioned chromosomes. Because the nucleolus organizer is restricted to a region near the centromere of the X chromosome, we conclude that non-ribosomal RNAs are also being transcribed.

The electron micrographs of the *otu* PNCs show that segments of the polytene chromosomes contain massive amounts of RNP (Fig. 3), that nuclear pores sometimes possess dense material in their centres (Fig. 5), and that localized deposits of granules cluster near the nuclear envelope (Fig. 3). Such nuages normally occur at the surface of nurse cell nuclei (Dapples & King, 1970, their fig. 6). These observations combined with the fact that ribosomes are abundant in the ooplasm of the mutant (Fig. 9c) indicate the ribosomal RNA is transcribed and transported to the ooplasm by PNCs of *otu* mutants, as is the case during normal *Drosophila* oogenesis.

During normal oogenesis of *D. melanogaster* the nurse cells begin degenerating during stages 11 and 12. The adjacent follicle cells are loaded with residual bodies, which suggests that these cells are engaged in heterophagy (Cummings & King, 1970). In the case of *otu*¹, the follicle cells adjacent to the PNCs in p-12 chambers contained few lysosomes and therefore had not received the cue to phagocytose the adjacent pseudonurse cells. There were some residual bodies in the PNCs, which indicates that some autophagy was occurring.

The cytoplasm of *otu*² PNCs contained organelles not seen in normal nurse cells. These tubular elements (Fig. 5) were sometimes attached to collecting vacuoles filled with dense material. Bowers (1964) observed similar tubular arrays in the pericardial cells of aphids. The tubular elements were often continuous with large vacuoles (see her fig. 5). Pericardial cells are thought to remove foreign compounds from the haemolymph, and the membranous vacuoles and their attached tubules may function in this process. Perhaps the tubular aggregates of the PNCs function in segregating endogenous, potentially toxic compounds from the rest of the cytoplasm.

The plasma membranes of PNCs seem to be fragile, since they sometimes fuse to produce binucleate cells (Fig. 3). The hydrostatic pressure within pseudonurse cells was evidently greater than that of the oocyte, since portions of adjacent pseudonurse cells were often forced into the oocyte.

*Transport of macromolecules from nurse cells to oocyte*

Electrophysiological studies have shown that the polarized movement of charged
macromolecules, which takes place within the egg chambers of the Cecropia moth, is caused by an electric current generated by potential differences between the oocyte and the nurse cells (Woodruff & Telfer, 1973, 1974). The equilibrium potential of the nurse cells is several millivolts more negative than that of the oocyte, and therefore macromolecules carrying a net negative charge are carried by electrophoresis to the oocyte from the adjacent nurse cells via the ring canals.

Presumably, molecules of nurse cell RNA are also carried into the Drosophila oocyte by this mechanism. PNC ribosomes are transported to the oocyte in otu^7/otu^7 chambers, but the rate of transport is probably slower in the mutant. The evidence for this conclusion comes from the relative sizes of basophilic deltas extending through the canals connecting the oocyte to its adjacent nurse cells in normal and mutant chambers (Fig. 6).

Vitellogenesis in normal and mutant oocytes

During normal Drosophila oogenesis the oocyte and the nurse cells grow at equivalent rates through stage 7. During vitellogenesis the oocyte grows about five times faster than previously. The nurse cells also grow at an increased rate (King, 1957) until late in stage 10, when they shrink as their cytoplasm is transferred to the oocyte. In otu^7, the construction of a micropylar apparatus brings this transfer to a premature halt at stage p-12.

During the formation of alpha yolk spheres, vitellogenins are internalized. In D. melanogaster these proteins are synthesized by the abdominal and thoracic fat bodies and by the columnar follicle cells surrounding the oocyte (Brennan, Weiner, Goralski & Mahowald, 1982). Each alpha yolk sphere is enclosed by a membrane composed of patches derived from pinosomes and Golgi membranes (Giorgi, 1980).

