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Cytodifferentiation in the Accessory Glands of *Tenebrio molitor.* XI. Transitional Cell Types During Establishment of Pattern

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ABSTRACT The bean-shaped accessory glands of male *Tenebrio* consist of a single-layered epithelium which is surrounded by a muscular coat. The epithelial layer, which produces precursors of the wall of the spermatophore, contains eight secretory cell types. Each secretory cell type is in one or more homogenous patches, and discharges granules which form one layer of the eight-layered secretory plug. Maturation begins in cell types 4, 7, and 6 on the last pupal day. A newly identified cell (type 8) in the posterolateral epithelium matures last. Cells of individual types mature in synchrony, and their secretory granules "ripen" in a sequence that is characteristic for each type. As the secretory cells of each patch mature, unusual short-lived cells appear at interfaces between patches. In some cases the secretory granules in these boundary cells have ultrastructural features which are mixtures of the definitive characteristics of granules in adjacent cell types. The transitional cell types disappear at 3–4 days after eclosion. Intermediate cell types are absent in the mature gland and boundaries between the patches are distinct. The transitional cells may form granules of intermediate structural characteristics as a dual response to cellular interaction with adjacent and previously differentiated secretory cells.

Insect reproductive accessory glands contain many secretory cell types and produce heterogenous secretory products (Leopold, '76). In mealworm beetles, some of these products form the spermatophore, an elongate sac which encloses the sperm during transit from male to female (Gadzama and Happ, '74; Dailey et al., '80). The wall of the spermatophore is formed by a molding of the viscous secretory mass in the upper ejaculatory duct. This viscous mass is largely derived from two secretory "plugs" produced by a bilateral pair of bean-shaped accessory glands (BAGs). Each plug comprises eight distinct layers, and each layer can be traced to a particular type of secretory cell. Collectively, the cells comprise the simple secretory epithelium of the gland. The various cell types are not scattered at random in the epithelium but occur together in discrete patches and occupy specific regions of the adult gland (Dailey et al., '80). Each patch contains only one of the eight cell types. Cell types in adjacent patches differ in the morphology of their secretory granules (Dailey et al., '80).

The maturation of the BAGs takes place over a period of less than three weeks. The glands arise from a mesodermal pouch in the last instar larva and primary organogenesis is completed before pupal ecdysis (Huet, '66). Cell proliferation and organ growth take place in the pupa, but the definitive cell types are difficult to recognize at that stage (Grimes and Happ, '80). By 5 days after adult ecdysis, all eight cell types are packed with secretory granules, and spermatophores are produced on a regular basis. We are interested in the hormonal control of this developmental sequence. In the present paper, we present a morphological chronicle of the process of terminal differentiation in the secretory cells of the BAG. This paper is particularly concerned with the maturation of each type of secretory granule, with the ontogenetic synchrony among the eight cell types, and with the fates of cells which lie at the interfaces between patches.

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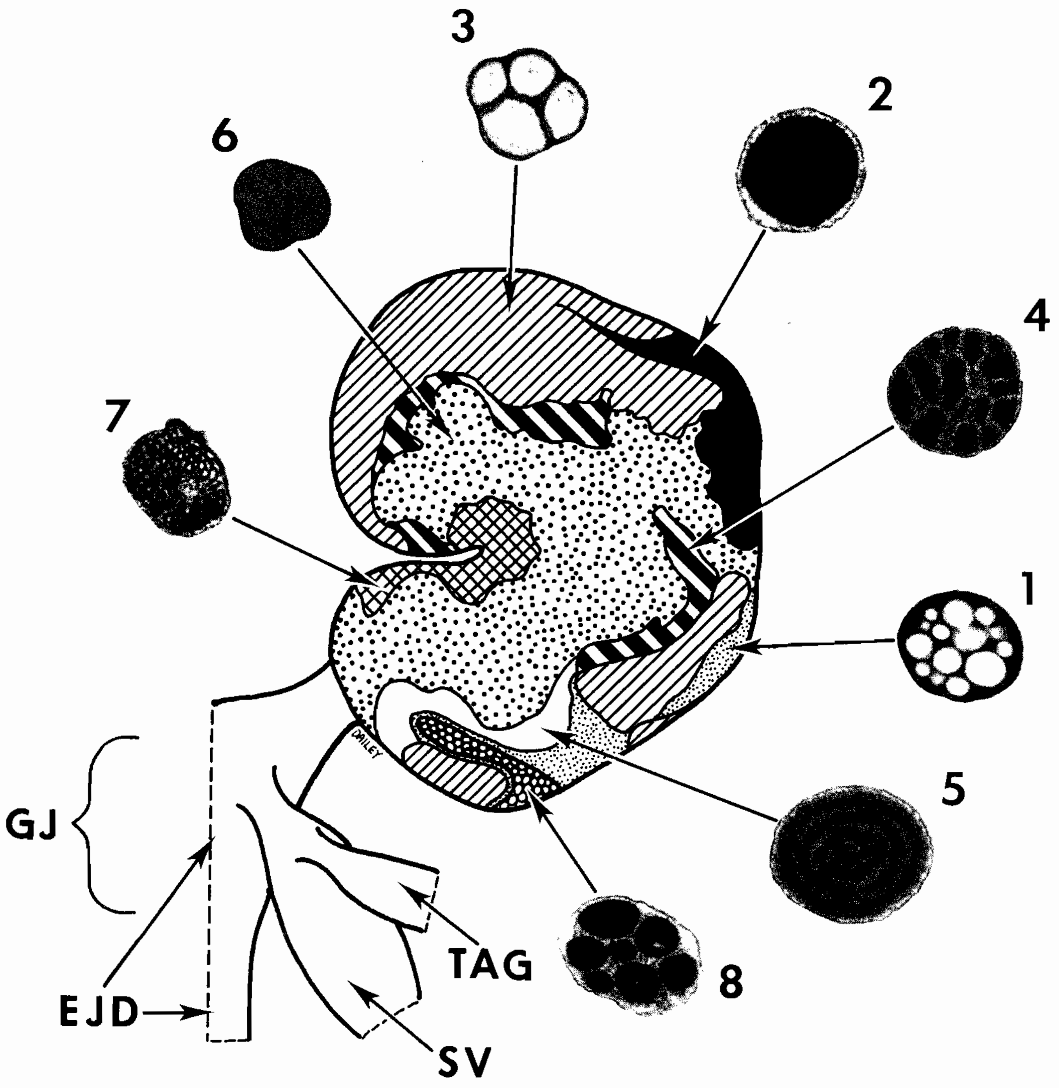
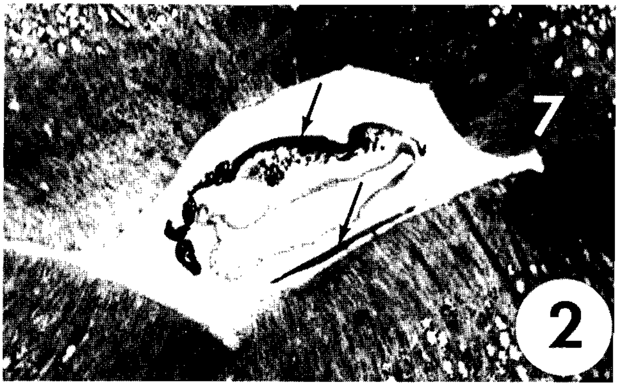


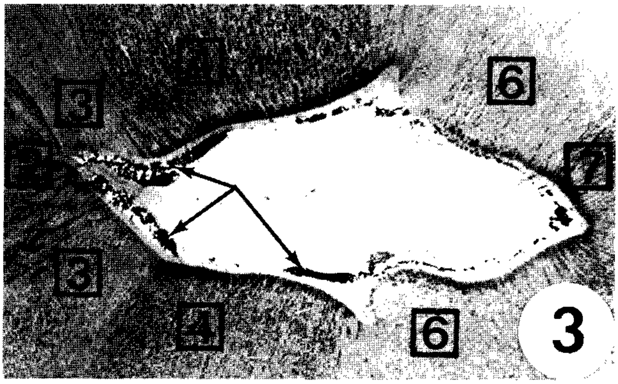
Fig. 1. Dorsal view of the right bean-shaped accessory gland (BAG). Cell regions are designated by number and adjacent electron micrographs depict the common granule type synthesized by cells of a particular region. The products of this gland pass into the grand junction (GJ) where they mix with those of the seminal vesicle (SV) and the tubular accessory gland (TAG) before flowing into the ejaculatory duct (EJD). (For further clarification of cell regions see Dailey et al., '80).

