

Cytodifferentiation of the accessory glands of *Tenebrio molitor*

IX. Differentiation of the spermathecal accessory gland in vitro*

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Summary. Spermathecal accessory glands from pupae of *Tenebrio molitor* were cultured in vitro in Landureau S-20 medium with or without ecdysterone at a concentration of 5 µg/ml medium. Morphological changes were examined by electron microscopy. Tissue taken from pupae that have not been exposed to a peak of ecdysterone in vivo is only able to differentiate in medium with hormone, and then only partially, while tissue taken from pupae that have experienced an endogenous peak of ecdysterone is able to develop maximally irrespective of the presence or absence of hormone. The specific ultrastructural changes that occur in vitro correspond to those occurring in the gland in situ during the normal course of differentiation, and are: the formation of the pseudocilium, cell retraction and formation of an end apparatus, and cuticulogenesis in the ductule and main lumen of the gland. Pseudocilium formation does not appear to be ecdysterone-dependent, while cuticulogenesis requires ecdysterone for initiation of the process. Deposition of cuticle is an expression of an earlier commitment of the cells to this process, and is initiated by elevated levels of ecdysterone. Ecdysterone is not required for completion of cuticulogenesis.

Key words: Cuticle – Ecdysone – Pseudocilium – Spermathecal accessory gland

Differentiation of the spermathecal accessory gland of *T. molitor* occurs in the pupal stage prior to eclosion to the adult. The cytodifferentiation of the gland, which has been described by Happ and Happ (1977), consists of 3 distinct phases: cellular proliferation, cell morphogenesis and cuticulogenesis. Development of the gland is similar to those of colleterial glands in general (Berry and Johnson 1975; Tashiro et al. 1976; Johnson and Berry 1977).

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In most hormonal systems *in vitro*, ecdysteroids have been shown to be required for development (Marks 1970; Milner and Sang 1974; Oberlander 1974) and, furthermore, ecdysterone is considered to be a trigger for cuticle deposition in insects (Gilbert and King 1973). In pupal *T. molitor* the ecdysteroid titre is known to rise to a peak level at day 4–5 (Delbecq et al. 1978), a time that corresponds to acceleration of pseudocilium formation and the start of cuticle deposition in the spermathecal accessory gland (Happ and Happ 1977). The *in vitro* culture system provides an ideal means by which to determine the precise effect of a hormone on its target organ. The tissue can be isolated in a medium that approximates those conditions encountered *in vivo* and the purified hormone can be applied in precise quantities at specific times. The response of the tissue can thus be monitored in a simplified system, in which potential interactions of tissue and hormone have been eliminated. Since the differentiation of the gland *in vivo* has been well-documented, the criteria by which to score development on an ultrastructural basis were available. The objective of our study was to identify hormone-dependent changes in the spermathecal accessory gland *in vitro*.

Materials and methods

Stock cultures of *T. molitor* were maintained at 25°C. Last instar larvae were picked from the stock cultures just as they eclosed to pupae. They were sexed and then maintained separately. Thus their precise ages were known.

Aged female pupae were surface sterilized with 70% ethanol prior to injection with ecdysterone. Ecdysterone (Calbiochem Ltd. U.S.A.) was prepared in a stock solution of 50% ethanol. The required concentrations of hormone were injected in a 1–2 µl volume using a Hamilton syringe, between the last two ventral sclerites. Control pupae were treated with 50% ethanol alone.

Two- and 4-day old female pupae were surface sterilized with two washes of 70% ethanol followed by a rinse in sterile distilled water. All procedures from this stage onwards were carried out under a laminar flow hood. The spermathecal accessory glands were removed from the pupae in sterile culture medium. Instruments for dissection were heat-treated, or passed through 70% alcohol and air-dried. Tissue was immediately transferred to fresh medium containing a streptomycin sulphate/penicillin mixture at a concentration of 500 units/ml, for 1.25 h. Transfers were done on small, sterile squares of nucleopore filter to avoid damage to the tissue.

The medium used was Landureau S-20 (Landureau 1976) at pH 7.0 and approximately 380 mOsm. The medium was filter-sterilized before use and foetal calf serum (GIBCO) was added at 0.5–1% (Landureau, personal communication). Ecdysterone was added to the medium before filtration. The stock solution of ecdysterone in 50% ethanol was used fresh, and assayed before and after use spectrophotometrically (at 243 nm where $\epsilon = 12,400$). The excised glands were cultured either in plastic Multiwell plates or in Falcon plastic organ culture dishes in a water saturated atmosphere at 25°C.

