JOURNAL OF MORPHOLOGY 172:97-112 (1982)

Cytodifferentiation in the Accessory Glands of *Tenebrio molitor.* X. Ultrastructure of the Tubular Gland in the Male Pupa

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ABSTRACT The tubular accessory gland consists of a simple secretory epithelium surrounded by a muscular coat. Over the pupal instar, the gland increases ten-fold in volume and 15-fold in length. Pupal development is divisible into a phase of mitosis and one of cell growth. During the mitotic phase, cytoplasmic membranes are sparse and nuclei move toward the luminal face of the epithelium to undergo division. In the cell growth phase, the cells become more columnar, a few stacks of rough endoplasmic reticulum are formed, and small dense secretory vesicles appear near the apical surface. The hormonal control of the developmental sequence is discussed.

The pupal stage of holometabolous insects is a period of extensive cellular reorganization and organ growth. The most obvious result of the pupal metamorphosis is seen in the remodelling of the surface cuticle and in the assumption of the adult body form. Equally important is the development of the viscera, which include circulatory, digestive, respiratory, excretory, and reproductive organs. The endocrine signals which coordinate the process of cuticle deposition (e.g., Wigglesworth, '70; Riddiford and Truman, '78) also affect visceral metamorphosis (Whitten, '68; Riddiford, '72). We are concerned with the impact of such signals on the maturation of the accessory reproductive glands of mealworm beetles *(Tenebrio molitor).* Recent work has defined many features of the fluctuating hormone titers (Delbecque et al., '78; Delachambre et al., '79; Weaver et al., '80).

Both pairs of accessory glands of male *Tenebrio* are composed of a simple columnar epithelium invested by a thin muscular coat (Jones, '67; Poels, '72; Gerber, '76). The larger pair of glands, the bean-shaped glands (BAGs), have seven distinct types of secretory cells (Dailey et al., '80). In the smaller tubular accessory glands (TAGs), there is but one type of secretory cell (Gadzama et al., '77). The products of both glands contribute to the spermatophore, a sac which transfers sperm from male to female (Frenk and Happ, '76; Black and Happ, unpublished observation; Happ et al., '81). Over the first week after adult ecdysis, the content of differentiation-specific proteins in BAG and TAG and of differentiation-specific antigens (at least in TAG) increases significantly (Black and Happ, unpublished observation; Happ et al., '81). Patterns of leucine incorporation shift so that at maturity over 50% of the total leucine incorporation is into the putative secretory proteins (Happ et al., '77, '81).

Reproductive maturation in *Tenebrio* is mostly confined to the 3-week interval from the beginning of the prepupal stage to a week after adult ecdysis. The paired male accessory glands originate from a mesodermal rudiment and are distinct from one another at pupation (Huet, '66). The BAGs undergo cell division, growth, and changes in shape over the pupal stage (Grimes and Happ, '80; Happ et al., '81). In the present paper, we describe the development of the TAGs in the male pupa.

MATERIALS AND METHODS

As last instar mealworms *(Tenebrio molitor* L.) pupated, they were picked from stock cultures and the sexes were segregated. Developmental age of the pupae was confirmed by morphological criteria (Delbecque et al., '78). Synchronously eclosed adults were collected and

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maintained in groups of ten or less males at 26°C. Colonies were fed Purina Chick Startena and potato or carrot.

Volumes of glands were determined with plasticene scale models. Pupae or beetles were dissected in saline, and the various aspects of the glands projected with a camera lucida. A plasticene model was trimmed and shaped until it conformed well to the outlines of a gland of known age (0-, 2-, 4-, 6-, 8-day pupa and 0-, 2-, 4-, 6-, 8-day adult), and then the model was checked by comparison with projections of three to six additional glands. The models were weighed and the volumes of the TAGs were calculated by adjustment to scale.

For determination of protein content, glands were dissected in saline, homogenized in distilled water, and the Lowry procedure was followed (Lowry et al., '51) using bovine serum albumin as the standard. For measurement of leucine incorporation, tritium-labeled leucine (0.5 μCi of 3,4, 5 ^{3}H-leucine, specific activity 60 μCi/mM) was injected into the hemocoel. After allowing 4 hours for incorporation, the animals were chilled, the glands were removed and homogenized, and the protein was precipitated and washed according to the procedures of Kennell ('67). All TCA washes contained 10 mM cold leucine. Application of Bartlett's test to the raw data indicated that the values for subsequent days were heteroskedastic; i.e., the variance was significantly heterogeneous. A log transformation was performed to obtain homogeneity of variance before the 95% confidence intervals were calculated (Sokal and Rohlf, '69). Any datum point which differed by more than three standard deviations from the corresponding daily mean was omitted from the final calculations; this procedure eliminated no more than one datum point for each day.

For electron microscopy, abdomens were opened in fixative (3% fresh Ladd glutaraldehyde in 0.1 M phosphate, pH 7.3) at room temperature, and the glands were placed in a fresh aliquot of fixative for 2 hours at room temperature. After a rinse in buffer, tissue was transferred to 1% osmium tetroxide (buffered with phosphate) for 1 hour at room temperature. Following dehydration in graded acetones, the glands were finally embedded in Epon 812. Thin sections were stained for 20 minutes in saturated uranyl acetate in 50% ethanol followed by 5 minutes in lead citrate (Reynolds, '63). Micrographs were taken on RCA-EMU-3D at 50 kV.

For counts of mitotic indices, metaphase cells were blocked for 4 hours by an injection of 2% colchicine (approximately 20 μg/gm body weight). After 4 hours, the gland complex was dissected, fixed in alcoholic Bouin's for 1-2 hours, dehydrated in graded ethanols, cleared in xylol, and embedded in Paraplast. Sections were cut 5-8 μm thick and stained with Delafield's haematoxylin. In a given area, 300-400 nuclei were counted and scored as mitotic or interphase so that the percentage in mitosis (mitotic index) could be calculated.

OBSERVATIONS AND RESULTS

In our cultures, the pupal stage lasts 9 days. At pupal ecdysis, the TAG is a short blind sac which starts as a basal ampulla attached to the BAG and into which the seminal vesicles open (Fig. 1). Over the succeeding 9 days, the TAG grows from 0.4 mm to 5-7 mm in length (Fig. 1). We used scale models to determine the changes in volume, as described in Materials and Methods. At pupation, each TAG has a volume of 6×10^{-3} mm^3; at ecdysis to the adult, its volume has increased to 1.4×10^{-1} mm^3, and by 8 days later, the maximum volume of about 8×10^{-1} mm^3 has been reached. The overall increase in volume fits a simple exponential model (Fig. 2). The protein content of the TAGs rises steadily until about 6 days after adult ecdysis (Fig. 3) as does the rate of leucine incorporation into TCA precipitable proteins (Fig. 4).

The simple secretory epithelium of TAG consists of closely packed columnar cells. In the young pupa, these cells are crowded together so that in sections the nuclei appear staggered and they occupy the outer four-fifths of the epithelium (Figs. 5, 9). The secretory cell nuclei are oval (4-5 by 8-9 μm), with their long axes oriented radially. Above these tightly packed nuclei of the secretory cells are the somewhat smaller, more rounded, nuclei of the muscle coat (Figs. 5, 6). The basement membrane, which separates the muscle cells from the secretory cells, is thin and of low electron density

Abbreviations

BAG, bean-shaped accessory gland
BM, basement membrane
E, secretory epithelium
EjD, ejaculatory duct
G, golgi complex
L, lumen
M, muscle layer
N, nucleus of secretory cell
NM, nucleus of muscle cell
RER, rough endoplasmic reticulum
SV, seminal vesicle
Tr, tracheoles
ves, Membrane vesicles

