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ULTRASTRUCTURAL CHANGES OF *DROSOPHILA* LARVAL AND PREPUPAL SALIVARY GLANDS CULTURED *IN VITRO* WITH ECDYSONE

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SUMMARY

Alterations in the ultrastructure of *in vitro* cultured larval salivary glands of *Drosophila melanogaster* in response to the steroid hormone ecdysone were studied in relation to complex changes in puffing patterns. We found that the changes in the fine structure of cultured glands reflected progression of the puffing pattern, and they paralleled those seen *in vivo*. We observed that glue secretion by exocytosis, the main function of salivary glands, took place between puff stage 5 (PS5) and PS7. Glue could not be expectorated under culture conditions but was slowly released from the lumen through a duct into the medium. After the cultured glands reached PS13/PS14, further progress of puffing and fine structural alterations required that the ecdysteroid titer be transiently extremely low or absent. Under *in vitro* conditions we did not observe the putative new secretory program(s) described for glands *in vivo* after PS12. However, ultrastructural changes which unambiguously indicated that an autohistolytic process had begun *in vitro* started to appear after PS17. Many salivary gland cells developed numerous features of progressive self-degradation between PS18 and PS21. Actual degradation of salivary glands *in vivo* seemed to be rapid, but *in vitro* degradation was never completed, probably due to a lack of exogenous factors from the hemolymph. Manipulations of ecdysone titer *in vitro* in the culture medium, known during the larval puffing cycle to cause premature induction of developmentally specific puffing patterns, did not affect the normal development of ultrastructural features of the cytoplasm and nucleus.

Key words: ecdysone; metamorphosis; Drosophila; salivary glands; electron microscopy.

INTRODUCTION

20-Hydroxy-ecdysone (20HE), a steroid hormone of insects and other arthropods, triggers many developmental processes including metamorphosis. In *Drosophila melanogaster* the activity of many genes in salivary gland polytene chromosomes is induced by the hormone at the time of metamorphosis. The sites of active gene transcription are seen as puffs which represent local decondensations of euchromatin (Beermann, 1956, 1972; Panitz, 1968; Berendes, 1972). A complex pattern of puffing changes occurs during the last few h of larval and early prepupal development of *Drosophila* which reflects the hierarchic regulatory network of genes involved in programmed cell differentiation.

In general, studies of puffing and related phenomena have led to several interesting findings, including heat shock syndrome (Ritossa, 1962, 1963, 1964; Tissières et al., 1974; Lewis et al., 1975), sequential gene activation by ecdysteroids (Becker, 1959, 1962; Ashburner, 1972b, 1973, 1974; Ashburner and Richards, 1976; Richards, 1976a, 1976b, 1976c), the correlation between specific puffs and production of secretory mucoproteins (Korge, 1975, 1977a, 1977b; Crowley and Meyerowitz, 1984), dependence of early ecdysone response on products of *Broad-Complex* (*BR-C*) genes (Belyaeva et al., 1980, 1981; Zhimulev et al., 1982), and the effects of ribonuclease on puffing activity (Ritossa and von Borstel, 1964; Ritossa et al., 1965). Investigations of the factors involved in chromosome puffing have been facilitated by *in vitro* techniques in which larval salivary glands have been explanted and maintained in saline or culture medium. The technique of culturing *Drosophila* salivary glands devised by Ashburner (1972b) allowed full control over complete larval and prepupal puffing cycles under *in vitro* conditions.

Most attention was devoted to the studies on sequential gene activation by ecdysones. Complete larval and prepupal puffing cycles were divided into 21 puff stages (PS), each characterized by a unique set of representative puffs which enabled precise determination of developmental period within larval and prepupal cycles (Ashburner, 1972a; Ashburner and Berendes, 1978). Unusually interesting hierarchic interactions of more than 100 puff-forming loci were revealed by analysis of polytene chromosomes (Ashburner, 1972b, 1972c, 1973, 1974; Ashburner and Richards, 1976; Richards, 1976a, 1976b, 1976c), and the combination of those findings with genetic tools (Walker and Ashburner, 1981) led to formulation of an ecdysone genetic model (Ashburner et al., 1974, 1990; Natzle, 1993). Subsequent molecular analysis of some ecdysone-regulated genes responsible for puffs such as *E74* for 74EF, *E75* for 75B, *BR-C* for 2B5, ecdysone receptor (*EcR*) for 42A, *βFTZ-F1* for 75CD, and *E78* for 78D (Feigl et al., 1989; Janknecht et al., 1989; Burtis et al.,

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1990; Segraves and Hogness, 1990; DiBello et al., 1991; Koelle et al., 1991; Lavorgna et al., 1993; Stone and Thummel, 1993; Talbot et al., 1993, von Kalm et al., 1994) proved the basic ideas of the model to be correct.

