THE EGGSHELL OF DROSOPHILA MELANOGASTER

I. FINE STRUCTURE OF THE LAYERS AND REGIONS OF THE WILD-TYPE EGGSHELL

LUKAS H. MARGARITIS*, FOTIS C. KAFATOS† and WILLIAM H. PETRIJ

* University of Athens, Department of Biology, Panepistimiopolis, Kouponia, Athens (621) Greece
† Harvard University, The Biological Laboratories, Cambridge, Massachusetts 02138 U.S.A.
‡ Boston College, Department of Biology, Chestnut Hill, Massachusetts 02167 U.S.A.

SUMMARY

The fine structure of the several layers and regional specializations in the Drosophila melanogaster eggshell has been studied by a combination of shell isolation procedures and ultrastructural techniques (conventional TEM, whole-mount TEM, SEM, HVEM, freeze-fracture electron microscopy utilizing rotary replication, shadow casting, optical diffraction and stereo imaging).

The main shell consists of five layers: the vitelline membrane (300 nm thick), the wax layer, the innermost chorionic layer (40–50 nm), the endochorion (500–700 nm), and the exochorion (300–500 nm). The vitelline membrane consists of irregularly organized particles. The wax layer appears to contain multilayered hydrophobic plates which split tangentially upon freeze fracturing. The innermost chorionic layer is composed of a crystalline lattice. The endochorion is made of a thin (40 nm) fenestrated floor composed of 40-nm fibres and an outer solid (200 nm) roof covered with a network of 40-nm strands. Intermittently spaced pillar connect these 2 parts. Similarities in the substructure of the floor, pillars and roof suggest that they may be composed of similar or identical structural elements.

The specialized regions of the shell are the 2 respiratory appendages, the operculum area and the posterior pole. The appendages exhibit 2 sharply distinct surfaces, a dorsal side with isolated 1.5-μm plaques and a ventral side with strands of 40–50 nm connected in a network with openings of 70–80 nm. The operculum area, which includes the micropyle and the collar, is distinguished by 3 unique types of cell imprints. The posterior pole contains 2 distinctive populations of cell imprints: the central area has very thin intercellular ridges and a thin, perforated, endochorionic roof, while the peripheral area contains mixed, thick and thin, intercellular ridges and serves as a transition zone to the main shell pattern. The pillars in the central area of the posterior pole have a distinct arrangement, forming one peripheral circle within each cell imprint.

An analysis utilizing structural and developmental criteria indicates that as many as ten different populations of follicular epithelial cells may be involved in the construction of the various regions of the Drosophila eggshell.

INTRODUCTION

Our interest in using the Drosophila eggshell as a model system for studies on developmental control mechanisms (see Kafatos et al. 1977) requires that we understand the shell architecture in considerable detail. This is especially true in order to determine...
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the mode of action of eggshell mutations (Gans, Audit & Masson, 1975; Mohler, 1977), and to infer the function of individual protein constituents of the shell. In addition, analysis of shell structure should permit inferences concerning the size and number of distinctly programmed populations of follicular epithelial cells which cooperate in producing the shell.

The structure of the *Drosophila* eggshell was originally documented by the work of King and his coworkers (for a review, see King, 1970). More recent information has been added by Quattropani & Anderson (1969), Klug, Campbell & Cummings (1974), Margaritis (1974), Margaritis, Petri & Kafatos (1976), Turner & Mahowald (1976) and Margaritis, Petri & Wyman (1979).

Our purpose here is to draw together new observations on *Drosophila* eggshell fine structure, which provide a more detailed description of this material than was previously available. This increase in information was made possible by new methods for obtaining isolated shell layers, and by the combined use of phase-contrast light microscopy, conventional transmission electron microscopy (TEM) of thin-sectioned material, whole-mount TEM, high-voltage electron microscopy (HVEM), scanning electron microscopy (SEM), freeze-fracture electron microscopy utilizing rotary replication, and optical diffraction techniques. Stereo micrographs were employed to clarify the 3-dimensional structure of the various regions and layers of the eggshell.

**MATERIALS AND METHODS**

**Preparation of tissues and shell layers**

Oregon R flies conditioned at 25 °C were lightly etherized and dissected in *Drosophila* Ringer solution (Ephrussi & Beadle, 1936). Ovaries or individual follicles were fixed as described below.

Purified endochorion samples were prepared by the distilled water method from mass isolated stage 14 follicles as previously described (Method I of Petri, Wyman & Kafatos, 1976). Vitelline membrane-bound oocytes (VMO) were separated from the chorion by the same method, or by gentle sonication after brief (20 min) fixation in 2 % glutaraldehyde (buffered with 0-08 M sodium cacodylate, pH 7.4). Laid eggshells (eggshells collected after hatching) were collected as described (Petri et al. 1976). Sheets of innermost chorionic layer (ICL) were separated from the remaining chorion by shaking laid eggshells in distilled water (Margaritis et al. 1979).

**Thin-section electron microscopy**

Immediately after dissection ovaries or follicles were placed in one of the following fixatives, washed in 4 % sucrose, 0.1 M sodium cacodylate buffered at pH 7.4, postfixed, dehydrated in ethanol and embedded in a modified Mollenhauer's resin (25 g Epon-812; 20 g Araldite-506; 60 g DDSA; and 3 g DMP-30). In all cases, the fixation buffer was sodium cacodylate at pH 7.4. The fixative-postfixative combinations used were one of the following:

(a) 2.5 % glutaraldehyde in 0.08 M buffer for 90 min at 4 °C, no postfixation (G fixation).
(b) 2.5 % glutaraldehyde in 0.08 M buffer for 90 min at 4 °C followed by 2 % osmium tetroxide in water for 60 min at 4 °C (G-Os fixation).
(c) 2 % glutaraldehyde, 1.5 % paraformaldehyde and 1.5 % acrolein in 0.08 M buffer for 90 min at 4 °C, followed by 2 % aqueous OsO4 for 60 min at 4 °C (GPA-Os fixation; Karnovsky, 1965).
(d) 1 % lanthanum nitrate with 2.5 % glutaraldehyde in 0.08 M buffer for 3.5 h at 4 °C, followed by overnight washing in 0.08 M buffer containing 1 % La(NO3)3 and 4 % sucrose (GLa fixation; Revel & Karnovsky, 1967).
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- 2.5% glutaraldehyde in 0.08 M buffer for 90 min at 4°C, followed by 4% aqueous KMnO₄ for 20 min at 4°C. 1% uranyl acetate in 70% ethanol was used during dehydration for 20 min (G-PM fixation).

Thin sections were cut with glass or diamond knives using MT-1 Porter Blum and Reichert OMU-2 ultramicrotomes and stained with uranyl acetate and lead citrate, unless otherwise stated. The grids were viewed using Philips EM 200, EM 300 and EM 301 (equipped with goniometer stage) electron microscopes, operating at 60 or 100 kV. Images were recorded on Kodak Electron Image plates or Dupont S-Ortho Litho plates.

**High-voltage electron microscopy**

Follicles were fixed in G-Os fixative, stained en bloc for 1 h in 2% aqueous uranyl acetate at 60°C and embedded in Epon-Araldite. Thick sections (1 μm) were stained with uranyl acetate and lead citrate, and covered with a carbon coat. Images were obtained in the JEM-1000 high-voltage microscope (MCDB, University of Colorado, Boulder) operating at 1000 kV, and recorded on Kodak 4489 plates.