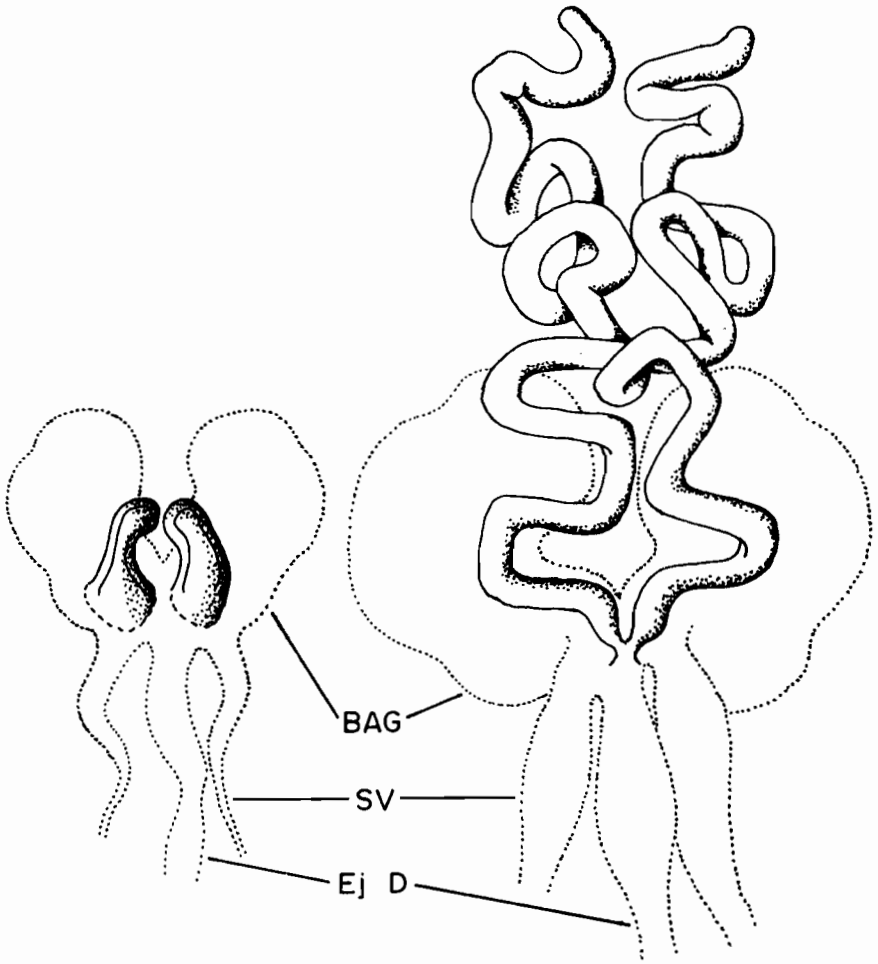


Fig. 1. The tubular accessory glands (TAGs) in 0-day pupae (left) and 9-day pupae (right). The outlines of the bean-shaped accessory glands (BAG), seminal vesicles (SV), and ejaculatory ducts (EjD) are shown for reference.

at pupal day 1 (Fig. 11). In the early and mid-pupa, the endoplasmic reticulum is sparse in the basal zone of the cells. The apical surface of the secretory cells contains large irregular lobules.

Growth over the pupal stage occurs first by cell division and then by enlargement. Mitotic figures, present from the beginning of pupation until the seventh day (Fig. 21), are confined to the apical zone (Figs. 6, 15, 20). We always saw the mitotic spindle parallel to the epithelial surface and oriented along the long axis of the gland. When cytokinesis takes place, the spindle remnants and the constriction which forms between two daughter cells usually lies 3–4 μm in from the lumen (Fig. 21). The spindle remnant persists as a bridge for some time. After the bridge has disappeared, dense fibrous material is found at the inner surface of the appressed plasma membranes and fibers are seen to run across the cell in a web-work at this level (Fig. 15). The plasma membranes are sinuous and always quite closely apposed (200–300 Å apart) (Figs. 16–19). The membranes themselves stain very darkly and often the intercellular space is filled with fine dense material (Figs. 17, 18). The belt desmosomes (zonula adherens) of the mature gland occupy the same level as did the spindle remnant bridges.

As cell division ceases (day 6–7), the epithelium and the muscle layer begin their late pupal differentiation. The gland continues to increase in volume, presumably due to cell enlargement. At day 6, the apical cytoplasm has many small irregular vesicles which appear in

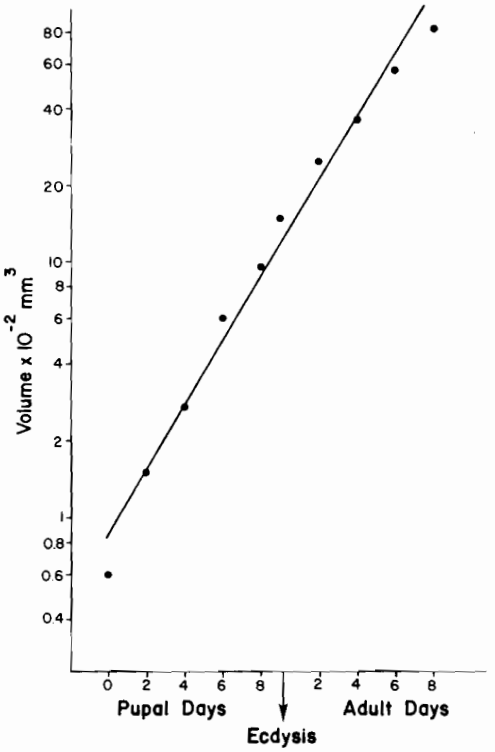


Fig. 2. Volume of individual TAGs, as determined by weighing the plasticene models. The regression equation on these values is log y = 0.125x − 0.0674 (r = 0.9921).

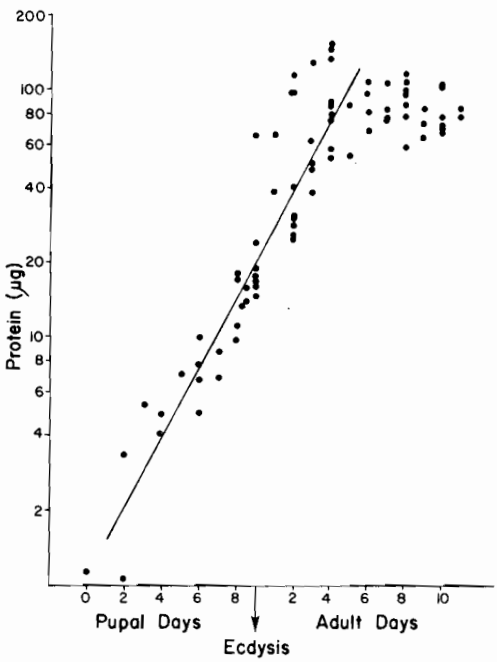


Fig. 3. Protein content of TAG pairs at various ages by Lowry assay. Individual datum points are shown (some were included in averages published earlier; Happ et al., '77). The regression equation on these points is log y = 0.1406x + 0.03826 (r = 0.922).

loosely ordered rows between the microtubules that run parallel to the long axis of the cell (Fig. 22). As in earlier ages (Fig. 14, 15), the vesicles seem to collect at the apex and then seem to be pinched off and shed into the lumen (Fig. 22). Presumably, these debris-filled extrusions serve to eliminate "spent" organelles and thus obviate the need for the isolation bodies (Locke, '69) seen in other insect tissue.

Microvilli begin to form at the apical surface of older glands. At 6 days, these are short and bulbous, usually with dense plaques like hemidesmosomes (Fig. 16). Over days 7-9, the microvilli become longer and more numerous until they appear as a labyrinthine brush border of thin folds, each about 1,000 Å in thickness and about 1 μm in length (Figs. 17-19). At 7 days, a layer of flocculent secretion is seen at the tips of the microvilli and lobular apical projections (Fig. 17) and by 9 days, it almost fills the lumen (Fig. 19). The subapical space in 8- and 9-day pupae contains a heterogeneous scattering of dense vesicles (Figs. 18, 19). Another common feature of the 8- and 9-day TAGs is an extensive infolding and interdigitation of the cell membranes in a zone 7-12 μm up from the apex (Figs. 18, 24). The much-folded cell membranes often appear to have an inner coat (Fig. 24). The cytoplasm at this level contains many small irregular membranous vesicles, and granular material, but not the dense vesicles seen near the apical border of the cells.