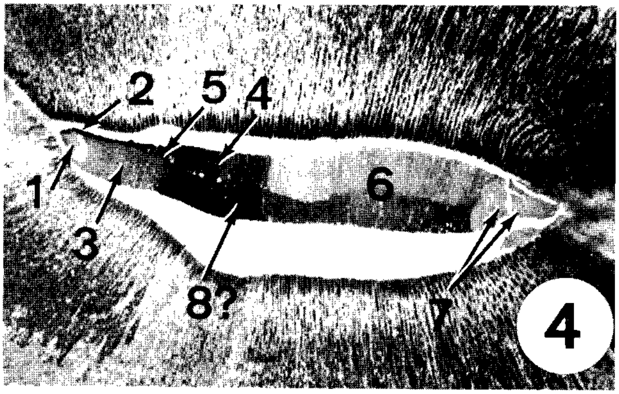
MATERIALS AND METHODS

Tenebrio molitor L. (Coleoptera, Tenebrionidae) were obtained from laboratory cultures, maintained on Purina Chick Startena supplemented with potato and carrot. Pupae were collected, sexed, and placed in an incubator at 26°C. In this study we used only 9-day pupae and the young adults. The developmental age of 9-day pupae was confirmed by examination of external morphological characteristics (Delbecque et al., '78).

After emergence from their pupal cuticles, adults were easily distinguished by their soft, white cuticle. Day and time of emergence were recorded, and the animals were placed in the 26°C incubator. At 6-hr intervals thereafter, adults were retrieved and bean-shaped accessory glands were removed by dissection in phosphate buffered saline. Prep-





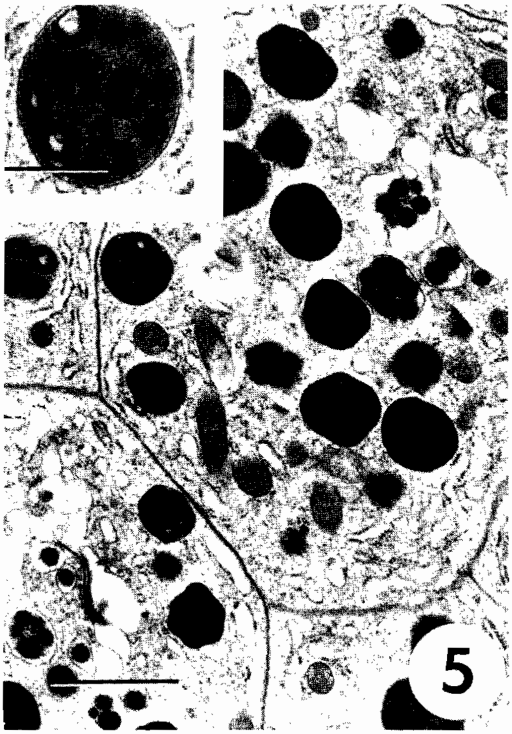


Figs. 2–4. Pupal and adult BAGs; epon thick sections (1 μm) stained with toluidine blue. All micrographs are oriented with the region occupied by cell type 7 at the right.

Fig. 2. Nine-day pupa. Arrows indicate the pupal secretory mass. Cell type 7 region numbered at right. ×660.

Fig. 3. Adult, 42 hr posteclosion. Arrows indicate secreted products of debris. Regions occupied by identified cell types are numbered. ×640.

Fig. 4. Adult, 78 hr posteclosion. Numbers indicate the secretory products of each cell type in the plug. The products indicated by "8?" appear to be the products of cell type 8. ×550.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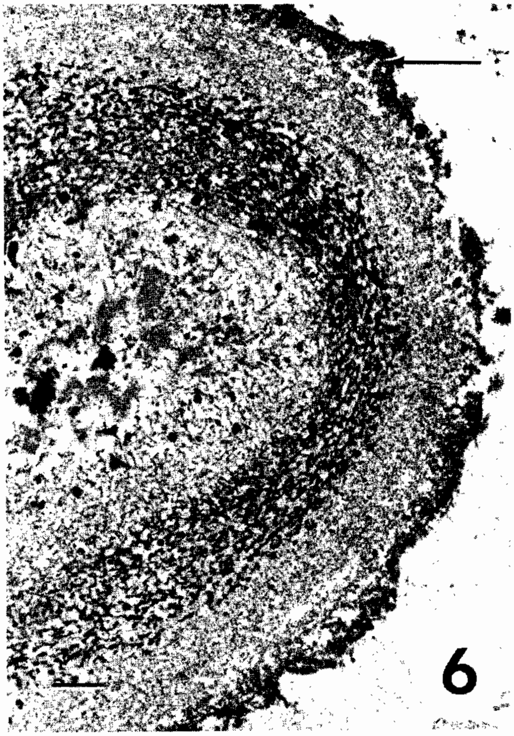
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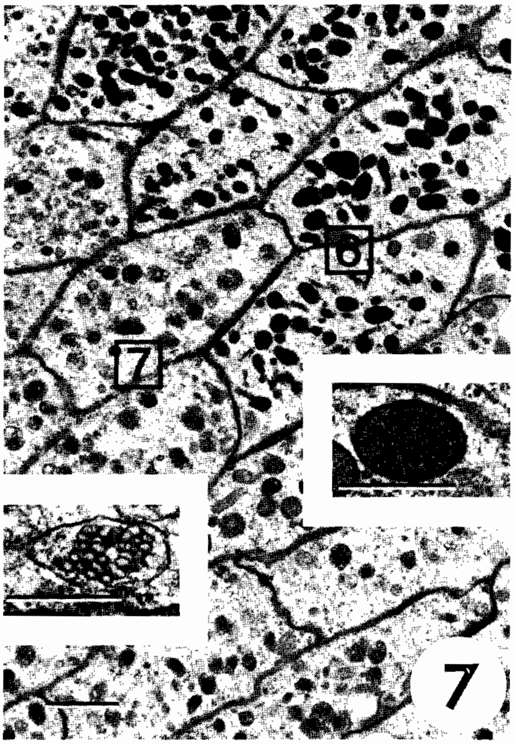
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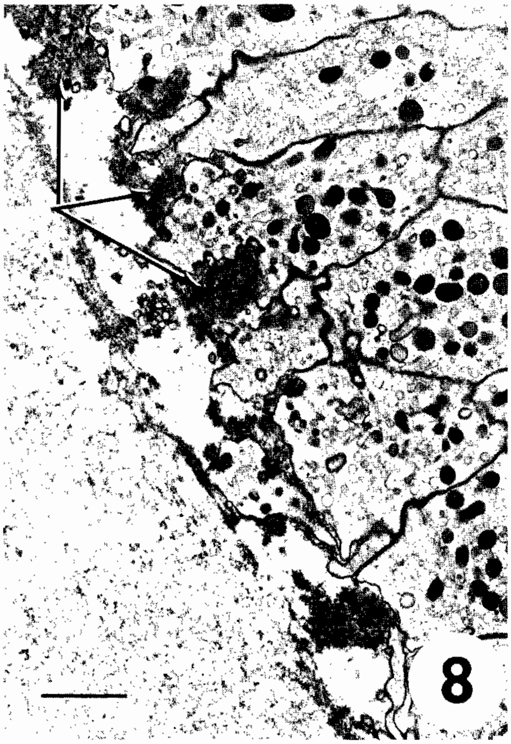
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aration of glands for electron microscopy followed procedures outlined by Dailey, Gadzama, and Happ ('80). Briefly, these included fixation in 3% glutaraldehyde for 1–3 hr, postfixation in phosphate-buffered osmium tetroxide for 1 hr, and embedding in Epon 812. Sections, cut with glass or diamond knives on a Reichert ultramicrotome, were stained with uranyl acetate and lead citrate, and were examined in a Philips EM200 electron microscope.

OBSERVATIONS AND RESULTS

In our previous study (Dailey et al., '80), we described seven distinct secretory cell types which contribute morphologically discrete products to the plug of the BAG. In tracing the ontogeny of each of these cell types in this study, we discovered another previously undetected cell type, herein designated type 8.