At termination of an experiment the glands were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), at room temperature for 2 h. Post-fixation was carried out using 1% OsO₄ in 0.1 M phosphate buffer for 1 h. En bloc staining in 2% aqueous uranyl acetate for 15 min followed post-fixation. Then the tissue was dehydrated in a graded series of acetones, infiltrated with an acetone:Epon 812 mixture in the ratio of 1:1, and finally embedded in fresh Epon. Pale gold to silver sections were collected on Formvar-coated grids and contrasted with 4% aqueous uranyl acetate and lead citrate before viewing with a Philips EM 200.

Results

The normal sequence of events in the cytodifferentiation of the spermathecal gland *in situ* is depicted in Figs. 1–4, 6–7. Briefly in the early stages of the pupa, the gland consists of undifferentiated cells (Figs. 1, 2). The cells of the gland are columnar and

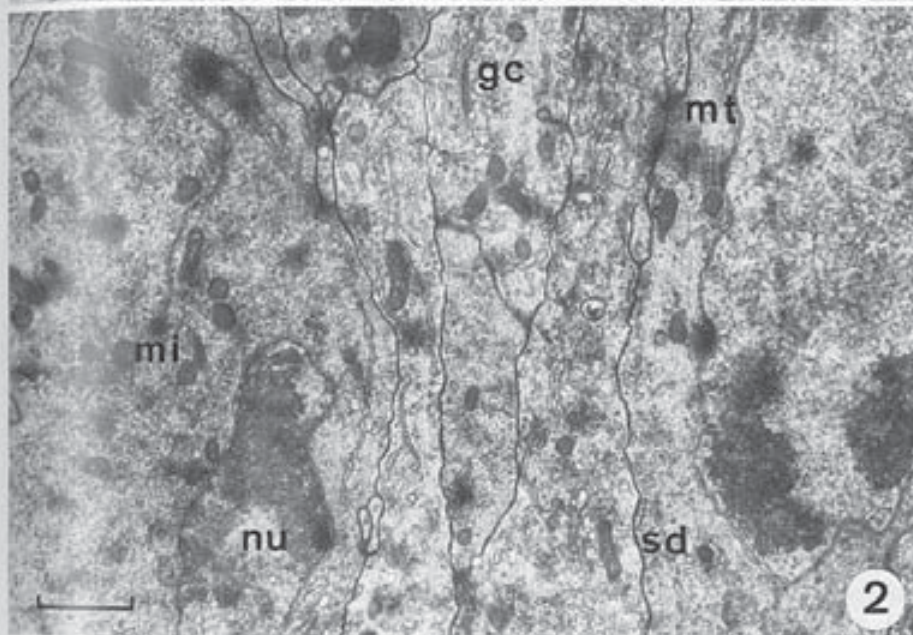
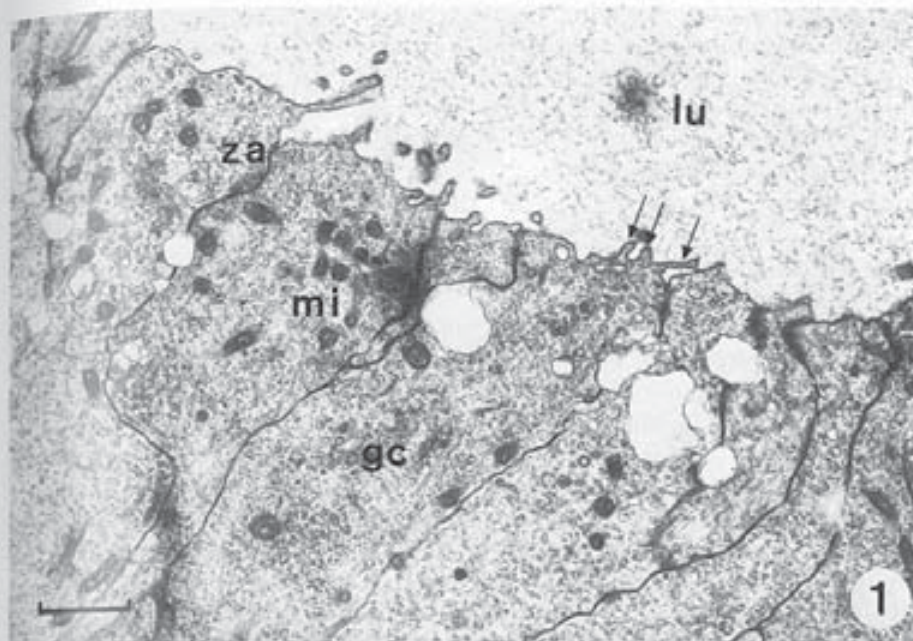


Fig. 1. Spermathecal accessory gland (SAG) of a 2-day pupa in situ. Note mitochondria (*mi*), Golgi complex (*gc*), and zonula adherens (*za*) at cell apex. Irregular projections occur at apical surface (arrows); lumen (*lu*). $\times 13,600$. Scale bar in micrographs = $1\ \mu\text{m}$ unless otherwise indicated

Fig. 2. SAG of a 2-day pupa showing numerous microtubules (*mt*), septate desmosomes (*sd*), mitochondria (*mi*), Golgi complex (*gc*), and irregularly lobed nuclei (*nu*). $\times 28,800$. Scale bar = $0.5\ \mu\text{m}$

the two cell populations seen at later times cannot be distinguished. Characteristically, the apical surfaces of the cells which extend into the main lumen of the gland are raised in many irregular projections (Fig. 1). Microtubules are numerous throughout the cells and run along the entire long axes of the cells in many cases. Free ribosomes, mitochondria and also secretory vesicles are present in the apical cytoplasm. Nuclei are located basally and are irregularly lobed. Golgi complexes are generally found near the nucleus (Fig. 2).