There is evidence for a correlation between the changes in puffing activity *in vivo* and specific developmental processes in *D. melanogaster*, studied at the fine structural level (Lane et al., 1972; von Gaudecker, 1972; von Gaudecker and Schmale, 1974). In addition, Poels (1972) and later Boyd and Ashburner (1977) showed that cultured glands are able to release accumulated secretion into the lumen during early stages (within 4–8 h) of ecdysone-induced puffing. However, no *in vitro* studies have previously attempted to correlate the puffing pattern in cultured glands with the complex ultrastructural changes of the cytoplasm during the larval/prepupal cycle and thus prove the physiological significance of the observed puffing pattern. We believed it of particular interest to examine also whether 20HE withdrawal, known to cause premature induction of late larval puffs, would result in premature ultrastructural changes of salivary glands, and also whether cultured glands could complete their development by autohistolysis under *in vitro* conditions. Our data show that changes in the fine structure of cultured glands do indeed reflect progression of the puffing pattern, and they do so on a time schedule similar to that seen *in vivo*, but prematurely induced progress in the puffing pattern cannot accelerate ultrastructural alterations of the cytoplasm. Salivary glands are unable to complete their disintegration *in vitro*, probably due to missing exogenous factors from the hemolymph.

Materials and Methods

Flies and tissue dissections. All observations and experiments were performed on wild-type strain *Canton S* of *Drosophila melanogaster*, obtained from Umea Drosophila Stock Centre, Sweden. Flies were cultured in 50 or 200-ml bottles at 22° C on standard agar–yeast–cornmeal–sucrose medium (Ransom, 1982) with the addition of nipagin to inhibit mold growth. Salivary glands of timed 3rd instar larvae and prepupae were dissected under a stereomicroscope in a saline medium (Becker, 1959), and freed of adhering fat body as much as possible. For *in vitro* experiments all salivary glands were at puff stage 1 upon initiation of culture; this was verified by cytological analysis of their chromosomes. Staging of glands has been performed in two ways. (1) The gland lobes were separated; one lobe was cultured *in vitro* and its contralateral lobe was used for chromosome examination to determine the precise developmental stage according to the puffing pattern (Ashburner, 1972b). This protocol is based on the observation that patterns of puffs of two sister lobes of salivary glands from a single larva are identical (Ashburner, 1970, 1971), and therefore one of them can be used as a control. (2) The gland lobes were not separated but cultured *in vitro*. After culturing, their lobes were separated; one of them was used for examination of puffing pattern, and the other, for light and electron microscopical analysis. To ensure the dissection of salivary glands from interecdysial larvae (PS1), fly food was enriched with 0.05% bromophenol blue (Maroni and Stamey, 1983), and only last instar larvae (at "wandering" stage) with blue contents in the gut (indicating the period prior to release of endogenous ecdysteroids) and with salivary glands 950–1000 μm long were taken for experiments.

To have *in vivo* controls simultaneously with *in vitro* experiments, we observed glands explanted from larvae without culturing in medium. They were processed in the same way as glands cultured in the medium. Results of our observations have been also compared with those of previous *in vivo* studies on the ultrastructure of *Drosophila* larval salivary glands (Poels et al., 1971; Lane et al., 1972; von Gaudecker, 1972; Harrod and Kastritsis, 1972a, 1972b; Berendes and Ashburner, 1978; Thomopoulos, 1987).

Organ culture. In keeping with the series of results on puffing obtained by the culture technique developed by Ashburner (1972a), dissected salivary glands were cultured in insect Grace's medium (Grace, 1962, 1966) diluted 5:1. Complete Grace's medium was purchased from GIBCO Ltd. (Rockville, MD, USA) or made up in the laboratory from components purchased from Calbiochem AG (Lucerne, Switzerland) or Merck (Darmstadt, Germany). Culture medium was aerated prior to use. Ten salivary glands were cultured in 100 μl of media in plastic plates (Farkaš, 1991). All gland culture experiments were done at 22 ± 1° C.