**Whole mount TEM**

Purified endochorion samples or laid eggshells were collected as monolayers on electron-microscope grids, and viewed either unstained, or stained with phosphotungstic acid or 2% sodium silicotungstate. ICL monolayers were picked up on naked 200-mesh EM grids and stained briefly with 2% uranyl acetate. Some samples were washed with distilled water after staining.

**Scanning electron microscopy**

Samples (laid eggs, purified endochorion or VMO) were fixed in G-Os. During glutaraldehyde fixation they were placed in Beem capsules, size 3, covered at both ends with Nytex cloth no. 102, so that solutions could be changed easily. After dehydration the capsules were placed in a Samdri PVT-3 critical point dryer (Tousimis Research Corporation). After drying, the samples were ‘dusted’ on SEM stubs covered with Scotch double-stick tape. Following light coating in a Hummer-II sputtering device (Technics Corp.) with gold-palladium and carbon, the samples were viewed in AMR 1000 and JEOL JSM-35 scanning electron microscopes operating at 30 kV. Images were recorded on Polaroid 55 film.

**Freeze-fracture electron microscopy**

Samples (laid eggshells, purified endochorion and follicles) were frozen on copper hats in Freon-12 and liquid nitrogen (Fisher & Branton, 1974). Some samples were fixed in 2.5% glutaraldehyde in 0.08 M sodium cacodylate buffer prior to freezing and others were incubated in 25% glycerol for 3 h after fixation and before freezing.

Freeze-fracturing was performed in a Balzers 500 unit modified for rotary replication (Margaritis, Elgsæter & Branton, 1977). Some samples were also single-direction shadowed. In the presented micrographs a rotary arrow designates rotary replication and the included number indicates the angle of heavy metal evaporation. Metal evaporation was performed with electron bombardment guns using Pt-C electrodes. The replicas were cleaned with sodium hypochlorite (commercial bleach or Chlorox) overnight and after distilled-water washing, were picked up on 300-mesh electron-microscope grids.

**Shadow casting**

Purified endochorion samples were picked up on 200-mesh grids, either right side out or inside out, shadowed as above, and viewed without digesting the endochorion.
Optical diffraction

Micrographs showing periodic structures were analyzed with an He-Ne vertical laser diffractometer (Brandeis University, Rosenstiel Medical Research Center, Waltham) as described previously (Margaritis et al. 1979).

Stereo images

Stereo pairs were made from 2 images obtained after tilting the specimen either with a goniometer stage or by using the tilting device of the Philips EM 200 and EM 300 microscopes. The use of a stereo viewer is recommended for appreciation of the third dimension.

RESULTS

The main layers of the eggshell

We shall first describe the structure in the main body of the eggshell, postponing until later consideration of the specializations found at the anterior and posterior poles. To aid the reader in properly orienting the various components of the mainshell, a diagram of a shell fragment showing all surfaces and layers is presented in Fig. 1. The reader should consult this diagram in parallel with the micrographs.

Stage 14 is the last stage of follicle maturation (King, Darrow & Kaye, 1956). It terminates with ovulation, i.e. rupturing of the follicular epithelium and release of the
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eggshell-covered oocyte into the oviduct. Four main eggshell layers can be observed by electron microscopy of conventionally fixed and thin-sectioned stage 14 follicles (Fig. 2). From the oocyte outward, these are the vitelline membrane (approximately 300 nm), the innermost chorionic layer (40–50 nm), the endochorion (500–700 nm) and the exochorion (300–500 nm). The endochorion is a complex, multilevel structure, and is best visualized in stereo images obtained by HVEM of thick sections (Fig. 5). This layer includes rounded cavities, between a thin (approximately 40 nm) and perforated inner endochorion or ‘floor,’ and a thick (200 nm) and continuous domed outer endochorion or ‘roof.’ The cavities are empty in laid eggs (Fig. 3) but at stage 14 they still contain a loose, flocculent material (Fig. 2). The floor and roof of the endochorion are connected by vertical pillars (Figs. 2, 3, 5). The outer surface of the roof shows protrusions (Figs. 2, 3) organized in a regular ‘roof network’ (Fig. 5). At sites which correspond to borders between secretory cells (King & Koch, 1963) the roof network is elaborated into elevated ridges (Figs. 3, 5). Pillars are never found immediately underneath the ridges (Figs. 3, 5). Phase-contrast views of flattened shells (Fig. 4) clearly show pillars uniformly distributed within polygonal areas which are set apart by the ridges. Since the ridges correspond to cell borders, each polygon represents the imprint of a single secretory cell.

Vitelline membrane (VM), wax layer, and innermost chorionic layer (ICL)

In sectioned material the VM appears as a continuous granular layer without clearly evident substructure. After freeze fracturing, it exhibits apparently unorganized particles on the fractured face (Fig. 8). The ICL is also continuous but thinner (40–50 nm) and characterized by periodic substructure (Figs. 9–18).

Between these 2 layers wisps of material can be discerned in sections (Fig. 2) and may correspond to irregular polygonal patches (0.5–1 μm across) which are evident by scanning electron microscopy of the exposed outer vitelline membrane surface in stage 14 follicles (Fig. 6). In laid eggshells, freeze-fracturing has revealed smooth fracture faces of a multilayer material lying between and in very close contact with the vitelline membrane and the ICL (Fig. 7), presumably the wax layer (see Discussion).

High-resolution images of sectioned ICL after conventional fixation and staining show 8–10 sublayers as horizontal striations, parallel to the oocyte surface and spaced at approximately 500 nm. Alternating lines differ in the intensity of staining (Figs. 9, 13). Appropriate tilting of the sections also reveals vertical striations with the same periodicity (Fig. 10). Thin-sectioned stage-14 follicles impregnated with lanthanum during fixation, reveal an array of electron-dense major (3 nm) and minor dots at an overall repeat distance of 10 nm, both parallel and normal to the oocyte surface; lanthanum also binds to the outer surface of the ICL (Figs. 11, 12). Comparison between conventionally fixed and lanthanum-impregnated ICL thin sections shows that the major lanthanum dots correspond to the lighter lines of the conventionally fixed preparations (e.g. compare the innermost line in Figs. 12, 13).

By convention, ‘inner’ faces towards the oocyte and ‘outer’ faces towards the follicular epithelial cells.
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Fig. 2. Thin-sectioned stage 14 follicle showing the vitelline membrane (vm), the innermost chorionic layer (icl), the endochorion complex, and the fibrous exochorion (ex) which has just been secreted by the follicular epithelial cells (fe). The endochorion consists of the inner endochorion (ie), the outer endochorion (oe), the pillars (p), and the protrusions forming the roof network (rn). A loose material is found within the cavities of the endochorion (c) and also between icl and endochorion. Indications of a different type of wispy material are found between vitelline membrane and icl (which are artifically separated). (View A). GPA-Os fixation. × 28000.
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Freeze-fractured, etched and rotary shadowed stage-14 follicle preparations demonstrate the existence of planar surface periodicity in the ICL (Fig. 14), in contrast to the non-periodic distribution of internal VM components (Fig. 8). Etching appears to be necessary to reveal the planar surface periodicity of the ICL; non-etched preparations always show the ICL in cross-fractured views (Fig. 7).