Figure 4 shows the 3-cell secretory unit (Happ and Happ 1977) which is formed after the completion of cell divisions. The innermost cell eventually produces a pseudocilium.

As the inpocketings of the epithelium develop, vestibules are formed in the gland. Cuticle deposition begins with the formation of more regular microvilli, at the tips of which there are dense plaques. A sheet of cuticle is laid down in the axial duct (Fig. 3) and in the vestibules (Fig. 6). Terminal stages of pseudocilium formation are shown in Fig. 7. The pseudocilium develops from the distal centriole. The pseudociliary microtubules are arranged in nine doublets without central microtubules. In later stages the innermost cell retracts leaving the pseudocilium in a cylindrical cavity. As the microtubules of the pseudocilium degenerate, the efferent ductule forms simultaneously (Fig. 7). The bounding plasma membrane is rippled and plaques of dense material are deposited at the indentations.

Treatment of pupae aged from 0–2 days with 2–4 μg ecdysterone prevented normal differentiation. Pseudocilium formation did not occur in either the hormone-treated or control glands, apparently due to a blockage of mitosis by a "wounding reaction". When 3-day pupae were injected with 2–4 μg ecdysterone normal development was not accelerated compared to control pupal glands which were responding to the endogenous peak of ecdysterone. In both cases pseudocilia and cuticle were formed in the same number of days as occurs in the normal pupa (8 days).

Glands explanted at day 2, cultured in medium with ecdysterone developed as shown in Figs. 5, 8–9. Regular, short microvilli with electron-dense plaques at their tips formed at the cell apices (Fig. 8). This represents an early stage of cuticulogenesis. As shown in Fig. 9, the remainder of the cells did not change and the 3-cell secretory unit did not form. When the culture was allowed to continue for a longer time cuticle deposition became more advanced and definite layers were laid down (Fig. 5). Glands cultured for equivalent lengths of time in medium without hormone failed to develop in this way (Fig. 11) and the irregular projections at the apices of the luminal cells, characteristic of the early stages, were retained. Figure 12 shows a pair of centrioles aligned in pairs, with their long axes perpendicular to the lumen, suggesting that the beginnings of organization of the 3-cell secretory unit had occurred. The cell containing the centrioles would correspond to the pseudocilium-forming cell, and the cells surrounding it resemble those found in concentric arrangement in the secretory units. This arrangement of cells was a rare occurrence in the cultured tissue and may have resulted from a group of cells that were out of phase at the time of explantation and in which further development was blocked *in vitro*.

Glands explanted at the 4th day of the pupal stage and cultured in medium with or without ecdysterone were able to undergo terminal stages of differentiation

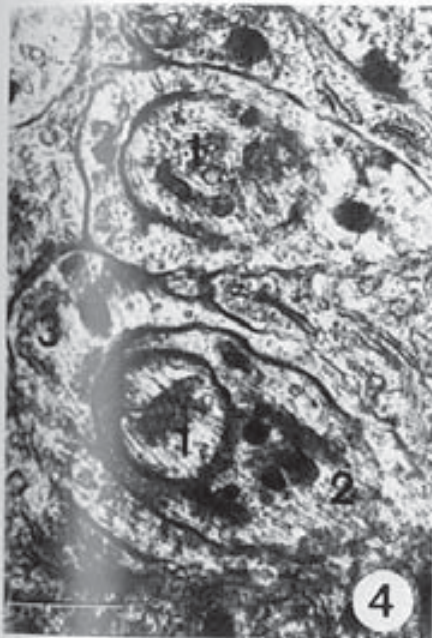
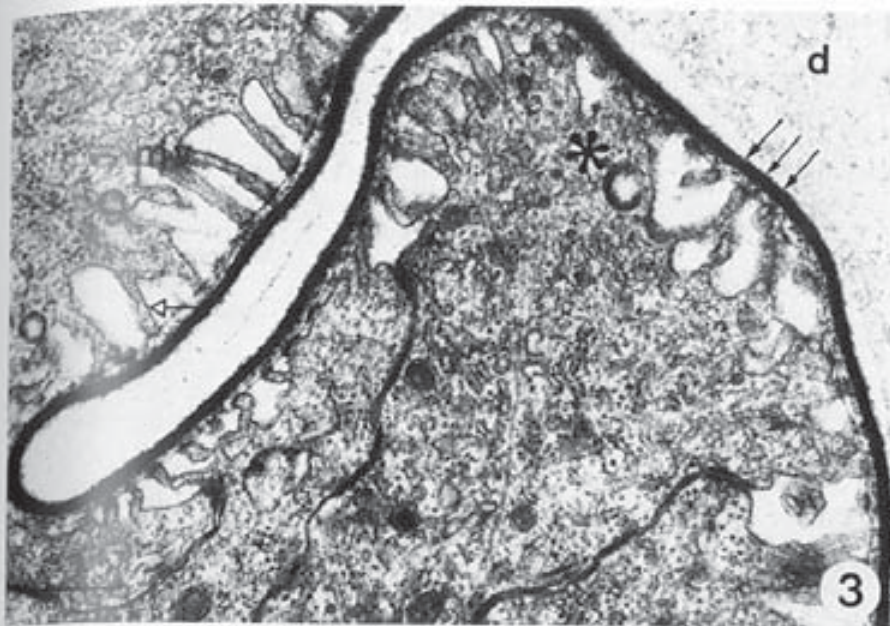