The basal and intermediate zones of the cells also show characteristic changes once cell division is complete. The nuclei lie toward the basal surface (Figs. 7, 8, 10) and the tall cells tend to be of the same cross-sectional area throughout. Parallel arrays of the endoplasmic reticulum are widely spaced and the flattened sacs usually run along the long axis of the cell (Figs. 13, 22). There are some small local interdigitations (Fig. 22) which suggest that the intercellular bridges run between the cells of the pupal BAG (Grimes and Happ, '80). The Golgi zones are well-developed in both the intermediate (Fig. 22) and the basal regions (Fig. 13). Especially in the basal zone, there are a number of loose ribosomes which are clustered together in polysomes (Fig. 12). In 8- and 9-day

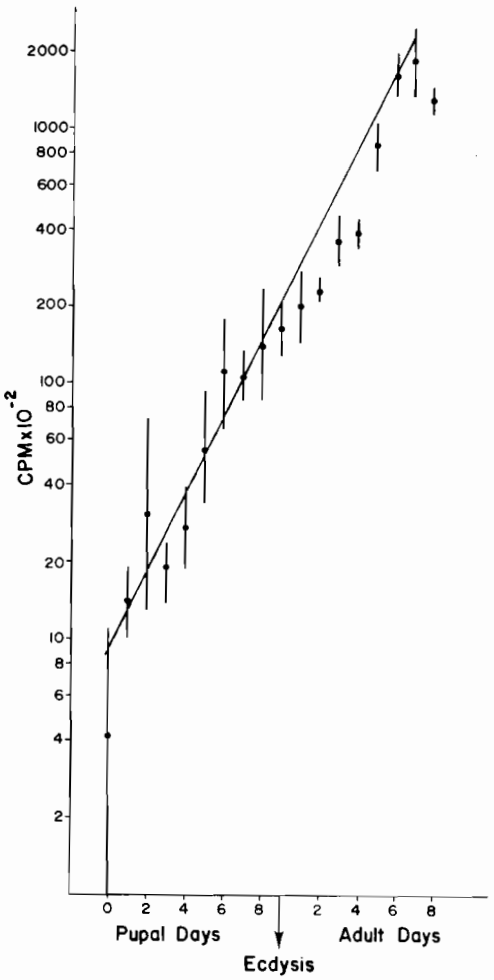


Fig. 4. ^{3}H-leucine incorporation into TAG-precipitated protein of TAG. A minimum of six gland pairs were examined at each day. The mean value and 95% confidence intervals are indicated. The regression equation for these means is log y = 0.142x + 0.944 (r = 0.978).

glands, the muscle layer is well developed and membrane plaques are found adjacent to the basement membrane (Figs. 12, 13). At 9 days, tracheolar branches can be seen running into the basal zone of the secretory epithelium (Fig. 13).

DISCUSSION

Although the increases in volume, in protein content, and in rates of leucine incorporation might seem to reflect a simple process of exponential growth, the morphological observations show that the development of TAG is divisible into five distinct phases: (1) *primary organogenesis* in the prepupa, (2) a *pupal mitotic phase,* (3) an interval of *pupal cell growth,* (4) *terminal differentiation* in the post-ecdysial adult, and (5) *sustained secretion* in the mature adult. Our earlier paper (Gadzama et al., '77) and the present work provide a morphological chronicle over the pupal phases of mitosis and cell growth and the terminal differentiation. The major features are summarized in Figure 25.

Intercellular bridges

During pupal differentiation, two routes are available for communication between adjacent cells in the secretory epithelium of the TAG: *spindle remnant bridges* and *fused membrane bridges. Spindle remnant bridges* are seen in many tissues during differentiation. These bridges link small clones and ensure their developmental synchrony. For example, spindle remnant bridges are found in the tergal glands of a cockroach (Sreng and Quennedey, '76) and the spermathecal accessory glands of *Tenebrio* (Happ and Happ, '77). In these two ectodermal glands, a stem cell divides to yield three or four daughter cells that jointly form an organelle or functional secretory unit. As the stem cell divides, the daughter cells remain attached to one another by broad spindle remnants like that shown in Figure 20 of the present paper. At least in *Tenebrio,* such spindle remnant bridges tend to remain at the site of the mitotic spindles, i.e., near the apical surface of the epithelium.

The *fused-membrane* bridges are rather more difficult to detect with confidence. We believe that they form when the plasma membranes of two adjacent cells become closely apposed and fuse to form a broad cytoplasmic connection surrounded by a bulbous membrane ring. In cross-section, this ring appears as a densely staining irregular vesicle in cross-section. The development of such bridges has been described in the rat cerebellum (Das, '77) and in fusing chick myoblasts (Shimada, '71). Similar intercellular bridges are seen (although not identified as such) in micrographs of the salivary gland of *Calliphora* (Berridge et al., '76) and they are common among the secretory cells in the pupal bean-shaped gland (Grimes and Happ, '80), and in the pupal tubular gland of *Tenebrio* (Fig. 23). In our previous study (Grimes and Happ, '80), we found these bridges after many fixation regimens (glutaraldehyde alone, osmium alone, sequential glutaraldehyde and osmium, and permanganate), and thus they are not likely to be fixation artifacts. In both the tubular and the bean-shaped