Each of the secretory cell types is distinguished by the morphology of its membrane-bound secretory granules (Fig. 1). Cell type 8 was previously misidentified as anomalously staining patch of type 1 cells (cf. Figs. 7 [asterisk] and 39 of Dailey et al., '80). Like other secretory cells of the accessory gland, type 8 cells are columnar, contain an ovoid nucleus just basal to their midpoints, and contain large numbers of secretory granules at their apices. Each type 8 granule is bounded by a unit membrane which surrounds a finely reticulate inner matrix. Within the matrix are two to seven ovoid or spheroid electron-dense bodies (see Fig. 5). These secretory granules have superficial similarities to the faceted granules of type 4 cells; however, the two types differ in that the inner dense regions of type 8 granules (see Fig. 5) are rounded, whereas those of type 4 are angular in cross section (see Fig. 9).

Maturation of the secretory epithelium

Granule morphologies were used as the criteria for maturation. The various secretory cell types do not mature in synchrony. In general, the first to mature are those in the inner region of the gland (type 4 followed by types 7 and 6), whereas the last to mature (type 8) occupy the posterolateral region. In all cell types, the granules collect in the apical third of the tall columnar cells before release to the lumen.

An irregular mass of fibrous and granular materials (Figs. 2, 6) accumulates in the lumen during pupal development of both the BAG (Grimes and Happ, '80) and the TAG (tubular accessory gland) (Happ and Happ, '82). Although some of these masses appear ordered into layers (Fig. 6), in most glands the lumen contains a disorganized mixture of products.

During the ninth pupal day and for 1–2 days after adult ecdysis, some definitive secretory products are passed into the lumen. At pupal day 9, the type 6 cells contain significant amounts of rough endoplasmic reticulum in their basal regions and active Golgi zones just basal to the nuclei. The simple secretory granules, characteristic of these cells, collect in the apical zone (Fig. 7). Secretory masses present in the lumen at this time bear close resemblance to cell type 6 secretory masses observed in mature adult secretory plugs (Fig. 8; cf. Dailey et al., '80).

A fine electron-dense feltwork, perhaps derived from the type 6 secretory product, coats the mass of debris in the lumen during the last pupal day (Fig. 6). At that time, secretory granules are observed in cell types 4 and 7. Most of these granules appear immature when compared to those in glands of animals which are 8 days or more past ecdysis. In cell type 4, the granule matrix first appears dense and homogeneous. Observation of glands in older animals suggests that the matrix is then successively divided into subcompartments to yield the faceted mature granules. About one-fifth of the type 4 granules show facets at 9 pupal days (Fig. 9). In cell type 7, the early granules appear frothy, as if filled with small spherical vesicles. The frothy granules accumulate at the apices of the cells

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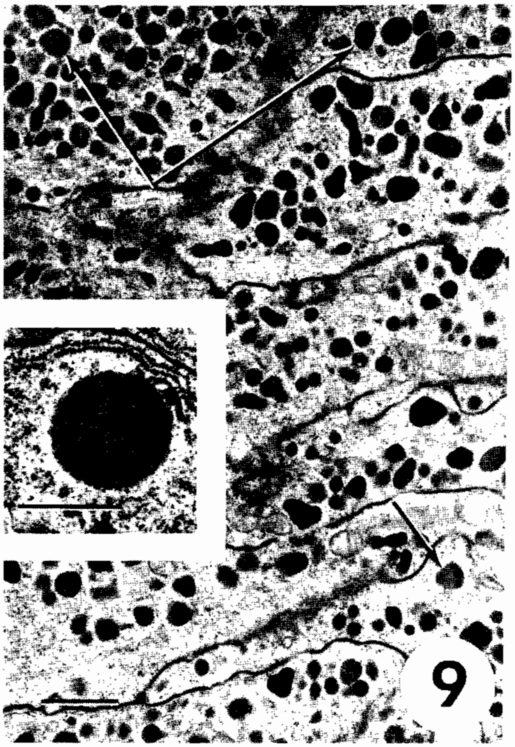
Fig. 5. Section through the midregion of type 8 cells. Inset illustrates rounded inner dense regions of a type 8 secretory granule. Adult 8 days posteclosion. Bar = 1 μm; inset bar = 0.5 μm.

Fig. 6. Mass of fibers and granules which occupy the lumen of the BAG in pupae and young adults. Arrow indicates fine granular material similar to cell type 6 secretory products observed in mature adult glands (see Dailey et al., '80). Adult, 1 day posteclosion. Bar = 1 μm.

Fig. 7. Border between patches of type 6 and type 7 cells. Insets depict high magnification micrographs of typical cell type 6 (inset, right) and 7 (inset, left) granules. Nine-day pupa. Bar = 1 μm; inset bars = 0.5 μm.

Fig. 8. Section of type 6 cells in 9-day pupa. Arrows indicate probable masses formed by cell type 6 secretory granules, the contents of which coalesce upon release into the gland lumen. For comparison of these secretory masses to the adult cell type 6 product refer to Dailey et al. ('80). Bar = 1 μm.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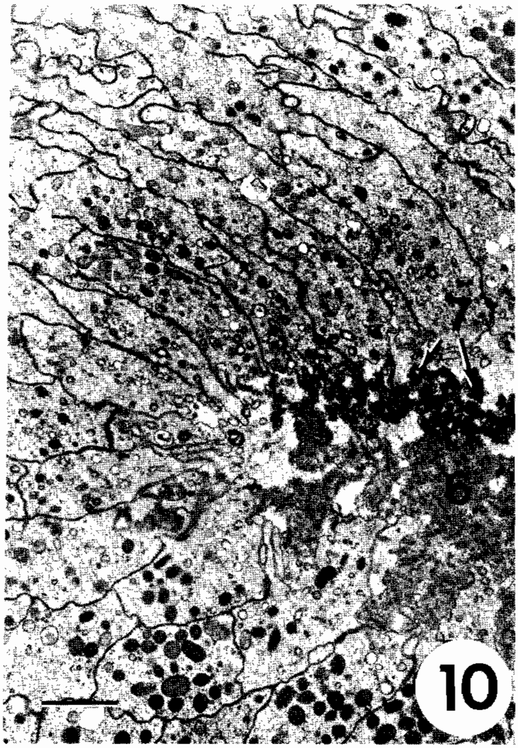
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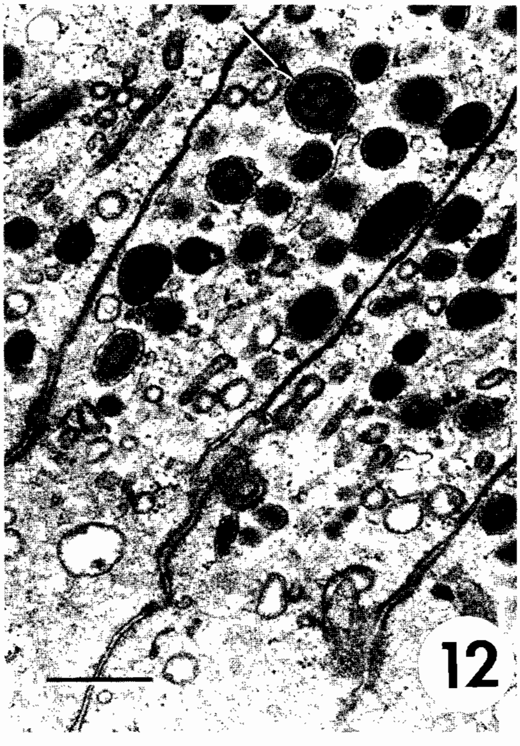
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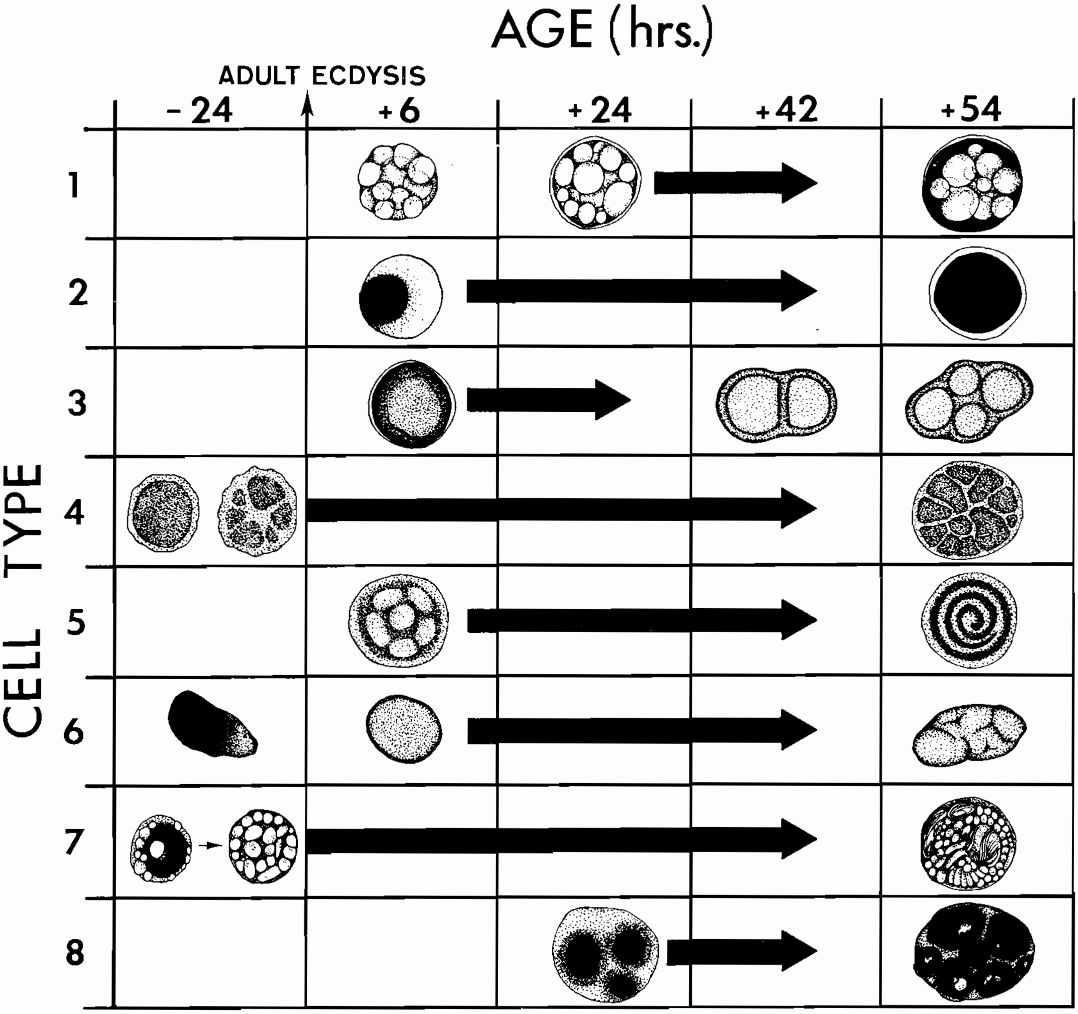