With care, sheets of pure ICL can be obtained from laid eggshells (Margaritis et al. 1979). The periodicity of flattened ICL layers after staining with uranyl acetate (Figs. 15, 17) indicates that the particles of the sublayers (Figs. 9-13) must be vertically aligned. Both heavily stained (Fig. 15) and lightly stained (Fig. 17) samples reveal tetragonal P2 planar periodicity as confirmed by optical diffraction (Figs. 16 and 18, respectively). The lightly stained preparations show striations at approximately 5.0-nm repeat distances in 2 directions, forming an angle of about 90° (Fig. 17). In addition, heavily stained preparations (Fig. 15) exhibit rows of major electron-dense 3-nm dots at a repeat distance of approximately 10 nm, resembling the lanthanum-impregnated ICL (Fig. 12).

The ICL appears to consist of crystallites, 1-3 µm across; their borders are evident where the crystal lattice changes direction (Fig. 15). Further details of this apparently crystalline structure remain to be established by 3-dimensional image analysis and reconstruction.

Endochorion

Endochorion can be isolated by shaking stage-14 follicles in distilled water (Margaritis et al. 1976). The preparation also contains insoluble vitelline membranes, but pure endochorionic shells can easily be selected manually (Fig. 19). TEM reveals (Fig. 20) that endochorions so isolated consist of all endochorionic structures recognized in intact follicles: inner and outer endochorion, pillars, roof network and ridges. Because it is devoid of other eggshell layers and follicular cells, isolated endochorion is ideal for detailed 3-dimensional study of the endochorionic structures.

At low magnification, SEM of the inner surface reveals that the inner endochorionic floor is flat and complex in appearance, including both solid and fenestrated regions (Fig. 25). The edges of cell imprints, which lack pillars, correspond to continuous
Fig. 6. Scanning electron micrograph of a vitelline membrane-bound oocyte, showing patches (pa) 0.5–1.0 μm in size on the surface of the vitelline membrane (vm). Part of the innermost chorionic layer (icl) still covers the vm. (View B3 and B4). × 22 000.

Fig. 7. Freeze-fractured laid eggshell. The cross-fractured icl and vm contrast with the presumably lipoidal, stacked "wax layer" (wl) plates which split longitudinally upon fracturing. These plates exhibit smooth surfaces as opposed to the particulate appearance of the icl. Ice is found to the right side of the icl only, suggesting impermeability. (View B3 and B4). G fixation followed by 25% glycerol. × 36 000.

Fig. 8. Freeze-fractured laid eggshell showing the particulate structure of the cross-fractured vm and the smooth surfaces of the split wax layer (wl). (View B4). G fixation. × 36 000.

Figs. 9, 10. Thin-sectioned stage 14 follicle showing at 2 different tilting angles the fine structure of the icl. Successive light and dense striations are resolved parallel (Fig. 9) or normal (Fig. 10) to the oocyte surface. A different type of regular substructure is detected in the inner endochorion (ie). (View A). GPA-Os fixation. Fig. 9, x 100 000; Fig. 10, x 110 000.

Figs. 11, 12. Thin sections through lanthanum-impregnated stage 14 follicle, showing dots of stain in a regular pattern within the icl, and concentration of stain on the outer surface (right) facing the inner endochorion. The repeat distance of the major 3-nm dots is approximately 10 nm in both directions; minor dots are found halfway between major dots. Observe that the innermost row of major dots (Fig. 12, arrow) corresponds to a lighter striation of the conventionally fixed sample (Fig. 13, arrow). (View A). GLa fixation, no other stain. Fig. 11, x 120 000; Fig. 12, x 180 000.

Fig. 13. Thin-sectioned stage 14 follicle showing the successive light and dense striations of the icl. (Compare and align with striations in Fig. 12.) (View A). GPA-Os fixation. × 160 000.

Fig. 14. Freeze-fractured, etched (−100 °C, 2 min) and rotary replicated stage 14 follicle showing an extensive periodic array in 2 directions (arrows) in a region between the vm and the endochorion, presumably representing one of the icl surfaces. (View B3 or C3). × 100 000.

Fig. 15. Isolated icl, stained with 2% uranyl acetate without wash. Two crystallites are evident, with slightly overlapping boundaries (asterisk) and different lattice orientations (arrows). Viewed from an angle, light and dense dots appear as alternating striations, separated by 5 nm and with a repeat distance of 10 nm (cf. Fig. 12). (View B3). × 180 000.

Fig. 16. Optical transform from a representative icl image prepared as in Fig. 15. The tetragonal lattice reflections extend up to 4th order, 2.5-nm spots.

Fig. 17. Isolated icl stained with 2% uranyl acetate and washed with distilled water. A fine, 5-nm periodicity is shown in 2 crystallographic directions (arrows), and is confirmed by the optical diffraction analysis (Fig. 18). (View B3). × 180 000.

Fig. 18. Optical transform of the image of Fig. 17, showing the basic reflections of a tetragonal lattice with a = 4.6 and b = 4.2 nm.
Fig. 19. SEM view of the dorsal disk of a purified endochorion with attached respiratory appendages (ra). Outer as well as inner surfaces (viewed through a tear) show imprints of follicle cells. (View B). × 190.

Figs. 20–43: Structure of the endochorion on the main body of the shell.

Fig. 20. Thin section through purified endochorion typical of preparations used for structural and biochemical studies. The inner endochorion (ie), outer endochorion (oe) and ridge (r) are identified; tcl and exochorion are absent from the purified endochorion. (View A). G-Os fixation. × 14000.

Fig. 21. Platinum-carbon shadowed preparation of the purified endochorion, showing the inner endochorion (floor). Note the bumpy substructure of both fenestrated and solid regions (arrows). (View C2). Whole mount TEM. × 75000.

Fig. 22. Oblique thin section through stage 14 follicle showing the endochorionic roof network (rn), composed of 35-nm diameter fibres arranged in polygons, approximately 160 nm across (extreme oblique section producing view similar to B2). GPA-Os fixation. × 30000.

Fig. 23. Platinum-carbon shadowed preparation of purified endochorion showing bumpy substructure of the roof network (arrows). (View B2). Whole mount TEM. × 75000.
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fenestrated bands 1 \( \mu \text{m} \) wide. These are naturally weaker than the rest of the floor and tear easily, revealing the internal structure of neighbouring pillars and cavities (Fig. 25). At high magnification, the solid regions of the floor reticulum are seen to be pitted (Fig. 29). Whole-mount TEM of shadowed preparations show fine bumps in both the solid and the fenestrated regions (Fig. 21), suggesting that these regions might represent different degrees of assembly of the same basic component(s).

SEM of the outer surface illustrates the continuous roof network, plus ridges which define the borders of cell imprints (Fig. 24). The unevenness of the underlying roof reflects the distribution of pillars, as best shown by a combination of whole mount and shadowing techniques (Fig. 28). At higher magnification, the roof network is seen to consist of approximately 160-nm polygons (Fig. 27), primarily hexagons and pentagons (Fig. 22), formed by 35–45-nm strands. TEM of shadowed whole mounts again reveals bumpiness in the strands (Fig. 23).

TEM of unstained whole mounts of endochorion permits simultaneous visualization of all endochorionic structures. At low magnification (Fig. 26) the pillars are seen to be 250–400 nm wide and spaced approximately 1 \( \mu \text{m} \) apart, covering approximately 14\% of the optical cross-section. The continuous ridges are particularly evident. High magnification (Fig. 30) highlights the contrast between the fine, regular roof network and the broad, irregular fenestrations of the floor.