20HE (β-ecdysone; Rohto Pharmaceutical Co., Osaka, Japan or Calbiochem AG, Lucerne, Switzerland) when used, was applied as a 0.5% ethanolic solution in Grace's culture medium at a final concentration of 5×10^{-6} *M*, or at the low concentration of 1×10^{-10} *M*. Ecdysone (α-ecdysone) was a gift from Zoecon Corporation (Sandoz Corp., Palo Alto, CA, USA), and it was used at a concentration of 1×10^{-8} *M*. To remove either ecdysone or 20HE, we briefly washed the cultured glands three times in 100 μl of hormone-free medium and subsequently cultured them without hormone. This simple procedure is called hormone washout or withdrawal and has previously been proved effective (Ashburner and Richards, 1976).

Light and electron microscopy. Salivary glands were fixed in 2% glutaraldehyde + 1% paraformaldehyde in 0.1 *M* cacodylate buffer, pH 7.2, for 2 h at room temperature, postfixed in 1% osmium tetroxide in 0.1 *M* cacodylate buffer, dehydrated in an ascending series of ethanol, infiltrated with propylene oxide, and embedded in Durcupan ACM resin (Fluka AG, Buchs, Switzerland) (Glauert, 1975) or Spurr's low viscosity medium (Spurr, 1969), the components of which were obtained from SERVA Feinbiochemica (Heidelberg, Germany) and Electron Microscopy Sciences (Fort Washington, PA, USA).

For light microscopy, stained semithick sections of 0.5 to 1 μm were used.

Ultrathin sections, made either on an LKB III Ultratome or a Reichert-Jung Ultracut ultramicrotome equipped with a glass knife, were contrasted with uranyl acetate (Watson, 1958) or uranyl formol (Mazza and Casale, 1979; Mazza et al., 1981) and lead citrate (Reynolds, 1963) as modified by Sato (1968), and examined in Tesla BS-500 or Jeol JEM-1200 EX electron microscopes operating at 80 or 60 kV, respectively. Photographs were taken with an ORWO EU2 film or AGFA 23D56 foil EM film. Whenever possible, we preferred to examine semithick sections under light microscope and the ultrathin section under electron microscope, with both types of sections cut from the same sample.

We should stress that the subjects of this paper were corpus and transitional cells of the salivary gland which are known to produce and secrete mucoproteins (Berendes and Ashburner, 1978). The collum cells, which possess polytene chromosomes as well but do not produce any secretion, will be the subject of another paper.

Results

Larval puffing cycle. Interecdysial salivary glands dissected from PS1 larvae when prominent puffs at 3C, 25AC, 42A, 58DE, 68C, 85E, and 90BC could be detected were actively engaged in the formation of numerous electron-dense membrane-bound secretory granules, which in a few h occupied most of the cytoplasm (Fig. 1 *a*). The nucleolus was very apparent (Fig. 1 *b*) and nuclear pores were numerous (Fig. 1 *c*). This period corresponds to the intense synthesis of secretory mucoproteins which are known to be components of the "glue" (Korge, 1975, 1977a, 1977b; Beckendorf and Kafatos, 1976). Rough endoplasmic reticulum was abundant and was present mostly as parallel stacks or concentric whorls (Fig. 1 *d*). The Golgi complex at this stage was large and its saccules appeared to elaborate small electron-dense vesicles, which seemed to be precursors of larger secretory granules with diameters up to 7 μm. During the first 4 h of culture in the medium with 20HE, when glands underwent a transition from PS1 to PS2 and reached PS4 (characterized by prominent early puffs at 2B5, 23E, 74EF, and 75B), the secretory granules grew and started to concentrate near the luminal membrane (Fig. 1 *e*). These secretory granules contained three structurally different components, one of them with a characteristic internal paracrystalline substructure (Fig. 1 *f*). Salivary glands which reached PS5 under culture conditions showed signs of secretion of mucoprotein granules into the lumen by exocytosis (Fig. 2 *a*) undistinguishable from that described for glands *in vivo*. The exocytotic event, when granule contents were released into the lumen, was not seen as frequently as would be expected due to the large amount of mucoprotein to be secreted. Glue secretion, similar to granule production, could be seen first in corpus cells at the distal end of the gland lobe and proceeded anteriorly towards transitional cells. By carefully examining many