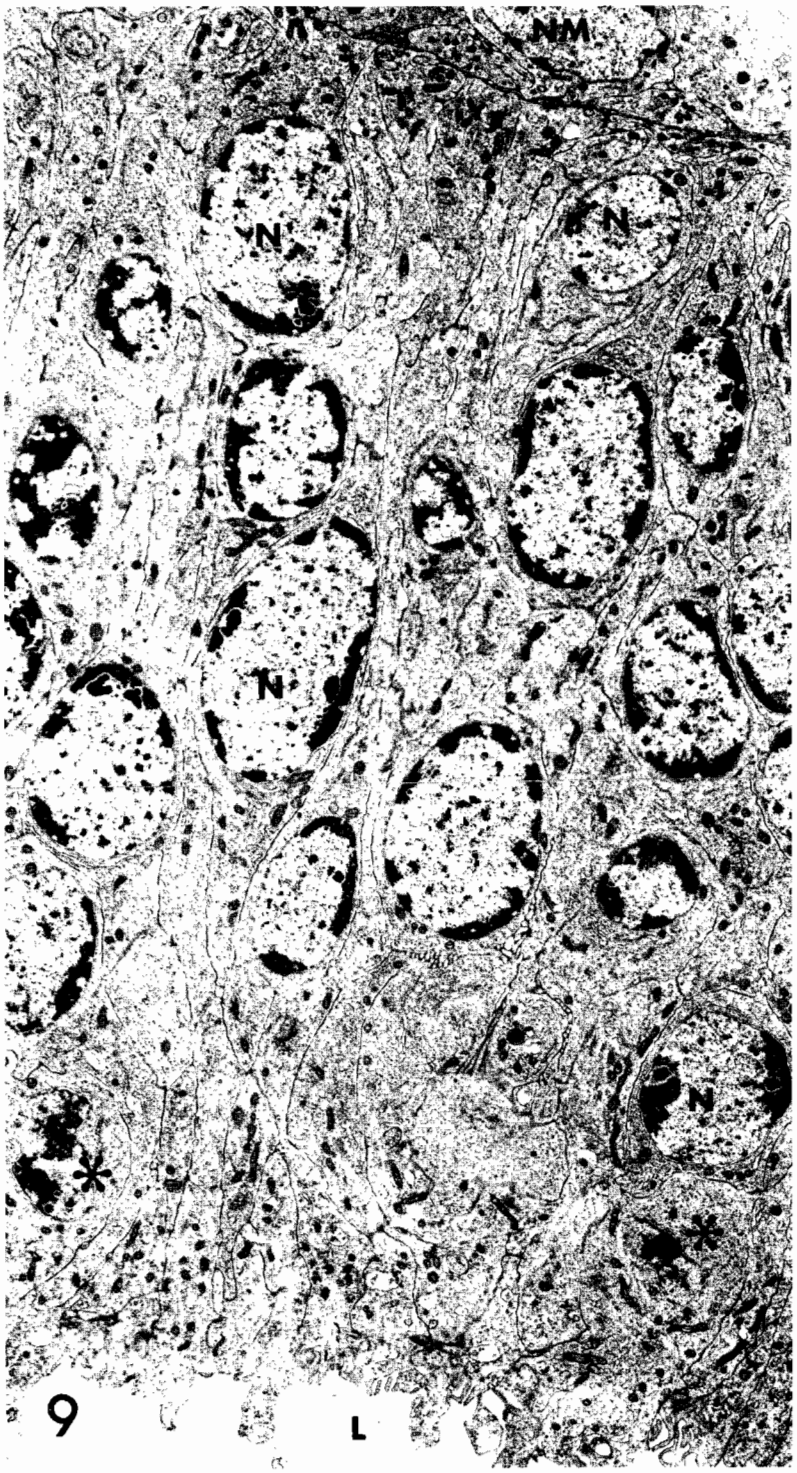
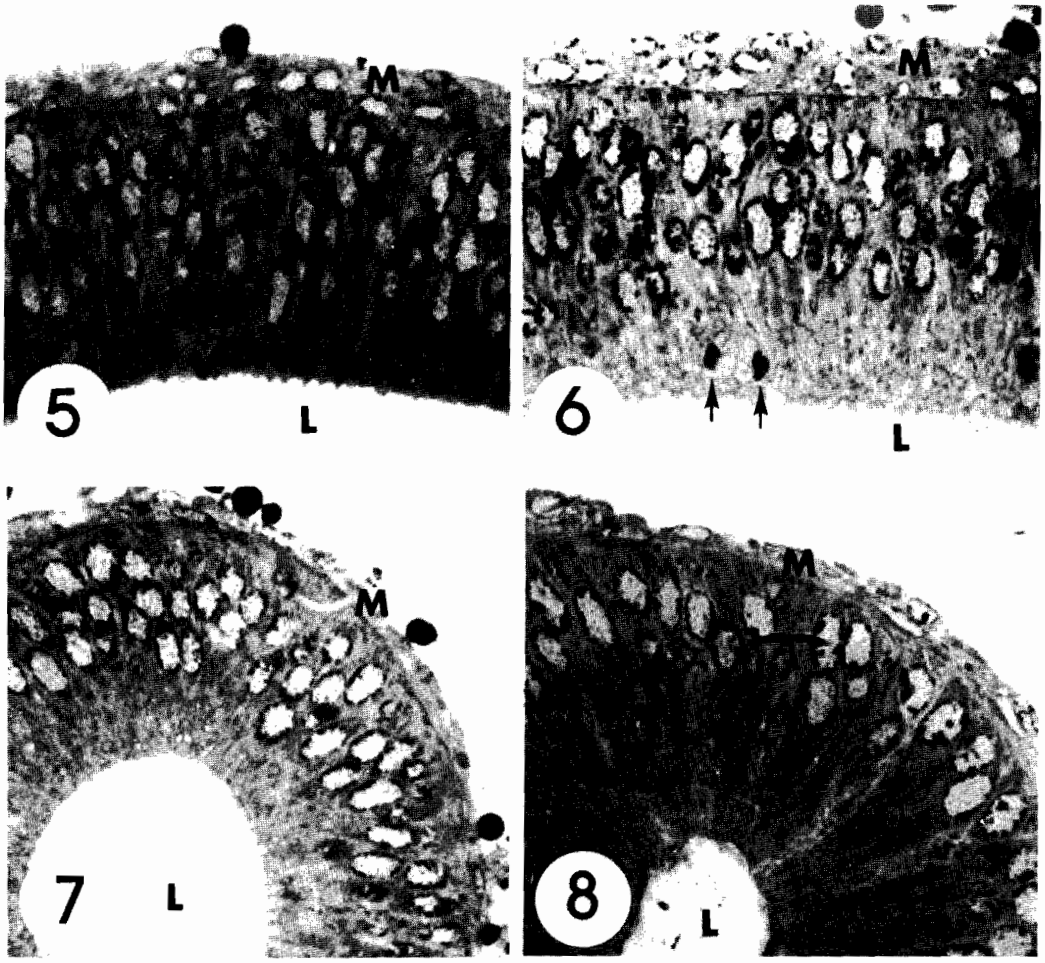


Fig. 9. A composite of low-magnification pictures of the TAG epithelium at 3 pupal days. Nuclei are distributed throughout the thickness of the epithelium. NM, nucleus of muscle cell; N, nucleus of secretory cell; L, lumen. Asterisks indicate cells undergoing mitosis. × 4,340.



Figs. 5–8. The secretory epithelium (E) and muscle cell layer (M) of TAG. L indicates lumen. Toluidine blue. × 900.

Fig. 5. Three-day pupa.

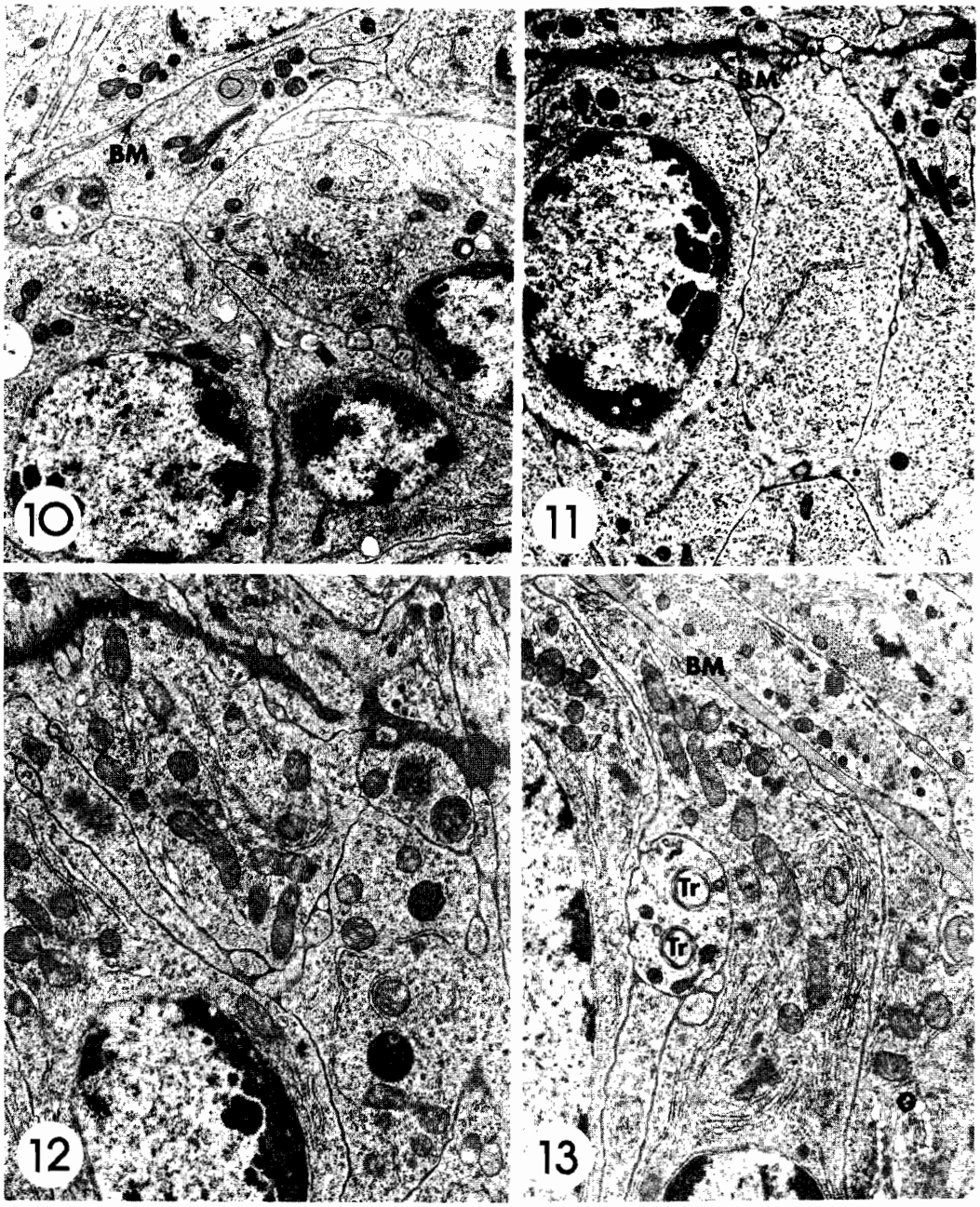
Fig. 6. Five-day pupa. Arrows indicate a cell in late telophase.

Fig. 7. Seven-day pupa.