The 3-dimensional organization of these endochorionic structures is best appreciated from stereomicrographs (Figs. 31–33). In freeze-fractured preparations (Figs. 31, 32), particles are evident in all fractured faces: floor, pillars, roof and roof network. Therefore, the bumpiness is not an exclusive feature of shadowed preparations (Figs. 21, 23) but might reflect an underlying substructure. Etching reveals that the lining of the endochorionic surfaces is continuous but bumpy (Figs. 31, 32), presumably because of the existence of the underlying particles seen in the fractured faces.

Examination of both sectioned (Fig. 34) and fractured (Figs. 35–37) endochorion at higher magnifications confirms the similar substructure at all levels. Rotary shadowing suggests that throughout the endochorion, coarse, 7–10-nm particles are spaced approximately 25 nm apart and interconnected by thinner filaments (Fig. 37). The particles appear similar in both etched and non-etched preparations (data not shown), suggesting that they are embedded in a non-volatile matrix. At high resolution (Fig. 34), TEM of sectioned material also shows coarse particles, comparable to those revealed by freeze-fracturing; in addition a periodicity, somewhat reminiscent of the ICL is evident, suggesting locally parallel orientation of constituent filaments or strings of fine particles (Fig. 34; see also Fig. 10). However, the endochorion and the ICL appear different when fixed with potassium permanganate (Fig. 39); the ICL then seems less electron-dense, suggesting a difference in chemical composition.

In the ovulated egg the lining of the endochorionic cavities, both surfaces of the floor and the outer surface of the ICL bind lanthanum extensively, whereas the inner surface of the ICL and the outer surfaces of the roof network do not (Fig. 38). Binding may be the property of the loose material which fills the endochorionic cavities and penetrates between endochorion and ICL at stage 14 (Fig. 2). Lanthanum also demonstrates that the pillar bases are porous, as suggested by the pitted appearance.
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of the floor as seen in SEM (Fig. 29), in ordinary TEM (Fig. 2) and following freeze etching (Fig. 31).

As seen both in purified endochorion preparations and in scanning electron micrographs of laid and extensively washed eggs (Figs. 19, 40, 41), the structure of the endochorion is essentially the same throughout the main body of the shell; this is also confirmed by TEM (data not shown). Only a subtle difference in the shapes and sizes of cell imprints exists within the main body: the imprints of the dorsal side are more elongate than those found ventrally (Figs. 40, 41). The most commonly occurring cell imprints are hexagons (55%) and pentagons (30-35%). However, imprints with 4 (5-9%), 3 (0-6%), 7 (7-9%) and 8 sides (0-0-6%) have been detected. Generally, the 3- and 4-sided imprints are found at the dorsal surface, just posterior to the bases of the respiratory appendages (Figs. 40, 42). In contrast to the main body, the anterior and posterior poles are highly specialized (Figs. 19, 42, 43), as discussed below.

**The operculum**

The dorsal aspect of the anterior pole is extensively specialized (Figs. 42, 43). It consists of a nearly flat plate or operculum (Turner & Mahowald, 1976) which measures 120 µm across, and bears a medial protuberance at its anterior end and 2 long appendages at its posterior end. The anterior medial protuberance is the micropylar apparatus.
Fig. 31. Stereo pair of freeze-fractured, etched (−100 °C, 5 min) and rotary replicated purified endochorion. The fractured areas exhibit particles, whereas the etched areas show a continuous but uneven surface covering. The surface unevenness may reflect the underlying structure. Ice has sublimed from the endochorionic cavities, revealing the surface features. Note the roof network, and also the gradual merging of the pillars with the floor. (Views A and B). Unfixed preparation, 6° tilt. × 16,000.

Fig. 32. Stereo pair of freeze-fractured, etched (−100 °C, 5 min) and rotary replicated purified endochorion revealing the configuration of the cavities shown to be empty (no ice sublimation can be detected from the area within the cavities). Observe also the solid and fenestrated regions of the inner endochorion (ie) and the unevenness of the internal surface of the outer endochorion (oe). (Views A and C). G fixation, 6° tilt. × 15,000.

Fig. 33. Stereo pair of whole mount inner view of purified endochorion showing the ridge (r), the roof network (rn), the torn inner endochorion (ie), and optical cross-sections of pillars. (Like Fig. 26). Unfixed preparation stained with 2 % PTA, 6° tilt. × 7000.
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through which sperm entry occurs (King & Koch, 1963). Its endochorionic component (Figs. 42, 43, 48, 49, 51) is an open-ended (Fig. 49) and hollow (Fig. 53) cone, into which fits a protuberance of the vitelline membrane (Figs. 48, 50).

The anterior and lateral margins of the operculum consist of a highly specialized (Figs. 43, 44, 54) transition between operculum and main body, the ‘collar’ (Klug et al. 1974). Here (Fig. 44) the inner endochorion is a thin continuous layer, unlike the normal floor in that it lacks fenestrations (Figs. 53, 54). Projecting above are 2 separate sheets of outer endochorion, dorsal and ventral, held together at the top by roof network and exochorion. The outer endochorion sheets are spongy (Fig. 54), consisting of a reticulum of structures reminiscent of pillar and roof fragments. Although no continuous roof exists, the roof network is well developed. The collar region has a continuous, normal ICL (Fig. 44 and data not shown) but a holey vitelline membrane (Fig. 46). Since the collar splits open (Fig. 43) to permit hatching of the larva, the holey vitelline membrane and bipartite outer endochorion appear to be adaptions to facilitate splitting; the continuous inner endochorion may form a transient barrier during embryonic life, analogous to, but flimsier than, the roof of the main body.

In the operculum, immediately around the micropylar apparatus, are one or two rings of indistinct small cell imprints (Area i; Figs. 42, 49 and 51); here the floor is less fenestrated (Fig. 53) than in the main body (Fig. 25). More posteriorly is another specialized region, Area ii which contains a total of approximately 30-40 cell imprints (Figs. 42, 43, 49). Here ridges of roof reticulum are highly developed (Figs. 44, 45, 51, 52). In the floor, corresponding to these ridges (Fig. 45), are broad continuous fenestrated bands (Figs. 53, 56), which surround nearly solid central regions (Figs. 44, 45, 53, 55). The solid regions appear to correspond to an amalgamation of floor, pillars and roof. Posterior to Area ii are the insertions of the 2 chorionic filaments (respiratory appendages); these are separated by Area iii, a band of approximately 10 to 15 laterally elongated cell imprints (Figs. 42, 43), smaller but otherwise similar to the Area ii imprints.

**The respiratory appendages**

The appendages are 250 μm long and largely cylindrical, but slightly paddle-shaped at the tip (Fig. 57); their diameter at the base is 15–20 μm. No cell imprints are apparent on either side except indistinctly near the base (Fig. 51). For much of their length, excluding the base (Fig. 42), the appendages have 2 sharply distinct surfaces (Figs. 57, 59). The ventral surface consists of a continuous fine network (Fig. 59) of 40–50 nm strands with openings of 70–80 nm, whereas the dorsal surface has isolated plaques (Figs. 59, 60) which are approximately 1.5 μm wide and cover 40–50% of the exposed surface area. Towards the base, the plaques merge into a continuous holey sheet (Fig. 58). The 2 sides of each appendage are interconnected via an extensive network of modified pillars (Fig. 59) clearly shown in stereo SEM (Fig. 67), whole mount TEM (Fig. 68) and freeze-fractured and etched (Fig. 69) views.