In addition to coated vesicles and developing alpha spheres, the cortex of vitellogenic oocytes also contains tubular elements (Fig. 8A, B), and these are often attached to immature yolk spheres. Telfer, Huebner & Smith (1982) maintain that cortical tubules arise as the membrane residues of pinosomes that have delivered their contents to the yolk spheres and have not yet been shaved off for recycling to the oocyte surface. While this may be true, mutant PNCs contain tubular arrays of similar morphology (Fig. 5). These tubules do not occur near areas of micropinocytotic activity and are present in chambers with or without oocytes. In the latter case the tubes cannot have arisen from oocyte pinosomes.

Alpha spheres were present in the ooplasm of females of all the mutant genotypes studied, and the surface of the pseudo-12 oocyte showed microvilli and coated pits, and pinosomes were present in the cortical ooplasm. We conclude that vitellogenins were synthesized, internalized by the oocyte, and concentrated to form alpha spheres in the normal way in otu^7 females. However, the total number of alpha yolk spheres contained in a p-12 oocyte is certainly less than that in a normal stage-14 oocyte.

Glycogen-rich, beta yolk spheres are first seen during stage 13 of Drosophila oogenesis. They form a gradient in size and number, since the anterior region of the oocyte contains the fewest and smallest spheres (Cummings & King, 1969). Wanson & Drochmans (1972) report that glucose 1-phosphate is converted to glycogen on
smooth-surfaced vesicles, and Johnson & King (1974) have observed vesicles of this sort in the centres of beta spheres. Koch & Spitzer (1982) demonstrated that D-[3H]glucose and D-[3H]-galactose, when injected into normal females, are rapidly incorporated into beta spheres. These results indicate that the vitelline membrane and chorion are permeable to sugars and that glycogen synthesis occurs in the oocyte, perhaps on membranous organelles previously supplied by the nurse cells. Beta yolk spheres never form in class 3 otu oocytes. The abnormally thick vitelline membrane and the deformed chorion may prevent passage of sugars to the oocyte, or the transfer of precursor organelles from the nurse cells may be blocked when the micropylar apparatus is formed at the p-12 stage.

**Secretion of oocyte coverings by follicle cells**

Autoradiographic evidence that the follicle cells were the source of the protein component of the vitelline membrane was first provided by (Cummings, Brown & King, 1971), and earlier evidence eliminating the oocyte as the source of the vitelline membrane came from observations of occasions in which vitelline membrane was secreted between follicle cells and cells other than oocytes. For example, vitelline membrane can form at the interface between follicle cells and ovarian tumour cells. This is true for the *fes* mutant (King & Koch, 1963, their fig. 7) and also for *otu*7 (Fig. 1A). Female sterile mutants with abnormally small oocytes generally have an abnormally thick vitelline membrane, and it is usually thickest at the posterior pole of the oocyte. This has been demonstrated in the case of *ty* (Falk & King, 1964, their figs 11, 13) and for *otu*7 (Fig. 7). Observations of this sort suggest that the follicle cells secrete a predetermined amount of vitelline membrane material, and in cases in which the area covered is subnormal the layer will be correspondingly thicker.

After the vitelline membrane is completed, the follicle cells secrete an endochorion and an exochorion. The endochorions of abnormally small eggs from mutant ovaries are often folded. Endochorionic peninsulas of the type illustrated in Figs 7 and 20 for *otu*7 also occur in *ty* oocytes (see King & Koch, 1963, their fig. 15; Falk & King, 1964, their fig. 13). The undulations of the endochorion suggest that the secretory surface of the follicle cell was thrown into folds during the time the layer was secreted and imply that the cells were compressed. The follicle cells of *otu*7 egg chambers also form exochorions (Fig. 20), and it follows that the columnar follicle cells of the mutant have all the synthetic capabilities of normal cells. We also know that these follicle cells undergo three or four endomitotic DNA replications prior to the p-12 stage (Rasch et al. 1984).

The respiratory filaments, commonly called the dorsal appendages, are anterior extensions of the chorion. They are secreted by two groups of follicle cells, each containing about 120 cells, that migrate centripetally between the oocyte and the nurse chamber during stages 10 and 11 (King & Koch, 1963). Mindrinos et al. (1980) have shown that a specific protein of high molecular weight is found in the dorsal appendages. It follows that the cells forming these appendages secrete different chorionic proteins from the other follicle cells. In this sense they resemble the border cells, which secrete a different type of vitelline membrane from the other follicle cells.
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Since dorsal appendages of nearly normal morphology are synthesized by otu1 homozygotes, it appears that the follicle cells in this region behave normally.