Fig. 13. The appearance of the most common secretory granules in each of the cell types during the period from 9-day pupa to 3-day adult.

Fig. 9. Cell type 4 with some mature faceted granules (arrows). Faceted appearance of granule matrix is shown in inset at left. Nine-day pupa. Bar = 1 μm; inset bar = 0.5 μm.

Fig. 10. Cell types 6 and 7 with apical secretions in the lumen. Secretions of type 6 form a homogeneous grainy matrix. The dense type 7 secretions are aggregated. Six hours after eclosion. Bar = 1 μm.

Fig. 11. Cells of type 6 at their midregion. Note inflated cisternae of the endoplasmic reticulum and the Golgi zones (G). Small arrows indicate secretory granules. Six hours after eclosion. Bar = 1 μm.

Fig. 12. Cell type 5 concentric granules (arrow) near apex of cell. Apical surface (not shown) at the lower left. Six hours. Bar = 1 μm.

and some are secreted into the lumen one day before ecdysis. As in the mature gland, the secretions of types 6 and 7 do not mix (Fig. 10).

The endoplasmic reticulum becomes more extensive and Golgi zones become larger in all cell types in the three days after ecdysis (Fig. 11). The changes which occur in the granules are summarized in Figure 13. At 6 hr after ecdysis, secretory granules are quite common in the first seven types of cells but are rare in type 8. However, granules in cell types 1–7 do not yet show the definitive adult morphology. Those in type 1 are somewhat

Fig. 14. Cell type 3 with several secretory granules. Endoplasmic reticulum is relatively sparse. Although studded with ribosomes shortly after eclosion, the endoplasmic reticulum of the mature adult is mostly smooth in outline. Six hours posteclosion. Bar = 1 μm.

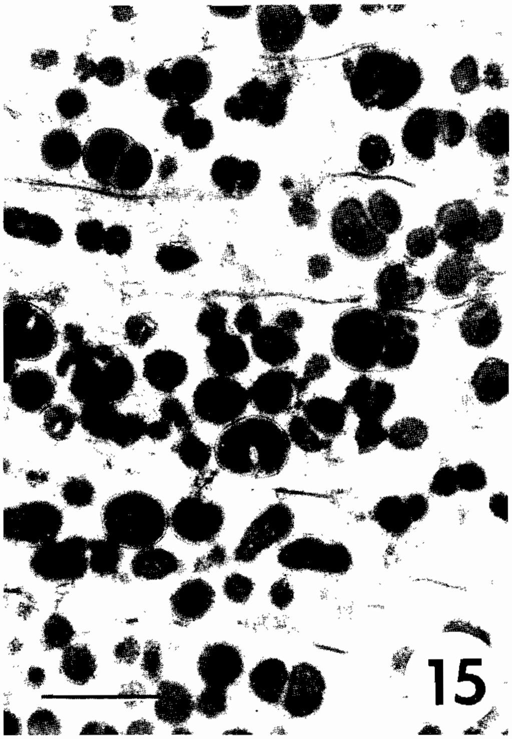


Fig. 15. Simple and bilobed granules in type 3 cells. Electron-lucent cytoplasmic matrix is characteristic of the apical region where granules accumulate prior to release. One day posteclosion. Bar = 1 μm.

multivesiculate, but lack the prominent electron-dense matrix (Fig. 13). At this age, granules in type 5 cells usually do not show the characteristic spirals seen in mature adults (Fig. 12).

In two respects, cell type 3 is unusual: its cisternal endoplasmic reticulum is *smooth* rather than ribosome-studded, and its multilobed secretory granules have a complicated peripheral structure (Dailey et al., '80). At 6 hr after eclosion, the type 3 cells still contain mostly rough endoplasmic reticulum. Those few secretory granules present are simple or, rarely, bilobed (Fig. 14). By 36 hr, smooth endoplasmic reticulum predominates and simple or bilobed thick-walled granules have formed (Fig. 15). By 52 hr, all cells have mature multilobate granules (Fig. 13).