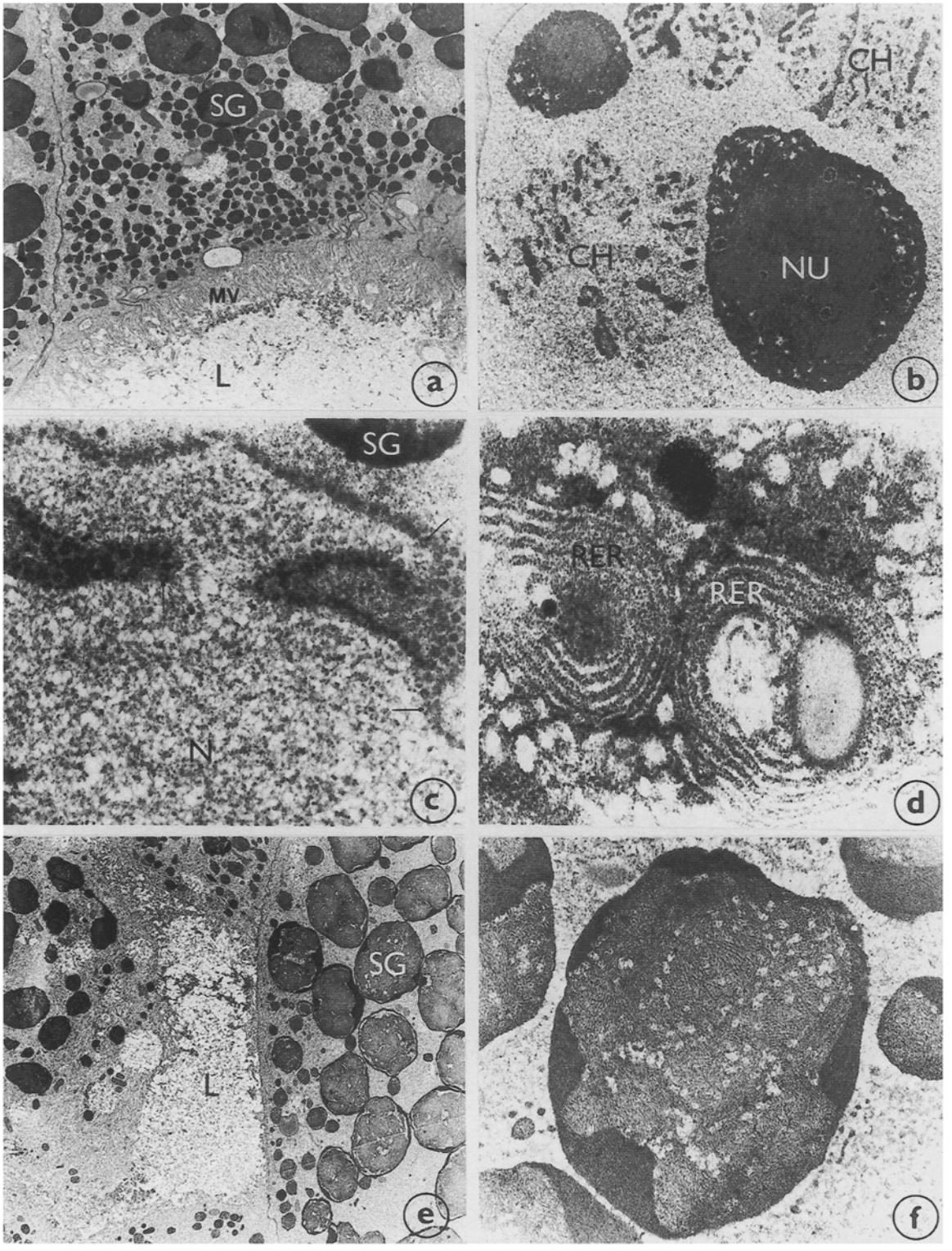

FIG. 1. *a*, The salivary gland of a PS1 larva showing cells actively engaged in production of numerous electron-dense secretory granules (SG) which gradually grow by fusion from less than 0.3 μm up to 7 μm. MV = microvilli; L = lumen. Magnification, ×5600. *b*, Large nucleolus (NU) (split into two pieces in this section) from PS1 gland. Polytene chromosomes (CH) are also shown. Magnification, ×19 800. *c*, Numerous pores (*thin arrows*) visible in nuclear membrane and its infoldings of PS1 gland which is synthetically active, as illustrated by the portion of secretory granule (SG) in the cytoplasm. N = inside of nucleus. Magnification, ×46 450. *d*, Detail of two whorls of circular rough endoplasmic reticulum (RER) from PS1 gland. The whorls contain various droplets and vacuole-like structures. Magnification, ×23 200. *e*, Mature secretory granules (SG) tend to concentrate around the lumen (L) of the gland which has reached PS4 by culturing *in vitro* with 20HE. Magnification, ×5600. *f*, Detail of large mature secretory granule from PS4 gland shortly before secretion. *Note* characteristic paracrystalline substructure. Magnification, ×30 100.