Surface scanning (Fig. 60) and transmission electron microscopy of sectioned
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Material (Fig. 61) show that the plaques are porous and apparently made up of thin, fused strands (Fig. 58), similar in thickness to the strands of the ventral side. The pores are 40-50 nm wide and penetrate into the base of the modified pillars (Figs. 61, 65, 69). They are similar to the pits of the normal endochorial floor (compare Figs. 58 and 29), and narrower than the pores of the ventral network (Figs. 59, 62, 63, 66). Conversely, the ventral network is reminiscent of the endochorial roof network in the main body of the eggshell, but denser (compare Figs. 63 and 31; also Figs. 59 and 27). The modified pillars end up directly on the network (Figs. 62, 63, 66) without the intervention of a solid layer analogous to the roof of the main body. Freeze-fracturing reveals internal particles in the plaques and pillars (Fig. 70), similar to those found in the main endochorion.

After the endochorial part of each appendage is formed, both sides become fully surrounded by exochorion (Figs. 65, 66). However, the exochorion is easily removed and has a patchy appearance in laid eggs (Fig. 64).

**The posterior pole**

The posterior pole of the eggshell is also specialized. Here a group of approximately 20–30 cells leave imprints distinctly smaller than those in the main body of the shell

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**Fig. 34.** High-resolution micrograph of thin-sectioned stage 14 follicle revealing parallel fibrillar substructure in the roof network (black arrow) and in the outer endochorion, oe (white arrow). In places particulate sub-structure (reminiscent of the 7-10-nm particles seen in freeze-fractured preparations) can be observed; the square outlines 4 such particles. Loose material bound on the inner surface, facing the endochorionic cavities (ec), is indicated by a double arrow. (View A). GPA-Os fixation. × 105 000.

Figs. 35–37. Freeze-fractured, etched (−100 °C, 2 min) and rotary replicated preparations of endochorion. The fractured roof network (Fig. 35) as well as the rest of the endochorion exhibit 7-10-nm diameter particles (arrows) spaced approximately 20 nm apart and interconnected with fine fibres (double arrows). Unfixed samples. (View A). Fig. 35, × 45 000; Fig. 36, × 50 000; Fig. 37, × 125 000.

Fig. 38. Thin section through ovulated follicle after lanthanum nitrate impregnation. Unstained areas were bleached with strong irradiation of the section in the microscope, to enhance visibility of lanthanum-binding sites. Electron density (arrows) is observed on all endochorionic surfaces except that of the roof network and on the outer surface of the id facing the endochorion. See also Figs. 11 and 12, which represent higher magnifications of the id from the same preparation. (View A). GLA fixation, no other stain applied. × 18 000.

Fig. 39. Thin-sectioned stage 14 follicle after GPM fixation. The electron-density differences between id and endochorion (id and oe) are suggestive of a difference in composition or packing of these layers. (View A). × 16 000.

Figs. 40–78: Structure of specialized regions.

Figs. 40, 41. SEM views of laid eggs showing the dorsal (Fig. 40) and ventral (Fig. 41) surface. Note the differences in shapes of follicle cell imprints. Generally, the imprints of the dorsal side are more elongate than those on the ventral side. Observe also that the imprints fade out near the posterior pole, which is marked with round holes, ra, respiratory appendage. (View B equivalent). × 200.
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The most peripheral of these contain fewer pillars (Figs. 71, 74) compared to the main body cells (Figs. 71, 75), and otherwise have a nearly normal surface appearance (Figs. 71, 72), transitional to that of the more central pole imprints. The latter, approximately 10-15 cell imprints in total, are even more specialized. Here the ridges of the roof network are not prominent (Fig. 72), and pillars are largely clustered in one peripheral circle per cell (Figs. 72, 74, 75). The roof is irregular and fenestrated (Fig. 47), and since it is unsupported it tends to tear within the circle outlined by the pillars (Fig. 72). In the posterior pole the floor is also unusual and in many places absent (Figs. 47, 73). As reported by King & Koch (1963) the pillars of the posterior pole are unusually tall. In a laid egg the pole is densely covered with exochorion, which obscures details of the cell imprints at the tip (Fig. 76).

The exochorion

The exochorion is seen in TEM to consist of loose fibres which tend to be oriented parallel to the oocyte surface (Figs. 2, 34). It usually appears to consist of 2 layers, the innermost being less electron-dense (Figs. 2, 38, 39, 47). It normally obscures the details of the endochorionic roof under the SEM, although the cell imprints are still evident, because the endochorionic ridges cause local elevation of the exochorionic layer (Fig. 3). As already mentioned for the appendages (Fig. 64), the exochorion is easily washed off after egg laying; it gradually becomes patchy (Fig. 77), revealing the underlying endochorionic roof network (Fig. 78).

Figs. 42, 43. SEM views of laid eggs showing the surface structure of the anteriodorsal region (operculum) exhibiting the collar (c), the micropylar cone (mc), the bases (b) of the respiratory appendages (ra), and the 3 areas distinguished by the type of follicular cell imprints (i, ii and iii). The operculum measures approximately 120 µm across, and is clearly different from the main body (mb). (View B eq.). Fig. 42, x 250; Fig. 43, x 150.

Fig. 44. The collar of a late stage-14 follicle, as seen in TEM. The id, inner endochorion (ie) and exochorion (ex) are continuous layers, whereas the prominent outer endochorion consists of dorsal (d) and ventral (v) halves. The halves have a spongy consistency (see also Fig. 54), and include highly developed roof network. Note the transitions to the normal endochorion of the main body (lower left) and the operculum (lower right). The elaboration of the roof network into a prominent ridge (r) and the coalescence of the endochorionic sublayers into a solid region (sr) in the operculum are shown, as well as part of a respiratory appendage (ra). (View A eq.). GPA-Os fixation. x 3300.

Fig. 45. Structure of the operculum (area ii) of a late stage-14 follicle, TEM. Above the id, the endochorion is coalesced into a solid region (sr), with interruptions (arrowhead) only under extremely high ridges (r) which represent elaborations of the roof network, penetrating through the exochorion (ex). (View A eq.). GPA-Os fixation. x 5500.

Fig. 46. TEM of a stage-14 follicle, showing holes (arrows) in the vitelline membrane underneath the collar (not visible). The oocyte (oc) is underneath the vitelline membrane. (View A eq.). GPA-Os fixation. x 17500.

Fig. 47. TEM of the posterior pole of the chorion from a laid egg. The floor around the porous base of a pillar (arrowhead) is missing, and the irregular roof is perforated (arrow). The thick exochorion (ex) shows 2 distinct layers. (View A eq.). GPA-Os fixation. x 11500.
Eggshell proteins

Eggshell preparations isolated by shaking stage 14 follicles in distilled water (Petri et al. 1976) are enriched in endochorion (Margaritis et al. 1976; see also Fig. 20). When such preparations are dissolved in urea and the proteins displayed on a 1-dimensional SDS polyacrylamide gel, 6 major protein bands, A1, A2, B1, B2, C1 and C2 are observed (Petri et al. 1976). Using higher-resolution 2-dimensional gels and highly purified samples we can resolve these 6 major bands into a total of 10 components (Fig. 79). The A1 and A2 bands are each seen to be composed of acidic (A1a and A2a) and basic (A1b and A2b) components. The B1 band resolves into 2 proteins (B1.1 and B1.2) with identical isoelectric points but slightly different molecular weights. The C1 band resolves into a major basic component (C1b) and a minor, more acidic one (C1a), while C2 is unresolved. In addition, at least 9 minor bands
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reproducibly co-purify with eggshell (Fig. 79, arrows) and are presumably bona fide shell components.