Fig. 3. SAG from 6-day pupa in situ. Note epicuticle (arrows); regular microvilli with plaques (*mv open arrow*); secretory vesicles fusing with plasma membrane (*asterisk*) and ductule (*d*). $\times 26,600$

Fig. 4. SAG from 5-day pupa in situ. The 3-cell secretory units (*1, 2, 3*) have assembled. Cell 1 will produce the pseudocilium. $\times 17,300$

Fig. 5. Apical surface of cells in the axial duct from 2-day pupal SAG cultured for 6 days in vitro with ecdysterone. Note that the epicuticle (*arrows*) has formed, and there is a dense layer (*den*). Secretory vesicles (*asterisk*) are found at the plasma membrane. $\times 20,000$. Scale bar = $0.5 \mu\text{m}$

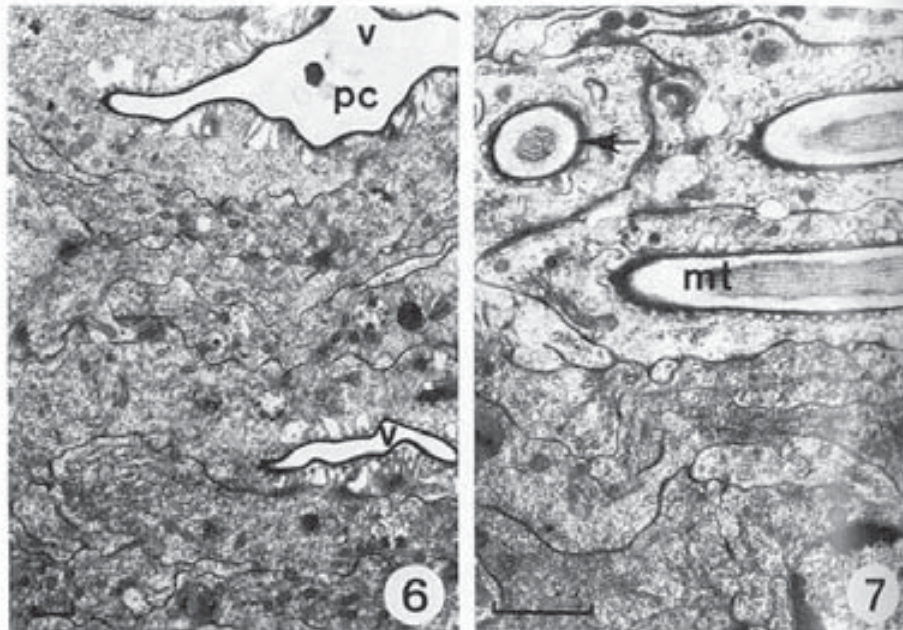


Fig. 6. SAG from 6-day pupa in situ showing vestibules (*v*) in the epithelium containing remnants of a pseudocilium (*pc*). $\times 6,300$

Fig. 7. Degeneration of the microtubules in the pseudocilia of SAG from a 6-day pupa in situ. Note microtubules (*mt*) and efferent ductule formation (*arrow*). $\times 13,600$

Fig. 8. SAG from a 2-day pupa cultured in vitro with $5 \mu\text{g}$ ecdysterone/ml for 4 days. Microvilli developed at the apical surface (*arrows*), and project into lumen (*lu*). Secretory vesicles are found near the apical membrane (*asterisk*). $\times 7,800$