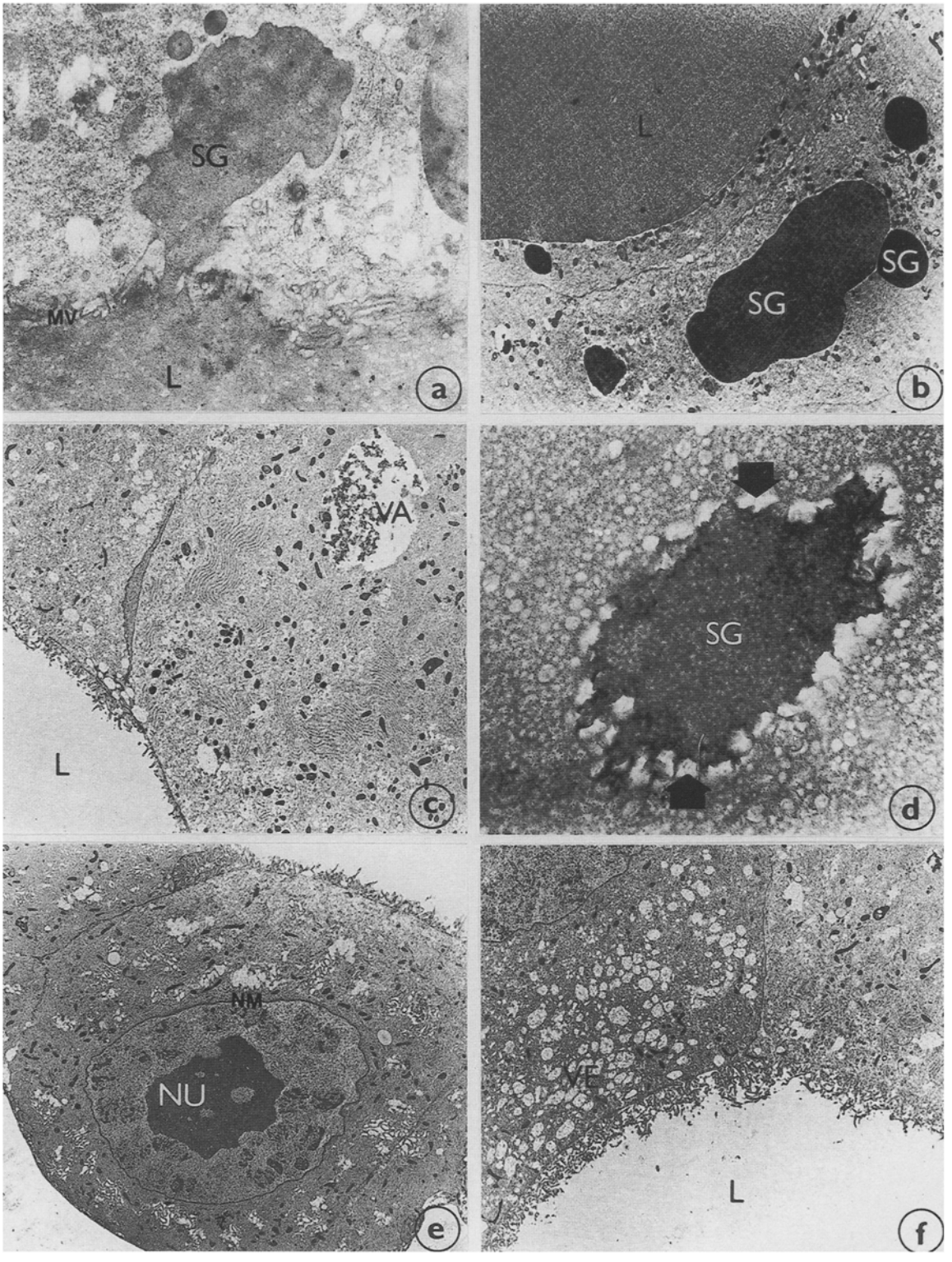

FIG. 2. *a*, Application of 20HE-initiated *in vitro* glue secretion into lumen (L) at PS5. Membrane of secretory granule (SG) fuses with the cytoplasmic membrane at the luminal surface, and its contents are released. The lumen is filled with a secretion which loses its paracrystalline substructure upon exocytosis. MV = microvilli. Magnification, ×10 300. *b*, Structure of the luminal portion of a salivary gland cell after reaching PS8 under *in vitro* culture. The lumen (L) is completely filled with amorphous secretion. Few granules (SG) are left over after secretion is complete, and they tend to continue to grow by fusion, becoming an unusual shape and attaining extreme size. *Note* that the lumen at this stage (so-called bloating) displays no microvilli. Magnification, ×5600. *c*, Type of vacuole (VA) which starts to appear after secretory glue is exocytosed from the cells and expectorated from the lumen. Electron-dense contents of these vacuoles are irregular in size and scattered throughout. L = lumen. Magnification, ×20 600. *d*, Vacuolization (*arrows*) of the periphery of ageing secretory granule (SG) in the salivary glands after secretion of glue into the lumen. This type of granules is present from PS9 through PS12/PS13. Magnification, ×24 100. *e*, Salivary gland cultured *in vitro* with the hormone up to PS14. The cytoplasm is cleared of any granules. *Note* apparent size of nucleolus (NU). However, the nuclear membrane (NM) has no dilated infoldings and thus displays a lowered number of pores. L = lumen. Magnification, ×4980. *f*, Vesiculation (VE) of the cytoplasm during PS16 to PS18 stages when salivary glands were incubated in hormone-free medium. The difference in the degree of vesiculation of the next cell is apparent and indicates already at this stage the possible discrepancy in the speed of ultrastructural alterations between neighboring cells. L = lumen. Magnification, ×10 300.