Fig. 8. Nine-day pupa.

glands, the fused-membrane bridges are at the midlevel in the epithelium, whereas the spindle remnant bridges are restricted to the apical zone.

Broad intercellular bridges link developing insect germ cells. These bridges arise because of incomplete cytokinesis and in some species the spindle remnant bridge is replaced by a structure called a fusome (Hirschler, '45; Mandelbaum, '80). In meroistic ovaries, the fusomes between several cells form a rosette which allows transfer of nutrients from nurse cells to the developing oocyte (Pollack and Telfer, '69). The sequence of events can be reconstructed from electron micrographs, which suggests that the spindle microtubules are replaced by an amorphous filamentous material (Mandelbaum, '80). Around the filamentous material, the fused membranes of the daughter cells form a densely staining inflated ring which appears as vesicles in section (Mandelbaum, '80). The fusome appears to be a special case in which the spindle remnant bridge is transformed into a fused membrane bridge. The special fibrous material that occupies the bridge may facilitate transport of RNA and protein from nurse cells toward the oocyte. In



Figs. 10–13. Basal region of secretory epithelium with muscle layer at top, above the basement membrane (BM).

Fig. 10. One-day pupa. × 4,600.

Fig. 11. Five-day pupa. × 6,800.

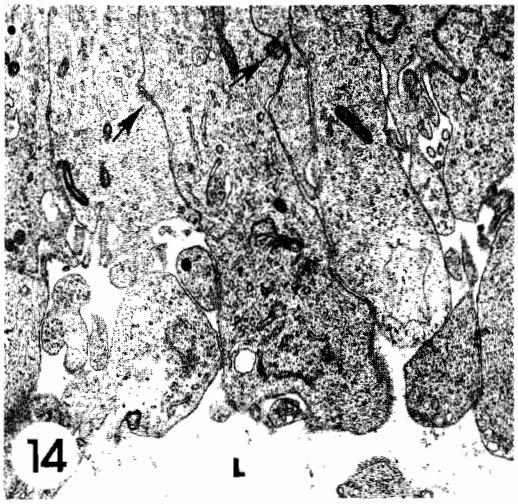
Fig. 12. Seven-day pupa. × 10,100.

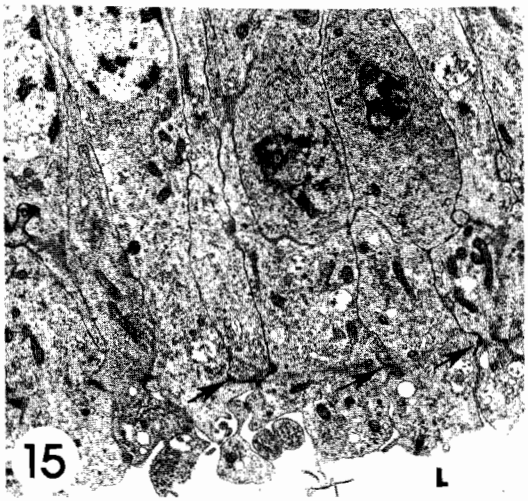
Fig. 13. Nine-day pupa. Tr, tracheoles, G, Golgi complex. × 10,400.

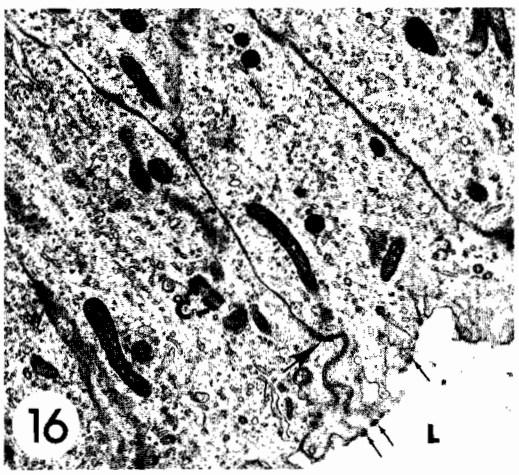
Figs. 14–19. Apical zone of secretory cells. L, lumen. Large arrows indicate the intercellular desmosones.

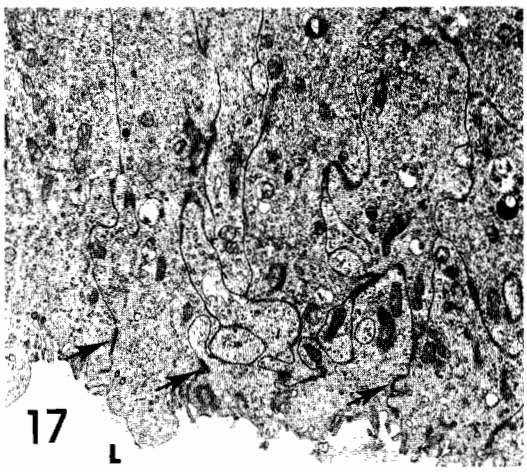
Fig. 14. Zero-day pupa. × 11,000.

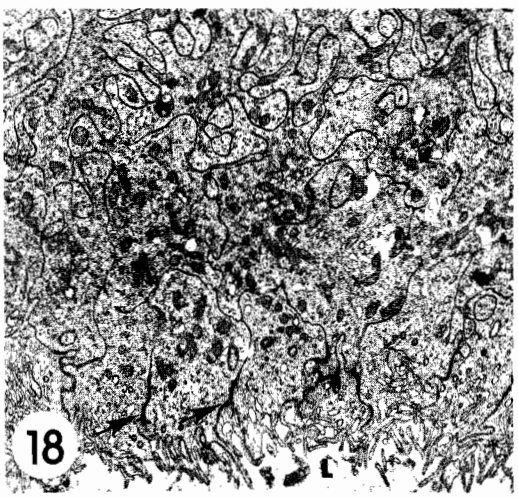
Fig. 15. Three-day pupa. × 5,900. Asterisks indicate daughter cells of a recent division.











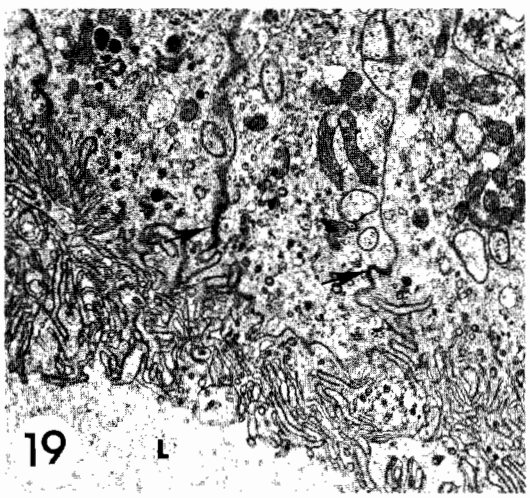


Fig. 16. Five-day pupa. Small arrows indicate membrane plaques at the tips of microvilli. C, centrioles. × 9,300.

Fig. 17. Seven-day pupa. × 5,800.

Fig. 18. Eight-day pupa. The upper half of the field shows the extensive cellular interdigitations. × 6,000.

Fig. 19. Nine-day pupa. × 14,800.