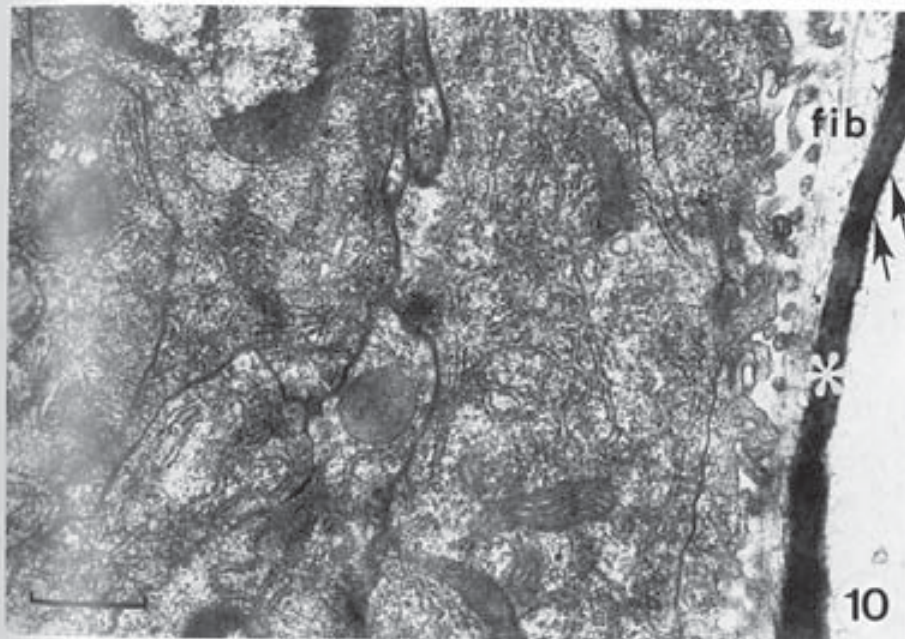
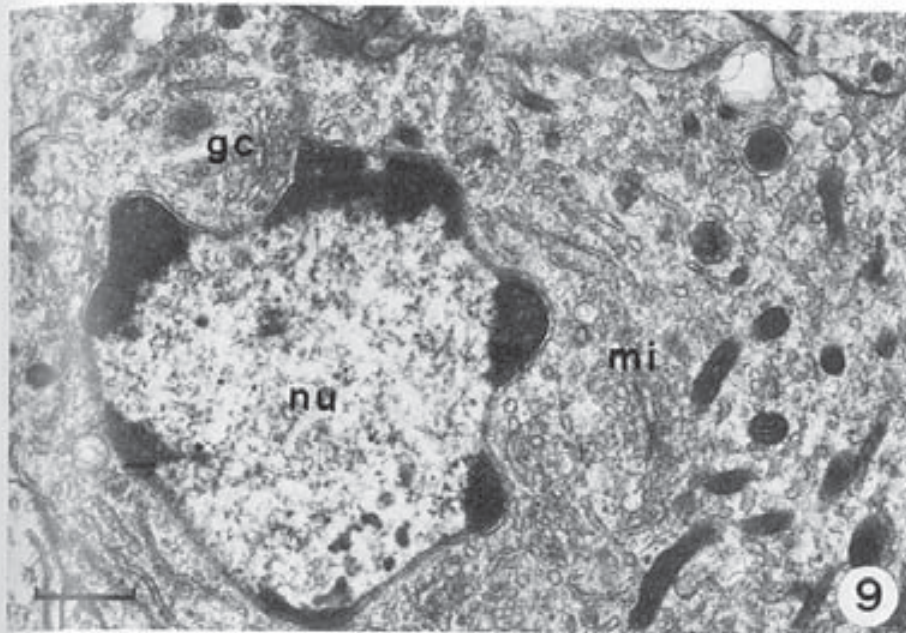


Fig. 9. Epithelium of 2-day pupal SAG cultured with ecdysterone in vitro for 4 days. Note irregularly lobed nucleus (*nu*), Golgi complex (*gc*), mitochondria (*mi*). There is no evidence of assembly into 3-cell secretory units. $\times 14,300$

Fig. 10. SAG from a 4-day pupa cultured for 4 days in vitro without hormone. Outer epicuticle (*arrows*), inner epicuticle (*asterisk*) and fibres (*fib*). $\times 33,300$. Scale bar = $0.5 \mu\text{m}$