serial sections, we observed that granule exocytosis, at least in corpus cells, could be seen mainly from PS5 through PS7 (the period represented by puffs 12E, 62E, 71CD, and 78D), but rarely later. The luminal surface of gland cells immediately after secretion showed a greatly reduced number of microvilli (Fig. 2 *b*). Accumulated secretion was gradually released from the lumen between PS9 and PS11, when new puffs 21F, 22C, 29F, 63E, 82F, 91D, and 98F were present, and previously active puffs 12E, 62E, and 78D, after reaching their maximum size, quickly regressed. Glue could not be expectorated *in vitro* in the same way as in intact larvae; it was slowly released into culture medium. This process can be easily monitored also under a dissecting or phase-contrast microscope on living glands.

After PS7/PS8 and onwards, the area occupied by the Golgi complex was reduced, the saccules did not contain electron-opaque material, and their appearance was rather vacuolated. Stacked cisternae of endoplasmic reticulum, however, remained numerous, thus indicating continuation of some synthetic activities. Many small and a few larger cytoplasmic vacuoles bearing flocculent material, which were already noted in PS1–PS5 glands, were still present during PS13/PS14. After secretion was released through the duct, the flocculent material could again be seen in the lumen. A new type of vacuole with irregular electron opaque material (bodies) was visible within the cytoplasm (Fig. 2 *c*).

Even though the process of secretion was complete by PS7/PS8, not all of the glue secretory granules were extruded; a few granules were almost always left over in the cytoplasm. While secreted glue remained in the lumen, granules remaining in the cytoplasm continued to grow (Fig. 2 *b*) and simultaneously some of them started to show vacuolization of the periphery (Fig. 2 *d*). Central parts of these granules still had a well- preserved paracrystalline substructure which allowed their easy identification. Such granules were identical to previously described electron-dense bodies with a vacuolated periphery. Some of the granules at this stage reached a larger diameter than granules in the secretory phase. The cytoplasm was cleared of remaining secretory granules by PS13/PS14, the stage when the activity of puffs at 34A, 46F, and 90BC culminated. At this time the luminal membrane again displated numerous microvilli, albeit not as many as in stages PS1 to PS4 (Fig. 2 *e*).