However, as many as 30–35 additional minor spots can be seen in stained 2-dimensional gels of highly concentrated, very pure eggshell samples prepared by Method II of Petri et al. (1976) and sonicated (Yannoni & Petri, in preparation). We do not know whether small amounts of exochorion and ICL proteins are represented in these gels; at least vitelline membrane proteins appear to be absent (Petri et al. 1976). Thus the possibility remains that the real number of distinct eggshell proteins may be significantly higher than the 19 described here. These 19 or more proteins, along with mostly neutral polysaccharides (Petri et al. 1976) and perhaps also mucopolysaccharides (Quattropani & Anderson, 1969) make up the Drosophila eggshell. (After submission of this manuscript, a paper by Waring & Mahowald (1979) appeared, reporting in detail the protein composition of eggshell preparations. Their results and ours are in general agreement.)

Fig. 57. SEM view of the paddle-shaped tip of a respiratory appendage from purified endochorion, showing the structural difference of the 2 surfaces: the dorsal side exhibits discrete plaques (d), while the ventral side is continuous (v). These 2 surfaces are examined in further detail in Figs. 58–70. × 500.

Fig. 58. SEM view of the dorsal side near the base of the appendage, from a purified endochorionic shell. Here the plates have coalesced into a layer with wide openings. Fused fibres are outlined by pits (arrow), resembling the fibres of the inner endochorion (see Fig. 29). × 19000.

Fig. 59. Detailed SEM view of the middle part of a respiratory appendage, showing the pitted (single arrow) dorsal surface (d), with plaques (pl) attached to modified pillars (p), and the ventral surface exhibiting a network (vn) composed of 40–50-nm fibres and 70–80-nm openings. The 2 surfaces are sharply distinct at the point of confluence (facing arrows). × 12500.

Fig. 60. Detailed oblique SEM view of the dorsal surface of a respiratory appendage. The attachment of the plaques (pl) to the modified pillars (p) is clearly demonstrated. The surface of the plaque exhibits 40-nm pits (arrow). × 19000.

Figs. 61, 62. Thin-sectioned purified endochorion showing the fine structure of both surfaces of the respiratory appendage, i.e. the dorsal (Fig. 61) and the ventral (Fig. 62). The dorsal side contains porous (arrows) plaques (pl), while the ventral side consists of a continuous porous network (vn). Modified pillars (p) insert at both sides. GPA-Os fixation. × 25000.

Fig. 63. Freeze-fractured, etched (−100 °C, 2 min) and rotary-replicated preparation of the ventral side of a respiratory appendage from purified endochorion. The ventral network resembles the roof network of the outer endochorion in the main body of the shell (see Fig. 31). It has 40-nm-thick fibres and 70–80-nm openings. Observe also the insertion of pillars (p), revealed by sublimation of ice. G-fixation. × 27000.

Fig. 64. SEM view of the dorsal side of a respiratory appendage from a laid egg. The surface features of the plaques (pl) are obscured in places by a fuzzy exochorionic layer, which also spans the gaps between plaques. × 5500.

Figs. 65, 66. Thin-sectioned stage 14 follicle. Both surfaces of the respiratory appendage, i.e. the ventral network (vn) and the plaques (pl) are covered by loosely fibrous exochorion (ex), which also enters the gaps between plaques. fc, follicle cells. GPA-Os fixation. × 15500.
DISCUSSION

Eggshell complexity and its origin

Differentiation has both a temporal and a spatial component. The eggshell represents an integrated manner the differentiated activities of the secretory epithelial cells, its complexity reflecting both temporal and spatial differences within and amongst these cells. In this regard 3 categories of complexity can be discerned.

Radial complexity (layers). As described in Results and summarized in Fig. 1, the main body of the eggshell consists of multiple, distinct layers and sublayers, progressively distant from the oocyte surface. Morphological analysis of developing follicles reveals that most of these structures are formed sequentially, although they may also undergo changes after deposition (King, 1970; Margaritis et al., in preparation). Thus, they largely reflect temporal chapters in the programme of differentiation of the secretory follicular cells.

Tangential complexity within cell imprints. This type of complexity has been detected as domains in the 'wax layer' and the ICL (patches and crystallites, respectively, measuring approximately 0.5 to 3 μm). Presumably the ICL crystallites are formed by self-assembly from a number of separate nucleation centres. Detailed developmental analysis of these 2 layers is necessary for evaluating their morphogenesis and possible relationship to each other.

The tangential complexity is obvious at all endochorionic levels. The periphery of each imprint, corresponding to the circumference of the overlying secretory cell, is marked by continuous fenestration of the floor, absence of pillars, and elaboration of
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the roof network into a ridge. The remainder of the imprint includes both solid and fenestrated regions in the floor, pillars and cavities at an intermediate level, and depressions alternating with domes in the roof, respectively. The most direct explanation for this type of complexity is that the secretory surface of each cell is locally specialized. However, developmental analysis is again necessary for critical evaluation of this hypothesis.

Regional differentiation of the eggshell. On a larger scale, almost all layers show differences between the main body of the eggshell and its specialized regions at the anterior and posterior poles. Thus, the vitelline membrane is specialized underneath the collar (Fig. 46), and the ICL appears to be absent within the micropylar cone (Fig. 50). The most prominent regional differences are found in the endochorion and involve all its components (floor, pillars, roof, roof network). Regional as well as radial complexities are summarized in Table 1. The regional complexity must reflect differences between corresponding subpopulations of follicular epithelial cells, as discussed later.

Functional significance of some eggshell layers and regional specializations

Wax layer. The wax layer is the least understood component of the Drosophila eggshell. Its existence was inferred from the observation that the oocyte is impermeable to water after ovulation, unless heated to 45 °C (King & Koch, 1963). Similarly,

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Fig. 71. SEM view of the posterior pole from a purified endochorion sample showing the specialized structure of 2 types of cell imprints, central (cc) and peripheral (pc), which are surrounded by ordinary main body cell imprints (mb). Note that the depressions, which reveal pillar insertions in the roof, are less abundant in the pc as compared to the mb cell imprints. (View B2 eq.). x 450.

Fig. 72. SEM view of the posterior pole at higher magnification. The central cell imprints (cc) are small and have very thin ridges. Their roof breaks easily. A circle outlines an underlying circular structure, interpreted as pillars (see Figs. 74, 75). The peripheral cell imprints (pc) are of more ordinary appearance, transitional to that of the main body cell imprints. (View B2 eq.). x 2200.

Fig. 73. SEM view of the inner endochorion of the posterior pole. The floor of the peripheral (pc) and central (cc) pole cell regions is considerably more open than the floor of the main body (mb) of the shell. The area corresponding to a single cell is outlined. (View C2 eq.). x 2200.