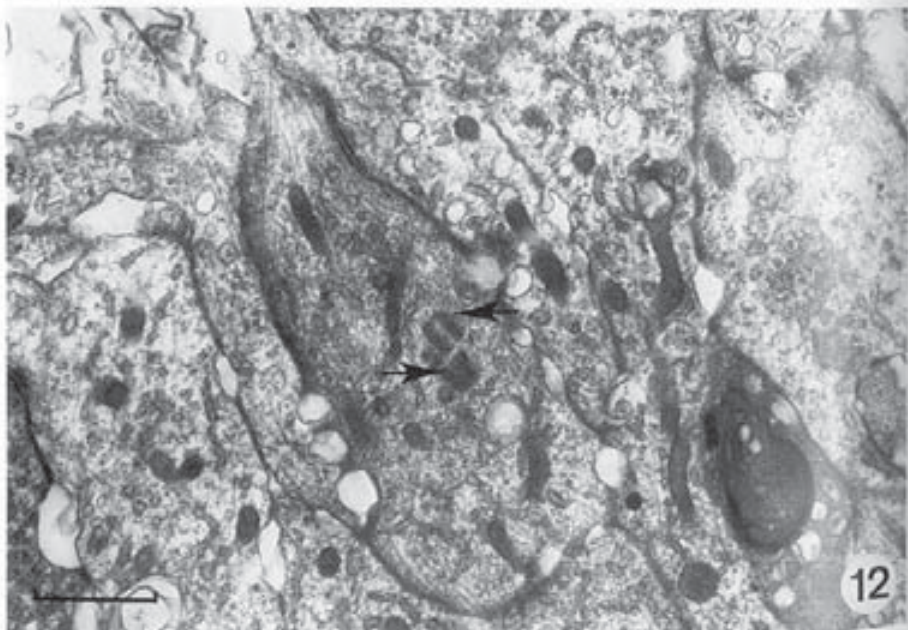
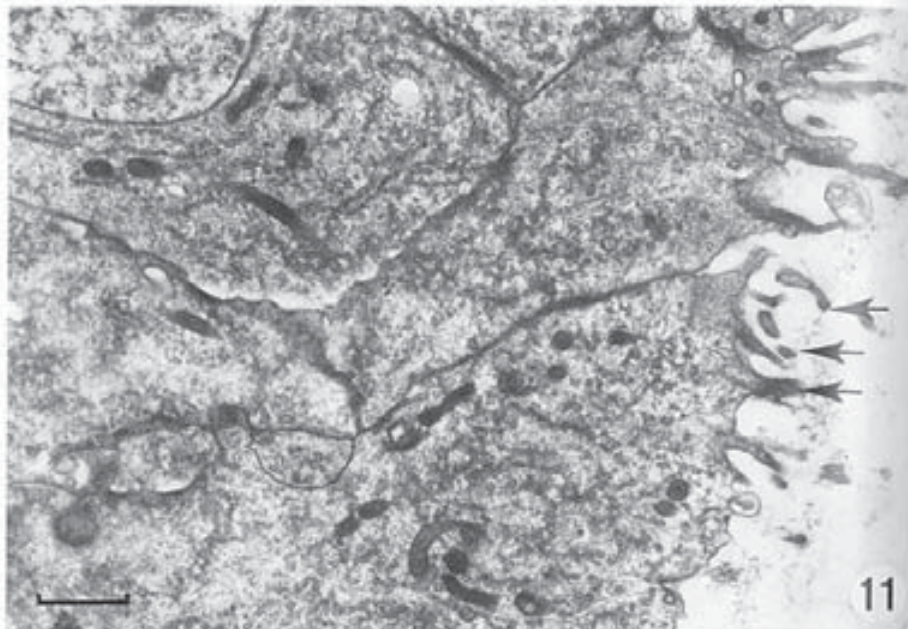


Fig. 11. SAG from a 2-day pupa cultured in medium without hormone for 6 days. Irregular projections at the apical surface of cells remain (*arrows*) and there is no rearrangement. $\times 13,000$

Fig. 12. SAG from a 2-day pupa cultured in medium without hormone for 6 days. The centrioles (*arrows*) are in the alignment such as is found in cell "1" from which the pseudocilium arises. Surrounding cells resemble the concentric arrangement found in the secretory unit. Instances such as these were found only occasionally. $\times 17,300$