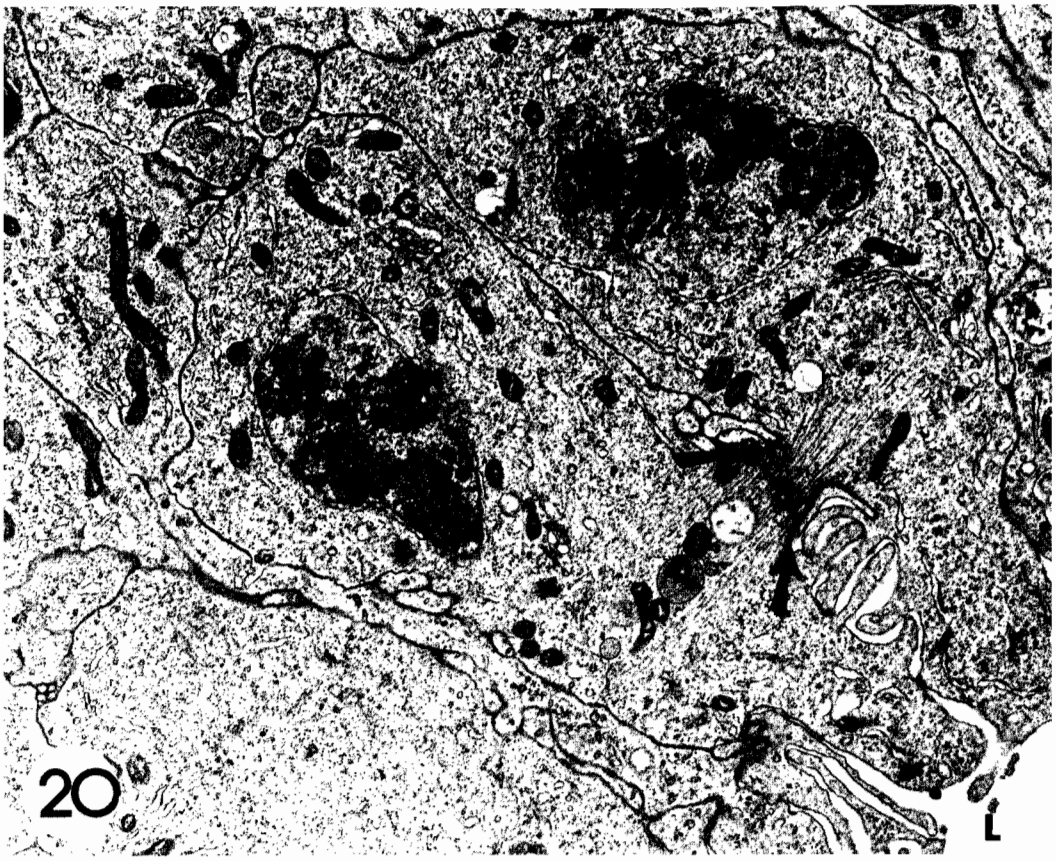
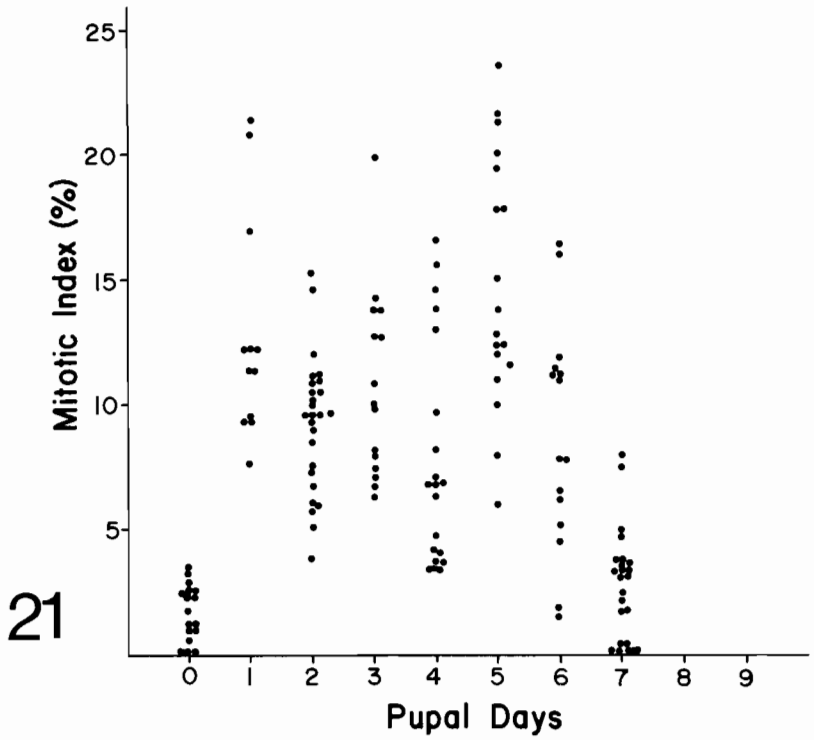


Fig. 20. Two daughter cells which are forming in a 1-day pupa. The chromatin is decondensing and the spindle remnant and midbody (between arrows) occlude the junction between the two cells. One-day pupa, G, Golgi complex. × 10,400.



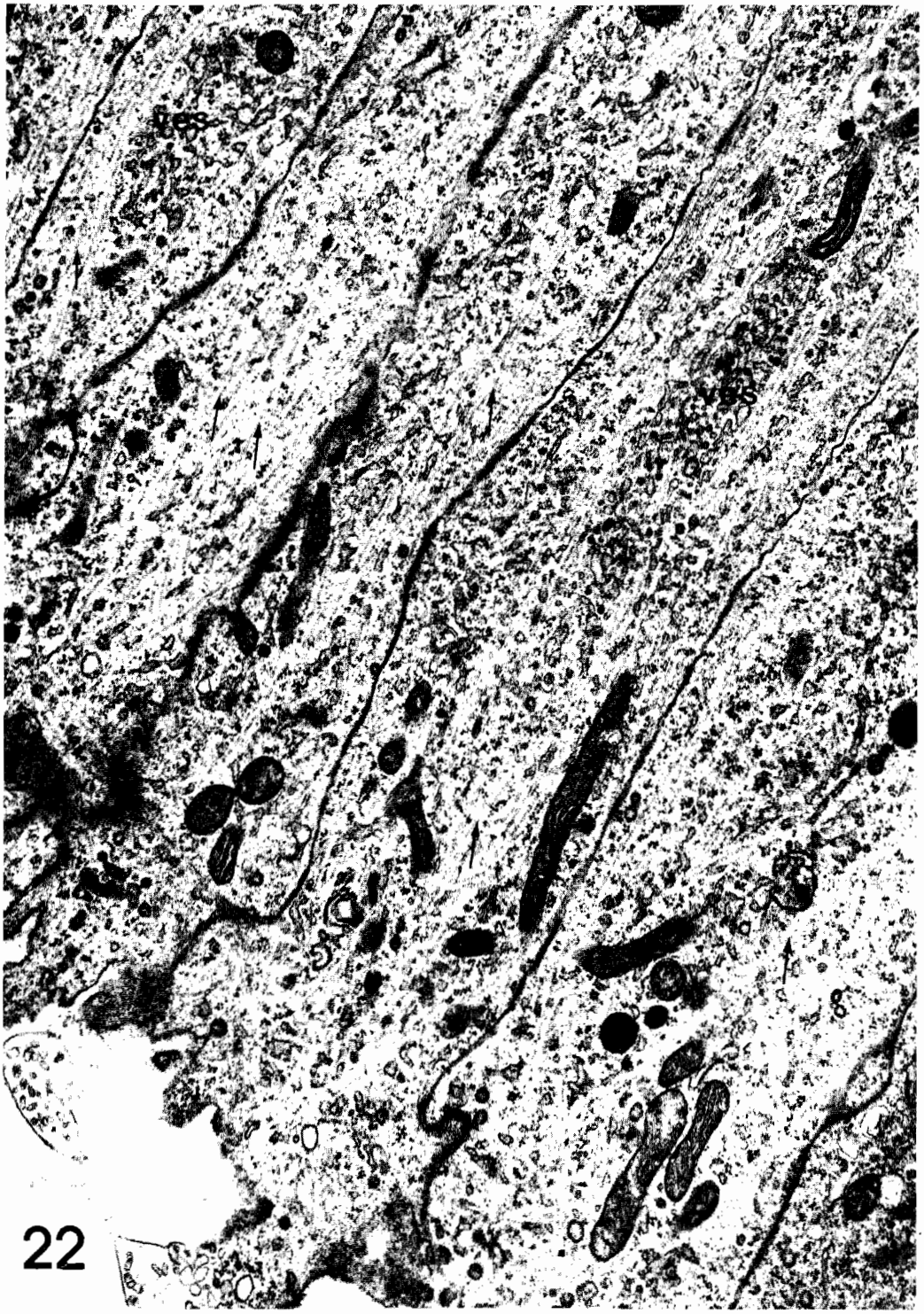


Fig. 22. Apical cytoplasm of secretory cells in TAG from 6-day pupa. The lumen is at the lower left. Small arrows indicate microtubules. Ves, membrane vesicles. × 18,600.

Fig. 21. Mitotic indices of cells in the secretory epithelium after 4-hour colchicine arrest.