**Formation of the micropylar apparatus**

The chorion is modified at the anterior pole of the oocyte to provide for the fertilization of the egg. King & Koch (1963) found that the chorion formed a non-compartmented protuberance (the micropylar cone), that the vitelline membrane extended into the cone, and that a canal (the outer micropyle) penetrated the tip of the endochorionic protuberance. In a subsequent ultrastructural study, Perotti (1974) showed that the canal continued through the vitelline membrane and that the vitelline membrane in the region of the inner micropyle had a different ultrastructure from the rest of the vitelline membrane. The lamellar material shown in Figs 10 and 11 is similar to that described by Perotti. She also noticed that the outer micropyle was lined with vertically striated, endochorionic fibres, and our observations (Fig. 18) confirm this. Striated fibres of this sort have been described in insect connective tissues containing collagen (see reviews by Ashhurst, 1968, 1982).

The lamellate material that characterizes the vitelline membrane in the region of the inner micropyle is first seen at the border cell-oocyte interface early in stage 10 (Fig. 16), so it is clear that these follicle cells secrete a different type of vitelline membrane from the others. In the otu1 oocyte during the p-12 stage, a plug of lamellate material lies in the neck of the protoplasmic tube connecting the oocyte to the nurse chamber. Droplets of dense material appear to be fusing to the base of the plug of lamellate material (see Fig. 10), and this suggests that the oocyte may be contributing molecules to the plug at this stage. So the vitelline membrane of the micropylar cone is structurally different from the rest of the vitelline membrane, and it contains molecules synthesized by specialized follicle cells and perhaps by the oocyte as well.

Electron micrographs, such as the one shown as Fig. 14, demonstrate that one border cell differs from the rest in that it generates a long projection that contains a bundle of microtubules. We propose that the secretions of the ring of border cells are deposited about this projection, and a cone-shaped protuberance jutting from the anterior pole of the egg shell results. When the process subsequently is withdrawn or degenerates, a canal is left behind, which the sperm enters during its journey of fertilization.

**Why are otu mutants of class 3 sterile?**

According to the hypothesis advanced by King & Riley (1982), the product of otu* is required at three different times during oogenesis in Drosophila. It is used in the lowest concentration in the earliest period, during which it is essential for oocyte viability. During period 2 it is needed in larger amounts to control the proliferation and differentiation of cystocytes. The gene product is required in the highest concentration during period 3, when it permits the oocyte to complete its growth. Under this scheme, mutants belonging to class 3 would produce sufficient functional product
to allow oogenesis to proceed past the first two critical periods, but not enough to get past the p-12 stage.

It is clear that the mutant oocyte grows abnormally slowly during vitellogenic stages. This defect could result from a reduced rate of transport of cytoplasmic materials from nurse cells to oocyte. We have observed sectioned canals connecting adjacent PNCs and between PNCs and oocytes, and have found no physical barrier to intercellular transport up to the p-12 stage. A barrier is produced by the forming micropylar apparatus, but this cannot explain why the oocyte grows slowly from stages 8–11. So the ou+ gene may play a role in generating the flow of cytoplasm, and the defect in the mutant could result from a reduced fluid flow or a reduced movement of charged molecules. However, the possibility that the slowed growth is due in part to a reduced rate of endocytosis by the oolemma has not been ruled out.

The developmental programs of the follicle cell components seem to be rigidly followed, in spite of the slowed growth of the oocytes. The results are vitelline membranes and chorions too large for the oocytes they enclose. According to this interpretation, the thickened vitelline membranes and folded endochorions are secondary effects. The formation of the micropylar apparatus by the border cells prevents the transfer of the remaining nurse cell cytoplasm, and the result is the typical p-12 chamber. We interpret this sequence of events to indicate that border cells are programmed to form the micropylar apparatus at a given time after they complete their migrations, irrespective of whether or not the transfer of nurse cell cytoplasm is completed. The lack of beta spheres in mutant oocytes may also be a secondary effect, caused either by a block in the transport of vital organelles to the oocyte from the nurse cells or by the barriers to the diffusion of sugars presented by the abnormal membranes that surround the egg.

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