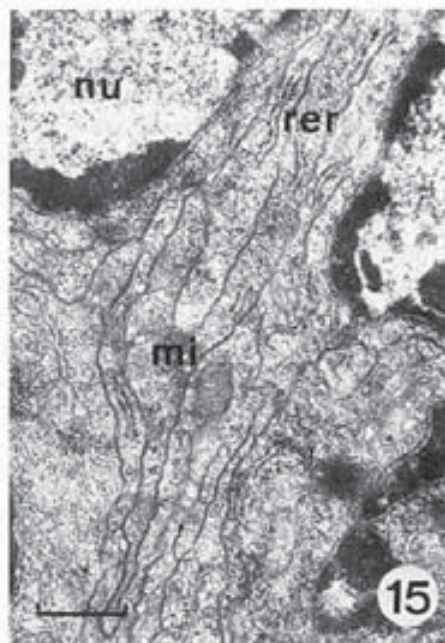
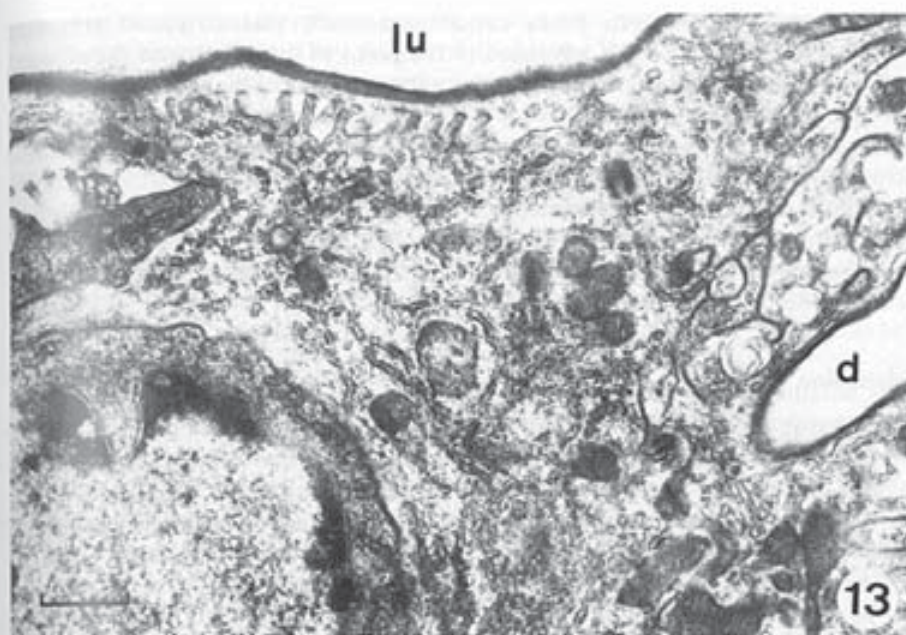


Fig. 13. SAG from a 4-day pupa cultured with ecdysterone for 4 days. Note cuticle lined lumen (*lu*) and efferent ductule (*d*). $\times 23,400$. Scale bar = $0.5 \mu\text{m}$

Fig. 14. SAG from a 4-day pupa cultured without ecdysterone for 4 days. Empty efferent ductules have formed (*d*), all cuticle lined (*arrows*). $\times 26,200$. Scale bar = $0.5 \mu\text{m}$

Fig. 15. SAG from a 4-day pupa cultured in vitro without hormone for 4 days. No general deterioration of tissue has occurred: mitochondria (*mi*); nuclei (*nu*); rough endoplasmic reticulum (*rer*). $\times 18,600$

(Figs. 10, 13–15). In both bases cuticle deposition was advanced (Fig. 10). Pseudocilia had formed and advanced to the point of retraction and the efferent ductule had formed (Figs. 13, 14). In culture the assembly of the cells and growth of pseudocilia continued irrespective of the presence or absence of ecdysterone. Similarly, cuticle deposition in the axial duct, vestibules and ductules was also achieved with or without ecdysterone.

The time course of development of the glands taken from 4-day pupae and cultured in vitro approximated that in situ (Fig. 15). In other respects, too, the medium proved suitable for culture of the tissue and for investigation of the role of ecdysterone in the differentiation of the pupal gland.

Discussion

Considering the development of the gland in vivo an initial phase of cellular proliferation by mitoses is followed by further growth and differentiation, as is typical of many differentiating tissues (White 1973; Johnson and Berry 1977).

As a result of these divisions two populations of cells are formed, the first of which gives rise to the secretory units and eventually cuticular ductules, and the second consisting of cells that produce the thick cuticle of the axial duct.

In basal S-20 medium, 4-day pupal explants differentiate normally but 2-day explants do not develop. At the time of explantation from 4-day pupae the glands have completed both the proliferative and differentiative divisions. The cells that would assemble to form the secretory unit had already been produced. But at 2 days only part of the proliferative divisions had been completed and the differentiative ones had not even started. We suspect that the reason for the failure in the differentiation of the 2-day gland may be due to blockage of cell division at explantation to the in vitro culture. Cell division is important in the acquisition of competence for metamorphosis in imaginal discs of *Drosophila melanogaster* (Mindek and Nöthiger 1973) and for transdetermination also (Gehring 1966, 1972; Tobler 1966; Mindek 1968; Wildermuth 1968).

The S-20 medium also proved suitable for investigation of the role of ecdysterone in the differentiation of the gland. The ecdysteroid content of *T. molitor* has been determined by Delbecq et al. (1978). The ecdysterone level is slightly elevated after pupal ecdysis and at day 3 begins to rise, reaching a maximum of 4000 ng/ml at day 4. The levels of the hormone then fall until they reach the basal concentration (100 ng/ml) at day 7–9. Since some of the developmental events occurring in the spermathecal accessory gland, especially cuticulogenesis, coincide in time with the peak of ecdysterone, it seemed probable that this hormone would be involved in the differentiation of the gland.