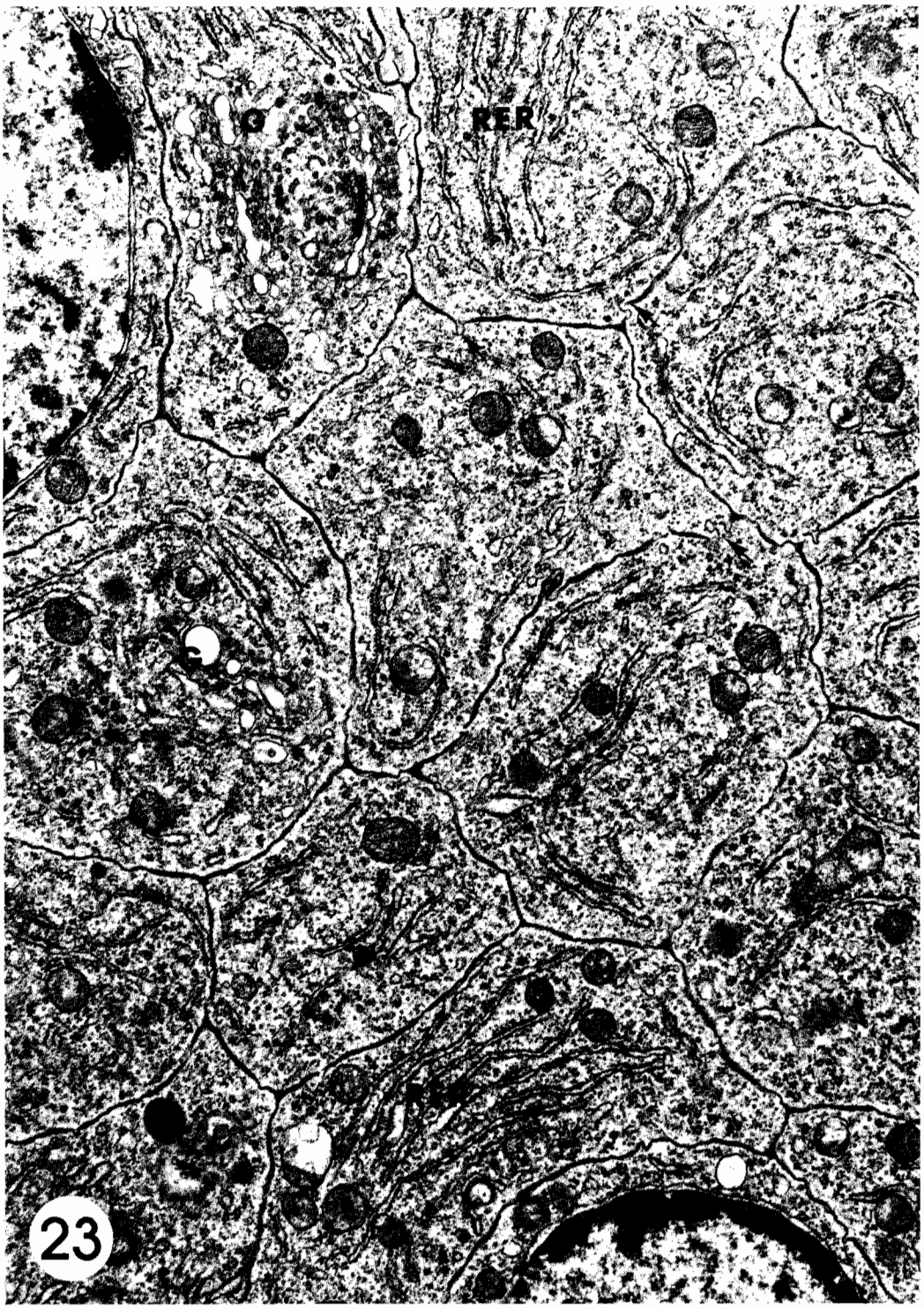


Fig. 23. A cross section through the midregion of the secretory cells in a 7-day pupa. Parallel arrays of rough endoplasmic reticulum (RER) and Golgi complexes (G) are common. Arrows indicate membrane outpocketings which may be the remnants of intercellular bridges. × 15,800.

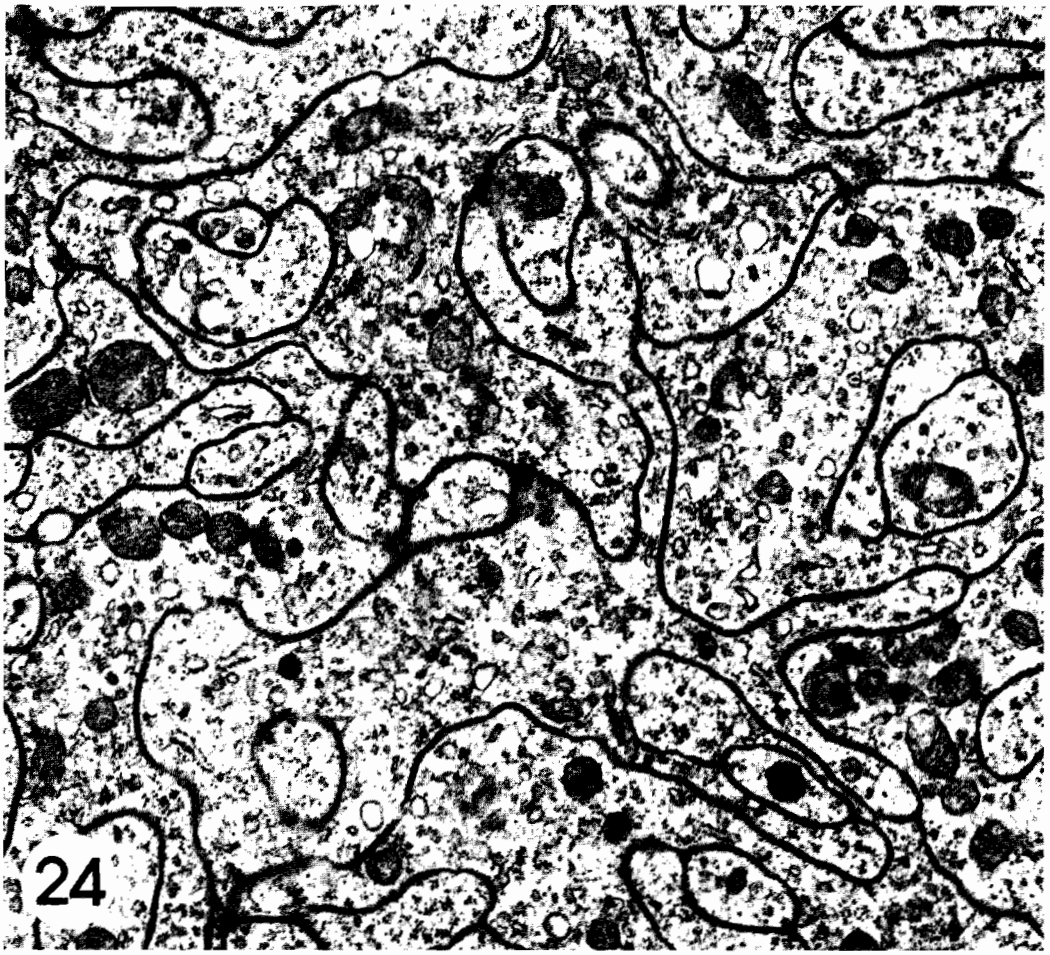


Fig. 24. A section through the secretory cells of 9-day pupal TAG about 8–19 μm above luminal surface. The cells are extensively infolded and thus interdigited (compare with Fig. 18). Note dense material between the cells as well as additional material appled on the cytoplasmic side. × 20,700.

contrast, the fused membrane bridges and spindle remnant bridges of the BAG and the TAG arise at different sites and lack the fibrous filler.

Phases in pupal tubular glands

The mitotic phase in the TAG and in the BAG (Grimes and Happ, '80) lasts until the seventh day while in the female spermathecal gland it only lasts until the fifth day (Happ and Happ, '77). The similarity between the two male accessory glands probably reflects their origin in a common mesodermal rudiment (Huet, '66). In contrast, the mitotic waves in the ectodermal female accessory gland resemble those of the ectodermal body epidermis (Besson-Lavoignet and Delachambre, '81).