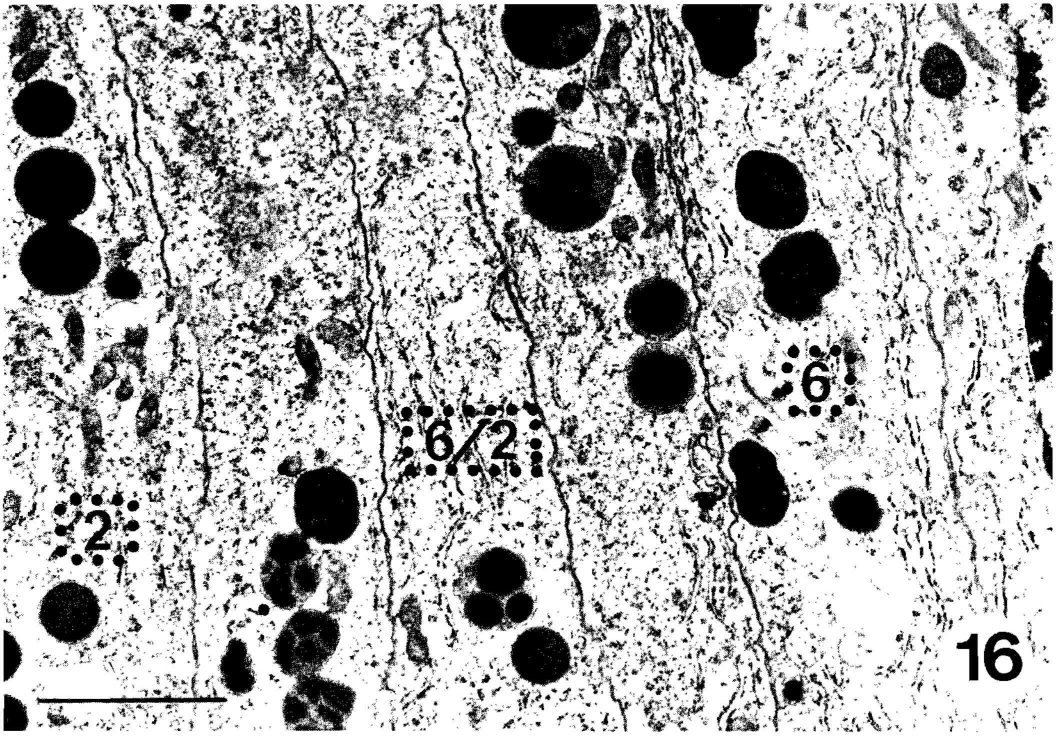
During the first two adult days, secretions gradually accumulate in the lumen around the debris and other materials which have persisted since the pupal stage. Two to three days after eclosion, the pupal debris and adhering secretory materials (Fig. 3) are discharged from the gland lumen. The cell types then lay down the eight layers which form the secretory plug (see Fig. 4).

Transitional cell types

In mature glands 4 days or more after eclosion, we very rarely find any cell which cannot be unambiguously identified as a particular cell type (1, 2, 3, etc.). The cells at boundaries between patches are either one type or the other. But in the 2–3-day adults, we observed a thin layer (3–6 cells) of transi-

Fig. 16. Transitional cell type 6/2 between type 2 (left) and type 6 (right). Transitional cells occupy a region three to four cell layers thick. Fifty-four hours posteclosion. Bar = 1 μm.

Fig. 17. Transitional cell type 2/3 between type 2 (left) and type 3 (right). These cells occupy a region six to seven cells thick. Fifty-four hours posteclosion. Bar = 1 μm.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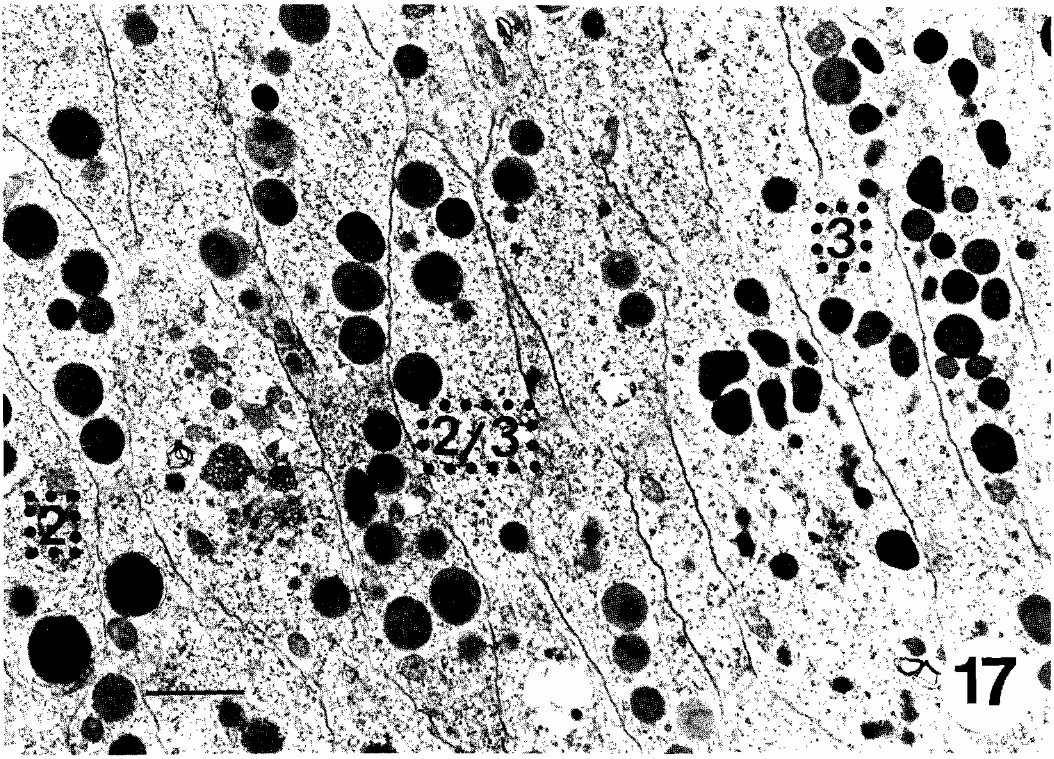
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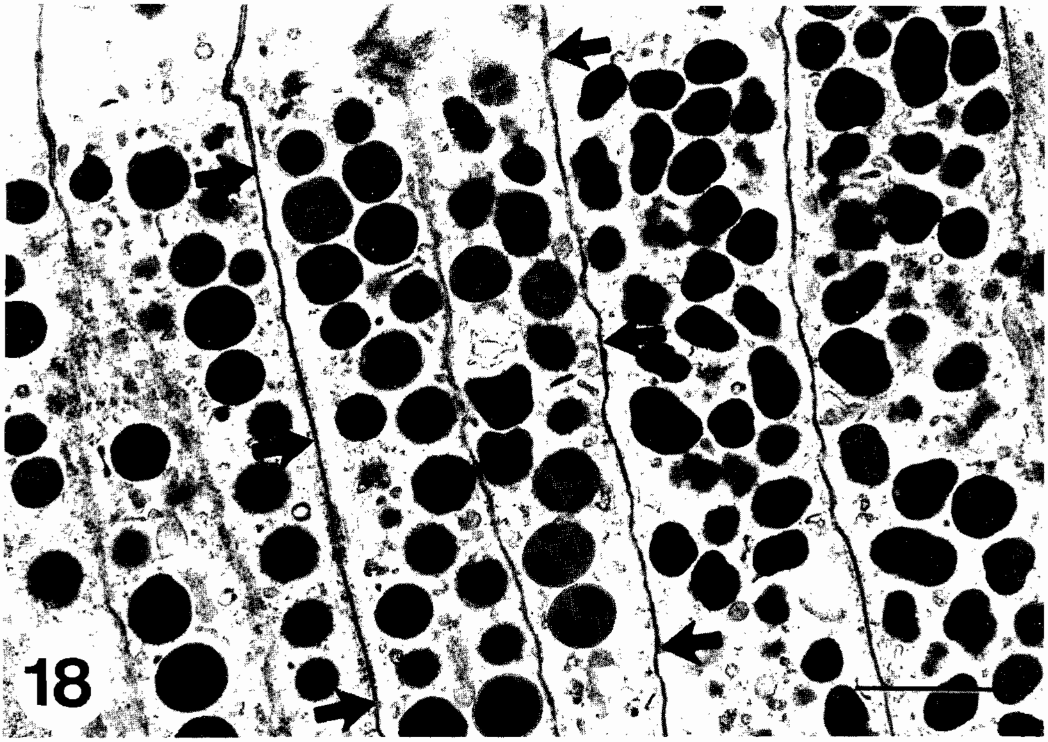