Figs. 74, 75. Phase-contrast views of the posterior pole of a hand-isolated endochorion from stage 14 follicle, at 2 different levels of focusing. Circles (arrows) in the central region (cc) consist of pillars and correspond to circular domains seen in the SEM views (Figs. 71, 72). Observe also that the scattered pillars of the peripheral (pc) imprints are less abundant than the pillars of the main body (mb) cell imprints. x 700.

Fig. 76. SEM view of the posterior pole from a laid egg. The surface features, including ridges of the endochorion have been obscured by exochorion, especially in the region of central pole cells (cc). However, the circular pillar domains are detectable as faint white circles (arrow), because during SEM imaging electrons in this region are strongly reflected, due to the underlying mass. (View B1 eq.). x 800.

Figs. 77, 78. SEM views of the surface from a laid egg showing 2 different degrees of coverage of the endochorionic roof network (arrow) by exochorionic material. The exochorion (0) can be removed rather easily by washing. (View B1 eq.). x 20000.
Fig. 79. Two-dimensional polyacrylamide gel analysis of purified *Drosophila* eggshell enriched in endochorion (Method II of Petri et al. 1976). The first dimension utilized a pH 3.5 to 11 ampholine gradient (left to right); the second (vertical) a 7 to 15 % linear gradient of acrylamide in a buffer containing urea and SDS (Yannoni & Petri, in preparation). Protein components were stained with Coomassie Brilliant Blue. Molecular weight standards (indicated by their Dalton values) and a sample of purified eggshell (labelled at the position of the 6 major chorion protein classes; Petri et al. 1976) were electrophoresed on the left side of the gel in the second dimension only. Eggshell components reproducibly resolved in 2-dimensional electrophoresis are indicated by arrows and those corresponding to the 6 major classes are identified. Unlabelled arrows indicate the 9 minor components which most routinely co-purify with eggshell. The position of a minor, low-molecular-weight, basic component which is difficult to photograph is indicated by the broken circle. Not all components are clearly resolved in this run.

Mahowald (1966) noted that ovulated eggs are impermeable to EM fixatives, even if dechorionated by hypochlorite treatment; they become permeable to fixatives after treatment with ether and other non-polar solvents. Hypochlorite-digested eggs are also impermeable to amino acids and nucleosides, but are rendered permeable by treatment with Triton X-100 (Sayles, Procunier & Browder, 1973) or octane (Limbourg & Zalokar, 1973). Although these observations do not establish the existence of a
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separate wax layer, as opposed to lipids impregnating the vitelline membrane, the idea of a separate wax layer is favoured by analogy with the insect cuticle (Wigglesworth, 1945; Beament, 1945), and because Davies (1947) demonstrated by abrasion experiments on blowfly eggs that waterproofing is due to materials at the surface of the vitelline membrane.

Table 1. Radial and regional complexity of the eggshell in Drosophila melanogaster

<table>
<thead>
<tr>
<th>Radial</th>
<th>Regional</th>
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<tbody>
<tr>
<td>Main body (mb)</td>
<td>Respiratory appendages (ra)</td>
</tr>
<tr>
<td><strong>Vitelline membrane (vm)</strong></td>
<td>Uniform layer 0.3 µm</td>
</tr>
<tr>
<td><strong>Wax layer (wax)</strong></td>
<td>Presumably hydrophobic plates</td>
</tr>
<tr>
<td><strong>Innermost chorionic layer (icl)</strong></td>
<td>Crystalline, 8-10 sub-layers 40 nm</td>
</tr>
<tr>
<td><strong>Inner endochorion (ie)</strong></td>
<td>Fenestrated, composed of fibres 40 nm</td>
</tr>
<tr>
<td><strong>Pillars (p)</strong></td>
<td>Solid but porous 0.5 µm</td>
</tr>
<tr>
<td><strong>Outer endochorion (oe)</strong></td>
<td>Solid but porous 0.2 µm</td>
</tr>
<tr>
<td><strong>Roof network (rn)</strong></td>
<td>Hexagonal-like 40-nm fibres</td>
</tr>
<tr>
<td><strong>Ridges of imprints</strong></td>
<td>Over-developed roof network 3 to 8-sided</td>
</tr>
<tr>
<td><strong>Exochorion (ex)</strong></td>
<td>Fibrous, very loose 0.3 µm</td>
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King (1964) and Quattrocchi & Anderson (1969) observed wispy material between the vitelline membrane and the ICL (see also our Fig. 2). These wisps may represent remnants of a more extensive layer which is largely extracted by conventional TEM procedures. Since most waxes have saturated aliphatic chains (Beament, 1955), they can be expected to remain unfixed by OsO₄ or KMnO₄, and to dissolve during
dehydration in ethanol or acetone. The best morphological evidence for an organized wax layer comes from freeze-fracturing (Fig. 7), which reveals smooth surfaces in a multilayer arrangement between vitelline membrane and ICL. Their smoothness suggests that they do not consist of proteins or other large molecules, and their tendency to permit longitudinal propagation of fracturing is suggestive of a hydrophobic nature (Branton, 1966). The existence, composition and morphogenesis of the wax layer should be further studied by cytochemical, freeze-fracturing and TEM studies using water-soluble resins to prevent extraction.

**Innermost chorionic layer.** The ICL is a polycrystalline layer, 40–50 nm thick and of unknown function. Its subunits have not only planar periodicity but also vertical alignment. We consider both the uranyl acetate overstaining (Fig. 15) and the lanthanum treatment (Figs. 11, 12) as negative staining; thus, the 10-nm periodicity observed by the 2 methods in all 3 dimensions indicates the existence of holes at the corners of a 10-nm cubic lattice. Because of the 5.0-nm spacing between lines in positively stained TEM preparations (Figs. 9, 10, 13) and in whole mounts lightly stained with uranyl acetate (Fig. 17), we infer that the ICL subunits might be packed 5 nm centre-to-centre as octamers within the 10-nm lattice. The actual shape and 3-dimensional arrangement of the subunits remain to be established by low-dose electron diffraction (Unwin & Henderson, 1975) and X-ray analysis of mass isolated ICL.

Our observations on the ICL (see also Margaritis et al. 1979) are the first evidence for the existence of crystalline chorion layers in Diptera. However, crystalline layers have been reported in 5 other insect orders (Orthoptera, Odonata, Neuroptera, Hymenoptera and Coleoptera; Furneaux & MacKay, 1972, 1976), with a cross-sectional appearance similar to that of the ICL, but considerably greater thickness: 350 nm in the beetle *Lyttta nuttali* (Sweeney, Church, Rempell & Frith, 1976), 1 μm in the Colorado beetle (deLoof, 1971), 800 nm in the cricket *Acheta domestica* (Furneaux, James & Potter, 1969), and 300 nm in the hymenopteran *Cardiochiles nigriceps* (Vinson & Scott, 1974). In non-Dipterous insects, the crystalline layer appears to be proteinaceous (Furneaux & MacKay, 1972). Similarly, we have observed that the *Drosophila* ICL is unaffected by ether but rapidly dissolves in hypochlorite, as does the proteinaceous endochorion (Margaritis & Lombard, unpublished observations). However, a difference in composition between ICL and endochorion is suggested by their differing reactions to KMnO₄ fixation (Fig. 39).