The phase of pupal cell growth appears to be mostly one of preparation for rapid terminal differentiation. The cell surface is thrown out into the many elongate microvilli and the lateral cell membranes are infolded so that, for part of their height, the columnar secretory cells are extensively interdigited and apparently tightly held to one another. These membrane expansions may be adaptations to allow postecdysial cell growth merely by infolding membrane to increase cell volume. The presence of the loosely stacked profiles of the endoplasmic reticulum and of the small dense secretory vesicles may be for production of the flocculent layer of secretory product of unknown function. At the final pupal day, tracheoles appear between the secretory cells.

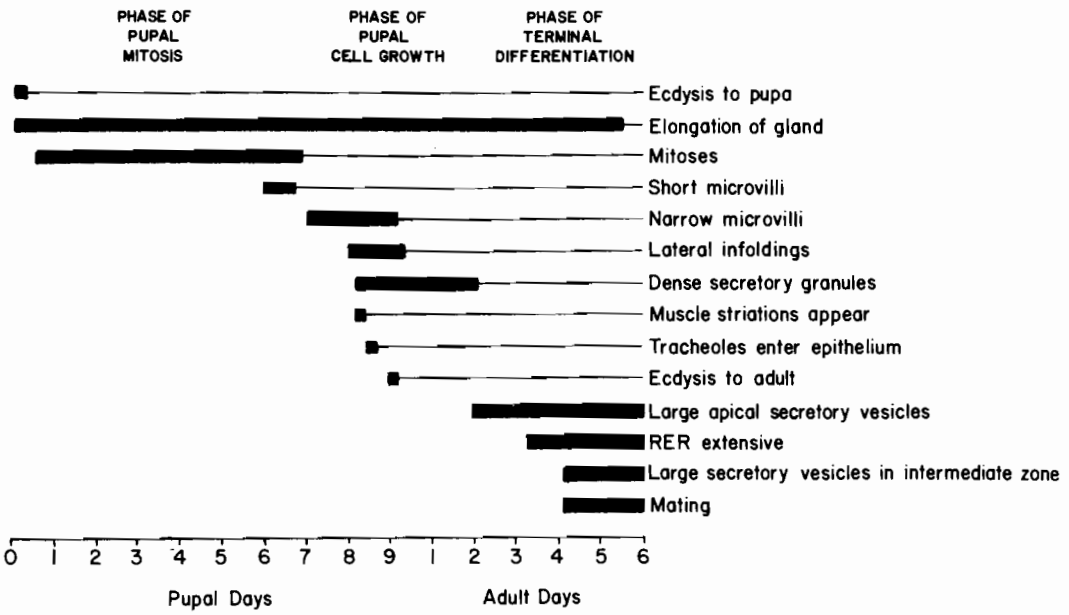


Fig. 25. Diagram showing the main events in TAG development during pupal and young adult stages.

A similar penetration of the tracheoles and their end cells occurred at the same time in the pupal bean-shaped glands (Grimes and Happ, '80). We interpret the tracheoles as an adaptation to support the high metabolic rate of the secretory tissue of the reproductive adult.

The patterns of protein synthesis change in concert with the morphological maturation. These biosynthetic changes are especially clear in the postecdysial adult TAG. Between ecdysis and 5 days later, the leucine incorporation data show a marked increasing emphasis upon four classes of soluble proteins of low molecular weight. Over half of the leucine used for protein synthesis is built into these differentiation-specific proteins (Happ et al., '77). Immunochemical and electrophoretic evidence indicates that these proteins are secretory, and at least two of them are very similar to soluble components of the spermatophore (Black and Happ, unpublished observation).

In the mammalian pancreas, differentiation can be viewed as a series of successive restrictions in developmental potential which culminate in terminal differentiation (Rutter et al., '68). This model can be extended to a variety of other tissues (Kafatos, '72; Rutter et al., '73). Of particular interest to the present study is the fact that in many tissues the accumulation is preceded by a period of low but constant rate of synthesis of specific proteins called the protodifferentiated state (Rutter et al., '73). Our morphological chronicle of development in TAG can be fitted to such a model; we need only assume that the phase of pupal cell growth is that protodifferentiated state. Thus the widely spaced stacks of rough endoplasmic reticulum (Figs. 12, 13, 23) and the small dense secretory vesicles (Figs. 18, 19) might be the cellular machinery for low level synthesis, storage, and perhaps export of secretory proteins. Our recent immunochemical data (Black and Happ, unpublished observation) support this interpretation; we detected low levels of two classes of the secretory proteins in late pupal TAGs. However, we do not yet have measurements of the rates of synthesis of these proteins.

The transitions from one phase to the next may be dependent upon hormonal signals or they may be tissue autonomous. During the period while the TAG is developing, several hormones act on ectodermal tissues and perhaps on the mesodermal TAG as well. These candidates for modulation of TAG development include juvenile hormone, ecdysterone, bursicon, and eclosion hormone. Eclosion hormone has yet to be demonstrated in *Tenebrio*, although its presence has recently been demonstrated in several orders of insects, including the beetle *Dysticus* (Truman et al., '81). Bursicon is released into the hemolymph just after pupal and adult ecdysis in *Tenebrio* (Dela-

chambre et al., '79) and could be involved in triggering terminal differentiation. Juvenile hormone titers are not known in pupal *Tenebrio*, but its levels begin to rise 2 days after ecdysis in adult females (Weaver et al., '80), and perhaps also in males. Ecdysterone rises sharply to a very high peak in the midpupa and then falls, to rise again slightly in the post-ecdysial adults (Delbecque et al., '78). The direct involvement of any of these hormones in TAG development remains to be established.

The times when cells change their patterns of gene expression, i.e., the transition points, can often be correlated with rounds of DNA synthesis. The importance of such mitotic events is emphasized by Holtzer's concept of the "quantal" mitosis (Holtzer et al., '72), which postulates that reprogramming occurs at certain rounds of DNA synthesis and that the new program is expressed only after mitotis to yield daughter cells with a new phenotype. Although in some insect systems, DNA synthesis is *not* necessary for reprogramming (references in Selman and Kafatos, '74; Kumaran, '78; Dyer et al., '81), a recent paper on the body epidermis of *Tenebrio* suggests that ecdysterone controls gene expression by controlling the cell cycle. Besson-Lavoignet and Delachambre ('81) report that most of the epidermal cells of prepupal and pupal *Tenebrio* are blocked in G_2 of the cell cycle and thus have 4C DNA content. The G_2 block is "associated with the beginning of cuticle deposition (and) can be correlated with a high 20-hydroxyecdysone titre." In the pupa, this block in the epidermis occurs at day 5, coincident with the major peak of ecdysterone. It is possible that such a control operates on the ectodermal spermathecal accessory gland of the female *Tenebrio*, for in this gland, mitoses stop also at day 5.

Some evidence indicates that the secretory cells of TAG have more than a diploid content of DNA and thus might also be in G_2 block. When paraffin sections of TAG are stained with Azure B, a metachromatic dye which allows microspectrophotometric measurement of DNA content (Flax and Himes, '52), the secretory nuclei stain much more intensely than do the nuclei of the muscle coat (Fig. 6: Gadzama et al., '77). Since the muscle nuclei must be at least diploid, the secretory nuclei are apparently of a higher ploidy. However, the rhythm of events in the mesodermal epithelia of the TAG and the BAG is definitely out of phase with the body epidermis. Mitoses in both the BAG (Grimes and Happ, '80) and the TAG (present paper) and ^{3}H-thymidine incorporation in the BAG (Grimes and Happ, '80) continue until day 7, well beyond the major ecdysterone peak (Delbecque et al., '78).

We will evaluate the importance of hormones to TAG development in future papers. In the present study, we have found morphological indices with which to score differentiation. Thus we can judge the impact of perturbation of hormones on the patterns of DNA synthesis and on the events of visceral metamorphosis.

ACKNOWLEDGMENTS

We thank Christine Yuncker and Margaret Grimes for technical assistance, Teresa Szopa for critical reading of an early version of this manuscript, J.J. Lenoir-Rousseaux for drawing Figure 1, Joyce Murray for expert secretarial assistance, and the National Institutes of Health (AI-15662 and GM-26140) for financial support.

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