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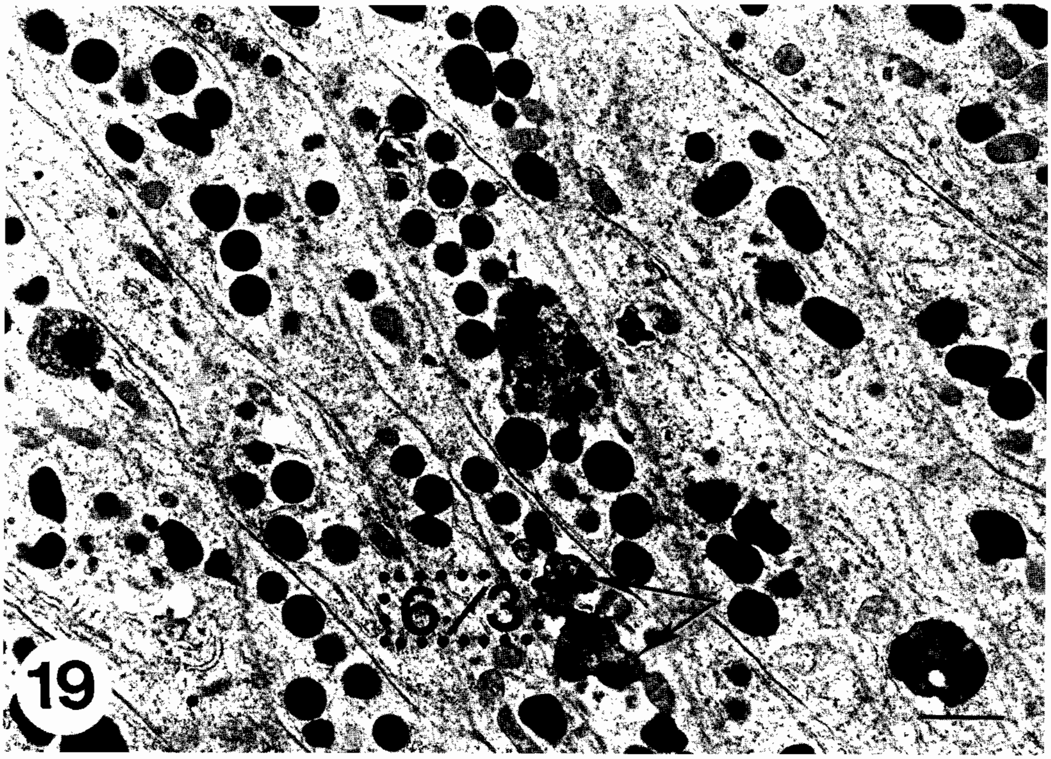

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tional cells at the boundaries between definitive cell types (Figs. 16, 17).

Secretory granules of these transitional types are of three classes: (1) similar to those of an adjacent definitive cell (transitional types 1/6, 1/8, 2/3, 6/7, and 6/8); (2) bearing limited resemblance to those of the definitive cells (transitional types 6/2 and 6/3); and (3) morphologically distinct forms (transitional types 1/3, 1/5). (Typical granules of transitional cells are compared with those of the definitve types in Figs. 22–24.)

Three examples of our classification scheme are presented. The granules of transitional type 2/3 are somewhat like those of the adjacent type 2 cells in having an electron-dense inner spherical structure. However, the lightly staining zone is wider and contains a finely particulate matrix in the transitional cell (Figs. 17, 23). Granules of transitional type 6/2 are heterogeneous, but some bear limited resemblance to those of type 2. In contrast to the granules of type 2 which have a single electron-dense spherical inclusion, those of transitional type 6/2 have several inclusions, some rounded and others angular (Figs. 16, 24). Transitional cell type 1/5 contains distinct granules which are unlike those of either cell types 1 or 5. Granules of both types 1 and 5 contain spherical electron-transparent zones, whereas in the transitional cells the electron-dense inclusions are spherical and are surrounded by a cell-dense matrix (Fig. 22).

The granules within these transitional cell types accumulate at the apices of the cells and may be secreted into the lumen in concert with definitive cell granules (Fig. 18). However, the transitional types are short-lived and so few in number that the products apparently do not form a significant part of the plug. No transitional cells were observed between type 4 and adjacent cells and any others. This may be related to the fact that cell type 4 forms an irregular band that generally isolates the faster-maturing inner cells (types 4, 6, 7) from the outer cells (types 1–3, 5, 8), whose maturation is delayed compared with the inner cells.

The transitional cell types do not persist for long. Some may be eliminated by cell death. However, we failed to observe significant numbers of dead and dying cells at the boundaries, although necrotic cells were occasionally detected (Fig. 21). Autophagic vacuoles, such as have been described by Locke ('81) were quite common in these cells at 2–4 days after eclosion (Figs. 19, 20), and it may be that many cells of transitional type undergo reorganization and then become committed to develop into one of the adjacent types.

With the disappearance of the transitional forms, the gland has completed its qualitative maturation. The gradual growth in volume continues until the eighth day. Except for an increase in the numbers of stored granules and the gradual accumulation of the complex aggregation of granules seen in the cells of aging beetles (Dailey et al., '80), no further changes of significance take place as the cells continue to secrete their products during the reproductive life of the male.

DISCUSSION

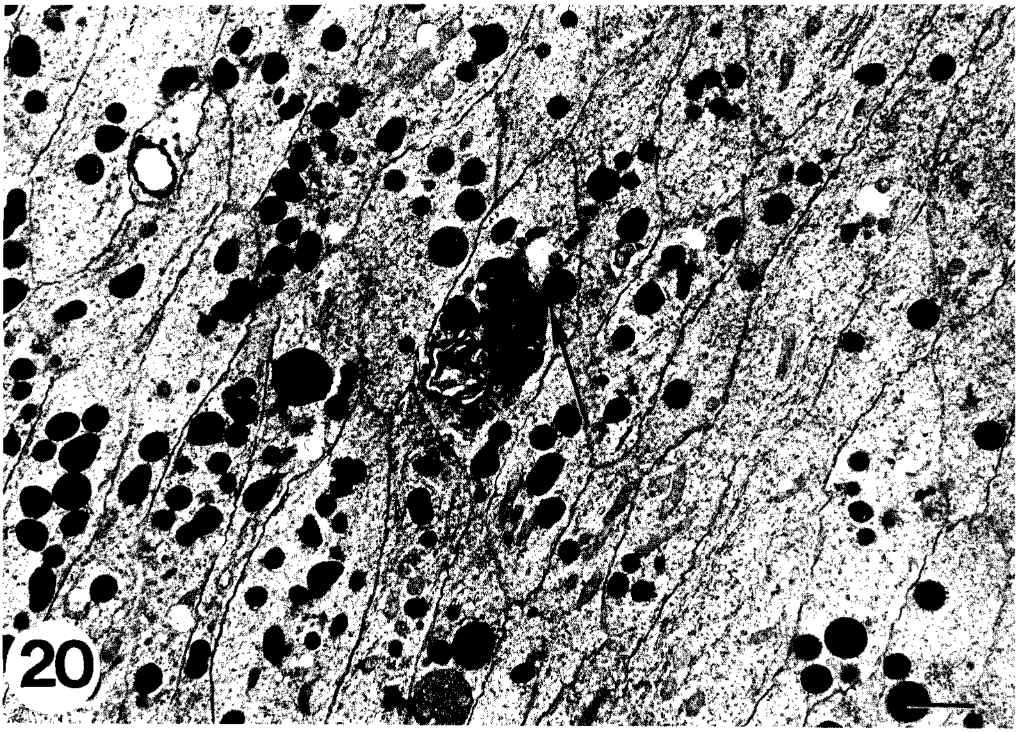
Maturation of the cell types

The major phase of cellular differentiation of the BAG takes place during a 4-day period beginning at, or just prior to, ecdysis. The morphological observations tell us little of the biochemical nature of the secretory products of each cell type — we know only what is rendered electron-dense with osmium, lead, and uranium. However, the time course of morphological maturation is correlated with a changing pattern of radioactive leucine incorporated into proteins resolved by two-dimensional polyacrylamide gel electrophoresis (Happ et al., '82). Of the adult-specific proteins, only a few are seen before ecdysis. Most adult proteins are detected at 2 days, and all are prominent at 4 days after eclosion. In parallel, the major pupal proteins (such as actin) have faded significantly by 8 days after eclosion. Thus, biochemical and morphological evidence reflects the rapid differentiation completed by 3–4 days after eclosion when the first spermatophore is formed.

Each cell type of BAG produces precursor granules having simpler morphology than their adult forms. By 54 hr after ecdysis, mature granules predominate. The complex

Fig. 18. Apical ends of type 6/2 transitional cells (cells between arrows). Lumen is at lower left. These cells probably release their products into the gland lumen in concert with secretory products of type 6 cells (right) and type 2 cells (left). Fifty-four hours posteclosion. Bar = 1 μm.