Most studies of the effects of ecdysterone in vitro have been concerned with imaginal discs, the epidermal tissue or primary reproductive organs (Fristrom and Yund 1976; Marks 1976, 1980; Oberlander 1976). In some systems ecdysterone is required not only for the initiation of differentiation but also for its maintenance. The imaginal wing discs of *Pieris brassicae* were cultured in vitro by Blais and Lafont (1980) who found that the response of the imaginal discs was dependent on the ecdysterone and was effected by the concentration of the hormone. Although all of the hormone concentrations used by them were within the physiological range

measured during metamorphosis (Lafont et al. 1976), they did not always induce imaginal differentiation. They suggested that basal levels of ecdysterone act on differential events like mitoses, and then the large peak that occurs during metamorphosis synchronizes cuticle secretion in the cells.

The spermathecal accessory gland of the 2-day pupa was able to lay down cuticle in vitro only if exposed to exogenous ecdysterone. We presume the control glands explanted at 2 days failed to develop because they had not been exposed to hormone in situ or in vitro. But when the glands which had grown for 4 days in situ were placed in culture, they secreted cuticle whether or not exogenous ecdysterone was added. The glands would have already experienced a rising concentration of ecdysterone which was sufficient to initiate cuticle deposition. Once the process had begun, ecdysterone was not required for its continuation.

The formation of the pseudocilium and rearrangement of the cells into the 3-cell secretory organules did not follow the same pattern. Pseudocilia never formed in 2-day explants, with or without ecdysterone, and always formed in 4-day explants, with or without ecdysterone. It is possible that ecdysterone has nothing to do with formation of pseudocilia.

It is also conceivable that its development depends on an unknown factor that is not present in the culture medium, e.g., bursicon. However, there is a strong likelihood that there has been an interference with differentiative divisions whereby the three cells that eventually assemble as the secretory unit are formed. This could occur as a direct action on the dividing cells or indirectly by affecting the production of ecdysterone. Low concentrations of ecdysterone may be required for the stimulation of differentiative divisions as was found for the imaginal wing discs in *P. brassicae* (Blais and Lafont 1980). Significantly, a "wounding" effect has been found in young pupae. When 0-2 day pupae are injected with inhibitors of DNA synthesis or of microtubule assembly, or with the corresponding solvents of these drugs, development of the pseudocilium was prevented, whereas cuticle deposition occurred by the end of the pupal stage (unpublished results). Cell division is considered to be an essential step of differentiation and pattern formation in many systems (Ursprung 1968).

The relatively high concentrations of ecdysterone used in both in vivo and in vitro treatments may have inhibited cell divisions. During the mitotic phase of gland development in the pupa the levels of ecdysterone rise from a low, basal concentration to a peak at day 4-5. The high doses of ecdysterone applied in vivo may have resulted in "hyperecdysonism" first described by Williams (1968). Similarly inhibition of development by high concentrations of ecdysterone have been observed in vitro (Milner 1977a, b; Blais and Lafont 1980).

An obvious change in competence of the spermathecal accessory gland to respond to ecdysterone occurs at some time between day 2 and 4 in vitro, and possibly earlier, in the prepupal stage. It primarily affects the cuticle-secreting cells, which are not competent to respond to the hormone at an earlier stage; the response at day 4 is limited or fixed, probably by ecdysteroid receptor sites. Such sites have been demonstrated in the K_c cell lines of *Drosophila melanogaster* (Maroy et al. 1978) and in the imaginal discs of *D. melanogaster* (Yund et al. 1978; Yund 1980). Changes in competence to respond to ecdysterone have been reported in many cases, for example, in the imaginal discs of *Drosophila* (Bodenstein 1943;

Oberlander 1969; Mindek and Nöthiger 1973; Schubiger 1974; Gateff and Schneiderman 1975; Kurushima and Ohtaki 1975), and in the cricket epidermis of *Manduca sexta* (Fain and Riddiford 1977).

The results that we have obtained show clearly that cuticle deposition, which is one of the main phases of differentiation in the spermathecal accessory gland of *T. molitor*, is ecdysterone-dependent. Pseudocilium formation, however, appears to be independent of the ecdysterone peak but is possibly hormonally controlled.

Although the foregoing investigation leaves a number of questions unanswered, it demonstrates an in-vitro culture system which, with some further modifications, will allow us to analyze the events occurring in the gland. It is especially important to consider and to mimic the fluctuations of the endogenous hormone in the insect in a culture system and to use physiological doses as demonstrated by Beckemeyer and Lea (1980). This type of study is being undertaken at present especially to determine whether basal levels of ecdysterone are essential for differentiative mitoses and to gain more details of the commitment and expression of characters by the cells of the gland under the influence of ecdysterone.

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