**Endochorion.** During choriogenesis the endochorionic cavities are filled with a loose, La³⁺-binding material, which disappears later, perhaps drying against the surfaces with which it is in contact and imparting to them their characteristic affinity for lanthanum. This material may consist of polysaccharides or glycoproteins, the anionic sites of which are known to have special affinity for La³⁺, La(OH)⁵⁺ and La(OH)₂⁺ (Boyd, Melnykovych & Fiskin, 1976; Macken, Eriji & Wooding, 1972). However, it should be noted that La³⁺ is also a probe for calcium-binding sites (Darnall & Birnbaum, 1970); Ca²⁺ is reported to account for 0.7% of the eggshell dry weight (Wilson, 1959). We presume that this loose material serves in part as a scaffold during construction of the endochorionic cavities.
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In laid eggs the cavities are thought to be air-filled (Hinton, 1959), undoubtedly facilitating respiration (see also Wigglesworth & Salpeter, 1962). The fenestrations of the floor of the main body, the endochorion of the operculum, and the floor and roof of the posterior pole may also have a respiratory function. The endochorionic cavities are presumed to remain air-filled even when the egg is submerged in water. In that case, the cavities would function in conjunction with the plastron (Hinton, 1959, 1968, 1969, 1970) of the respiratory appendages. Air trapped among the network of pillars within the appendages would be able to exchange gases easily with the surrounding water, through the extensive surface area between plaques (Figs. 59, 60) and possibly through the pores of the plaques themselves (Figs. 60, 61, 70) and the pores of the ventral surface network (Figs. 62, 63). The posterior pole might also serve as a secondary plastron, since its tall pillars create large air spaces (King & Koch, 1963), and its holey roof may tear easily, creating large holes (Figs. 19, 40, 72; Kluge et al. 1974). Gases would diffuse between the plastron(s) and endochorionic cavities of the main body, which are in close contact with the embryo.

Under dry conditions, the holes of the operculum and posterior pole (Figs. 44, 47) might be quite important for respiration, since gas diffusion from the appendages must be relatively limited by their narrow cross-sectional connection to the main shell.

The operculum also serves as a trap door for hatching of the larva through a break (Turner & Mahowald, 1976), which is presaged by the unusual construction of the endochorionic collar.

Structure-function analysis of eggshell constituents

With a detailed knowledge of the eggshell structure in wild-type Drosophila melanogaster, it is now possible to undertake a structure-function analysis of its components, using a combined morphological, biochemical, genetic and phylogenetic approach. This analysis should help identify with specific structures and functions the various chorion proteins (Fig. 79) and other constituents. Favourable material for such an analysis are the numerous available chorion mutants (Gans et al. 1975; Mohler, 1977), as well as the Hawaiian Drosophila species which occupy diverse ecological niches (Carson, Hardy, Spieth & Stone, 1970). Most of the methods used in this report such as whole mount TEM and SEM of isolated endochorion permit rapid screening of structural variants.

The shell preparations, which according to 2-dimensional gel electrophoresis contain 19 or more proteins, are highly enriched in endochorion. If all these proteins are in fact constituents of the endochorion, their sequential production over a 5-6 h developmental period (Petri et al. 1976) seems puzzling, since freeze-fracturing, TEM and SEM suggest that all parts of the endochorion are similar in substructure and presumably in composition. This apparent paradox must be resolved by a careful correlation between the changing pattern of chorion protein synthesis and the progressive formation of endochorionic sublayers and regional specializations. Ultimately, monospecific antibodies against individual eggshell proteins must be used for localization experiments.
Fig. 8o. Tentative categorization of follicular epithelial cells in Drosophila. Categories of cells are shown by boxes, their origins by lines, and the type of eggshell structure they produce by thick arrows. In parentheses are indicated the approximate number of cells in each category (estimated either from our examination of the mature chorion, or from the work of King and collaborators; see text for references). The number of columnar follicular cells that form the main shell, operculum and posterior pole areas are derived from unpublished (Petri & Margaritis) counts of the total number of cell imprints per mature shell. The processes and stages of development during which the cell categories become evident are also indicated in parentheses. Lack of information is shown by question marks or by dashed lines. For example, we are not certain whether all cells which intercalate between nurse cells and oocyte at stages 10–11 develop into anterior pole cells, and vice versa whether all anterior pole cells originate from that migration. We are also not certain how the posterior pole cells are derived (directly from the columnar cells, or from the main body subpopulation). The categorization of respiratory appendage cells into ventral and dorsal groups is based on unpublished observations of Galanopoulos & Margaritis; the exact roles of these 2 groups are not fully established yet (dashed arrows). It is not known whether the border cells might produce any type i imprints, in addition to the micropylar cone.
Regional differentiation of follicular cell populations

Our best estimate is that the mature shell is formed by approximately 1000 cells. The regional differentiations of the eggshell, some of which have also been documented by developmental studies (King & Vanoucek, 1960; King, 1970), reveal the existence of distinct subpopulations of cells (Fig. 80).

According to the developmental studies, when mitoses end at stage 6 of oogenesis, approximately 1000–1200 epithelial cells are found in each follicle (King & Vanoucek, 1960). At stages 8–9 most of these migrate posteriorly and come to form a columnar monolayer over the oocyte, while a few cells (probably 50–100 according to the data and diagrams of King & Vanoucek, 1960, and Cummings & King, 1969) remain behind and stretch into a thin squamous covering of the nurse cells. During stages 9 and 10, a group of 6–10 ‘border’ cells, initially at the extreme anterior pole, migrate between the nurse cells and come to lie next to the anterior dorsal part of the oocyte, where they eventually form the micropylar apparatus (King & Koch, 1963; Cummings & King, 1969).

Toward the end of stage 10, approximately 300 cells from the main, columnar population (estimated from the data of King & Vanoucek, 1960, as reinterpreted by King & Koch, 1963) migrate anew and intercalate between the nurse cells and the anterior end of the oocyte, which were previously in direct contact. Of these anterior pole cells, 180–240 (King & Koch, 1963) later aggregate to form the 2 dorsal appendages (stages 11 and subsequently), while the rest apparently form the operculum. (Our own preliminary estimates suggest a smaller number, 30–50 for each appendage.)

Even the cells which remain in situ at stages 10–11 and cover the main body of the oocyte are not uniform. At stage 10, the cells along the dorsal aspect are taller than those along the ventral aspect (King & Koch, 1963; see also diagrams in Cummings & King, 1969). As revealed by their imprints in the mature eggshell, the dorsal cells also tend to be elongated in the anterior-posterior axis (Fig. 40), in contrast to the ventral cells (Fig. 41). It should be noted, parenthetically, that a dorso-ventral polarity also exists at that stage within the oocyte (the germinal vesicle is located anteriadorsally) and is ultimately manifested during gastrulation (Turner & Mahowald, 1977).

The cell imprints in a mature chorion confirm and extend this categorization. The posterior pole cells appear to belong to 2 different populations (peripheral and central), while the cells of the operculum belong to as many as three (I, II, III). The cells which produce the appendages would also seem to belong to 2 distinct populations (ventral and dorsal), judging by the 2 distinct surfaces in each mature appendage. The cell categories first revealed by the morphology of the mature shell should now be studied developmentally.

In summary (Fig. 80), the eggshell appears to be constructed by as many as 10 different populations of follicular epithelial cells. Although some of them may belong to a continuum (dorsal and ventral main body cells; the 3 categories of operculum cells; main body and posterior pole cells), these categories do correspond to structural differences, and should prove useful in interpreting the defects observed in eggshell mutants, as well as the physiology of follicle development.
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