Fig. 19. Two type 6/3 transitional cells undergoing the early stages of autolysis. Arrows indicate autophagic vacuoles which were observed only in transitional cells. Fifty-four hours posteclosion. Bar = 1 μm.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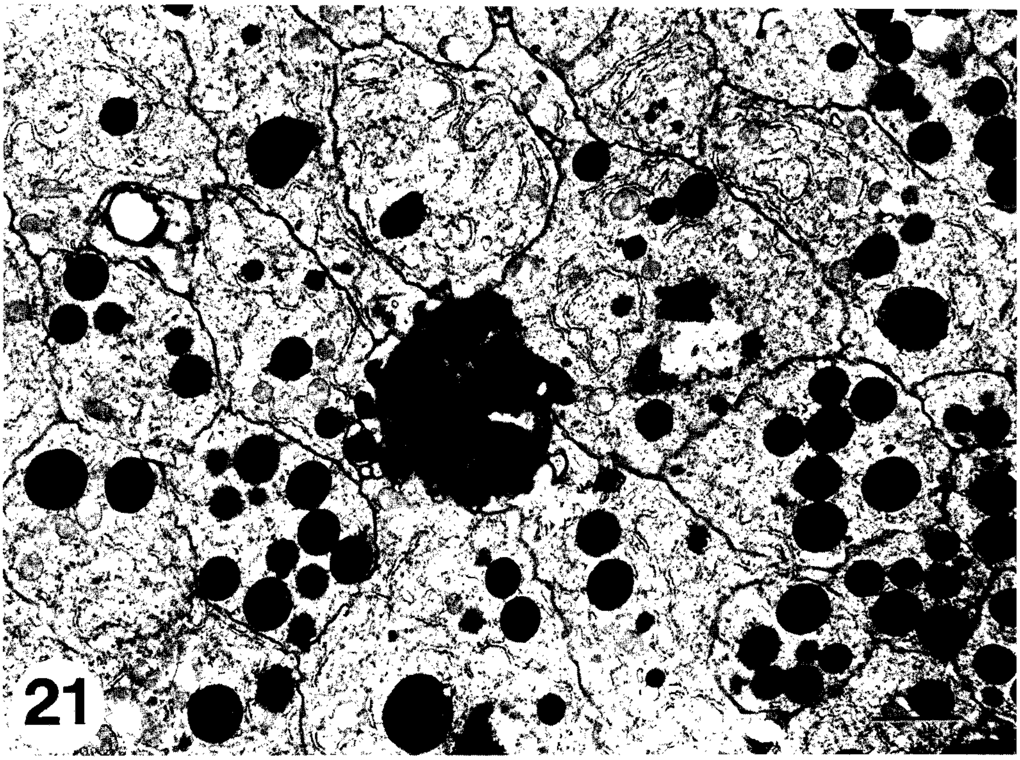
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granular forms described previously in older adults (Dailey et al., '80) probably result from fusion of morphologically simpler individual granules. These granules do not appear until after 8 days following adult ecdysis. Przybylski ('67) and Sjöstrand ('62) reported a similar maturation of granules during cytodifferentiation of the acinar cells in the chick pancreas. According to Przybylski ('67), zymogen granules in the acinar cells increase in structural complexity, number and size with developmental age. The earliest granules, called prozymogen granules by Sjöstrand ('62), appear to be miniatures of the mature adult type. Further investigation in other developing secretory tissues containing several distinct cell types will probably reveal precursor forms in the early synthesis of granules.

The coordinated maturation of all eight secretory types at or just after the time of ecdysis suggests the action of a common synchronizing signal. The signal is not likely to be a rise in ecdysterone since the major ecdysterone peak occurs in the midpupal stage. Apolysis in *Tenebrio* occurs on the fourth day of the 9-day interval between pupal ecdysis and adult ecdysis (Delachambre et al., '80). This "pharate adult" stage (as defined by apolysis of the sternal epidermis) begins many days before the first precursor granules appear in the BAG. Both bursicon and juvenile hormone are possible candidates as signals for differentiation. Bursicon titers rise just before eclosion and fall on the first day of adult life (Delachambre et al., '79). Bursicon could be affecting the onset of differentiation in types 4, 6, 7 and/or the rapid growth of the secretory machinery immediately after ecdysis. Juvenile hormone begins to rise in female *Tenebrio* at 2–3 days after eclosion (Weaver et al., '80). If a parallel increase in circulating juvenile hormone occurs in males, it may trigger the discharge of the secretory debris from the lumen of the BAG, and also promote terminal cellular differentiation.

Fig. 20. Autolysis of type 6/2 transitional cell. Arrow indicates fusion of secretory granule with vacuole. Fifty-four hours posteclosion. Bar = 1 μm.

Fig. 21. Section through midregion of 6/2 transitional cells, avoiding the nucleus, and characterized by profiles of rough endoplasmic reticulum. Similar necrotic remnants were observed in several boundary zones. Fifty-four hours posteclosion. Bar = 1 μm.

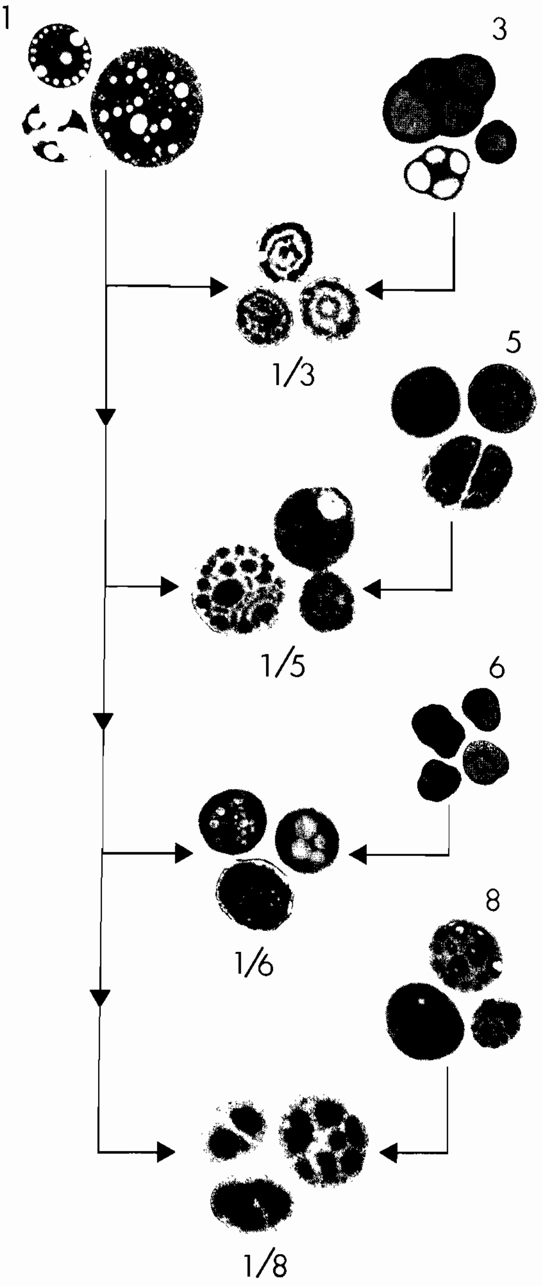

Fig. 22. Secretory granules from electron micrographs of transitional cell types found between the borders of cell types 1 and 3 (1/3), 1 and 5 (1/5), 1 and 6 (1/6), and 1 and 8 (1/8). Transitional granules 1/3 and 1/5 forms are distinct in character from the border cells, whereas forms 1/6 and 1/8 bear close resemblance to granules produced by one of the adjacent cell types.

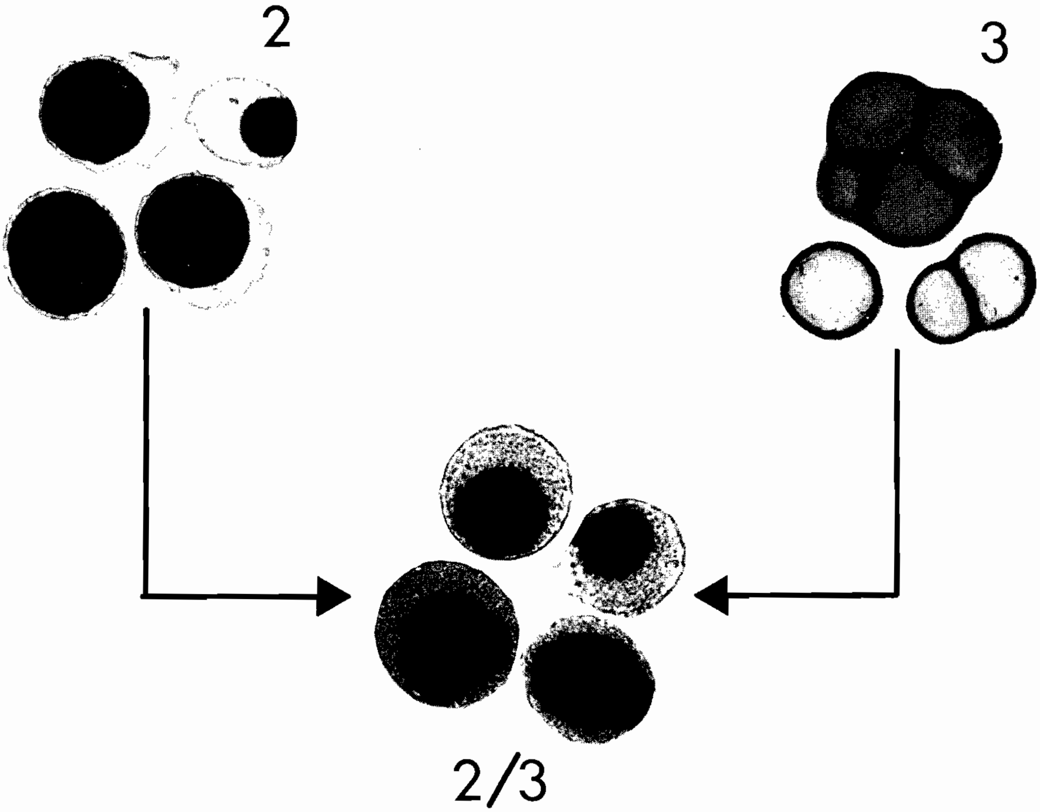

Fig. 23. Secretory granules from electron micrographs of the transitional cell type 2/3 found in the border region of cell types 2 and 3. Note similarity of these transitional granules to those of cell type 2 secretory granules.

Intermediate cell types and pattern consolidation

Pattern in organs or systems requires differences in cellular structure. The differences may be very marked, such as those between a neuroblast and a neuron after an asymmetric mitosis. Alternatively, cells may differ from their neighbors, near and far, by a subtle and gradual shift in positional values (Lawrence, '73). In the mature BAG, neighboring cells are of the same cell type except at the sharp interfaces between the patches. It might be argued that the commitment toward a particular cell type in the accessory gland was made early in development, and that its overt expression follows the mitoses and growth of the descendent clone or polyclone as has been described in the epidermis of *Drosophila* (Crick and Lawrence, '75). If inherited commitment through a cell lineage were the only major factor in the emergence of pattern, in the BAG, transitional cell types would not be expected at the interpatch boundaries. Their presence argues for the importance of cell-cell interactions well after mitoses are over.

Intercellular bridges sometimes form between the plasma membranes of closely apposed cells. We previously reported the presence of broad (0.5–2.0 μm) intercellular bridges, termed 'fused membrane bridges' (Happ and Happ, '82) in the 5–7-day pupal BAG (Grimes and Happ, '80). Fused membrane bridges are always worrisome, since they might be artifacts due to breaking and reannealing of membranes during fixation or processing. However, they are seen in the pupal BAG after several different fixation procedures with aldehydes, osmium, or permaganate (Grimes and Happ, '80). If the fused membrane bridges persisted through-

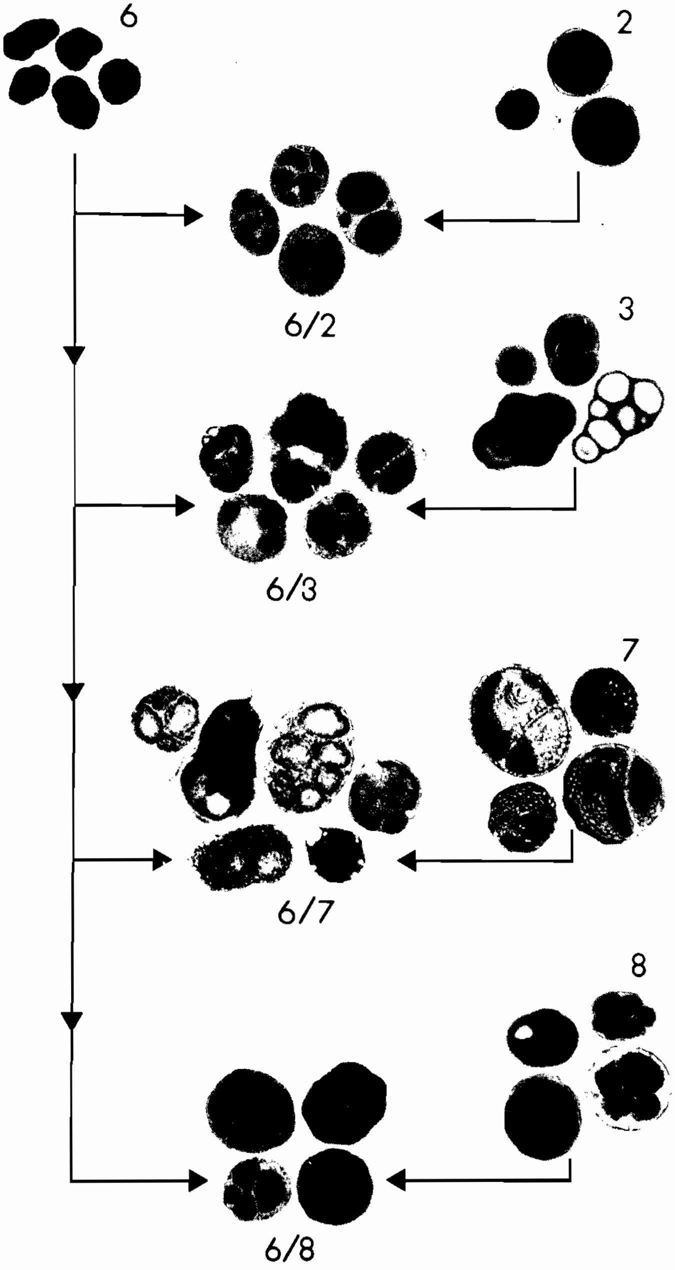

Fig. 24. Secretory granules from electron micrographs of transitional cell types occurring betwen borders of cell type 6 and 2 (6/2), 6 and 3 (6/3), 6 and 7 (6/7), and between 6 and 8 (6/8). Transitional forms 6/2 and 6/3 synthesize granules bearing limited resemblance to those of one or another of the adjacent definitive cells. Intermediate granules of 6/7 and 6/8 are quite similar to those of one or another of the established border cells.

out the pupal stage, they might allow secretory granules to move between neighboring cells and thus lead to the intermediate granular forms described in this paper. However, the bridges disappear well before the appearance of secretory granules or their immature precursors. Therefore, these connections are not channels for intercellular movement of the granules, but they could allow other modes of cellular interaction.

Gap junctions are common in insect tissues (Lowenstein, '81) and the permeability of such junctions within the sternal epidermis of *Tenebrio* is under hormonal control (Caveney, '78). We have not identified gap junctions in the BAG, but they could be associated with the terminal bars which ring each cell near its apex or with the septate junctions above these desmosomes (Dailey et al., '80). By such junctions or other routes, we believe that intercellular morphogens diffuse between adjacent cells and influence their structure. In this way, intermediate granular forms would be produced in cells in contact with two differentiated secretory cells.

One insect model for an ordered change in secretory cell type is the epidermal cell of the body surface, which can sequentially produce the cuticle either of one metamorphic stage or of another. Intermediate cuticles, with characteristics of two stages, are uncommon in nature. When Willis and colleagues ('82) treated metamorphosing insects with juvenile hormone analogues, composite cuticles sharing features of two successive metamorphic stages were deposited by a single epidermal cell. These authors concluded that the cellular reprogramming normally occurring at metamorphosis had been interrupted and stabilized at an intermediate point by juvenoids. Unlike the composite cuticle, which results from administration of exogenous hormone, the transitional granules in the BAG seem to be a normal feature of pattern consolidation in development.

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