Prepupal puffing cycle. With the continuous presence of 20HE in the medium, the larval puffing cycle proceeded from PS1 to the prepupal puffing pattern at PS13/PS14 which took about 12 to 14 h to complete; the puffing sequence induced by continuous culture with 20HE, however, did not progress beyond that stage (Ashburner, 1972b). As described by Richards (1976a, 1976b, 1976c) further progress of puffing in cultured glands requires that the ecdysone titer be transiently very low or absent for at least 3 h. During this period, while mid-prepupal puffs (represented by 52A, 63E, and 75C) were active, the late prepupal puffs (e.g., 62E, 74EF, 75B, and 93F) acquired competence to respond to elevated 20HE and thus to complete the second puffing cycle by reaching PS21. The time course from PS13/14 to PS21, *in vivo* as well as *in vitro*, was 9 to 11 h. To learn whether ultrastructural changes of salivary gland cells also progress as they do *in vivo*, glands in which progression from the completed larval puffing cycle to the prepupal cycle was initiated by temporary absence of 20HE in the medium were processed for light and electron microscopy. During the period of no or very low ecdysone titer, when mid prepupal puffs 52A, 63E, and 75C were active (PS16 through PS18), the first signs of vesiculation of the cytoplasm were seen (Fig. 2 *f*). The nuclear membrane, the outline of which previously was circular, became folded and crenated (not shown). There was also an irreversible decrease in the number of nuclear pores, which may indicate a reduction in overall synthetic activity. If glands were kept in hormone-free medium for more than 3 h and late prepupal puffs (22A, 62E, 74EF, and 93F) could not be induced, no other structural changes in the cytoplasm occurred.

When 20HE was added after the hormone-free period, late prepupal puffs (PS19) and strong vacuolization of the cytoplasm were noted within 1 to 1.5 h (Fig. 3 *a*). The luminal cell membrane might become interrupted in several places and sometimes cytoplasmic components and organelles could be found in the lumen. The apical membrane and outside basal lamina remained uninterrupted. During this period, nuclear blebbing became apparent. The degree of vacuolization of individual cells might be different, and two neighboring cells, one with very progressive vacuolization plus degeneration of cytoplasmic structures, the other with slower development, were often found (Fig. 3 *b*). When culture of the glands which reach PS21 was continued for another 3–6 h, all cells became highly vacuolated (Fig. 3 *c*) and no difference in degree of degeneration between individual cells was noted. Moreover, vacuoles reached extreme sizes. The outline of previously folded and crenated nuclear membrane was again circular. Although no other structural changes in gland cells were then recognized, glands became more fragile upon any manipulation, e.g., the gland proper would break if these glands were transferred by forceps placed on the ducts. This procedure could be normally performed on glands just a few h before this stage. We did not observe complete breakdown of salivary glands *in vitro*. Even when PS16/17 glands were dissected from the prepupa, they did not complete breakdown in culture medium.

Manipulations of ecdysteroid titer. If PS1 salivary glands were cultured for 12 to 16 h in the absence of 20HE, their Golgi complex became smaller (Fig. 3 *d*), the areas of rough endoplasmic reticulum were reduced, and free ribosomes in cytoplasm appeared more abundant. However, we observed that if PS1 glands were dissected directly to medium containing low concentrations of 20HE (10^{-10} *M*) or ecdysone (10^{-8} *M*) and maintained in such medium for 12 h, the Golgi stayed large and active and endoplasmic reticulum still occupied large areas (Fig. 3 *e*). In the presence of these low hormone concentrations, neither induction of early puffs nor secretion of mucoprotein granules into the lumen was observed. Addition of 20HE up to 5×10^{-6} *M* anytime during the culture in low hormone medium resulted in normal structural alterations of salivary gland cells, preceded by the puffing pattern.

Late larval puffs, represented by 21F, 22C, 29F, 63E, and 82F, normally occurring at 9–11 h of continuous culture, could be prematurely induced by hormone washout 5 or 6 h earlier. These late puffs are typical for the period between PS9 and PS12. Nevertheless, at the moment of hormone washout (4 h of culture), salivary glands were in PS4 or between PS4 and PS5. To find out whether hormone removal which accelerated appearance of PS9 to PS12-specific puffs could also accelerate development of salivary glands at the cellular level, we investigated the ultrastructure of glands in which premature puffing of the above mentioned loci was induced by 20HE withdrawal. The late puffs were induced within 0.5 h of hormone withdrawal and reached their maximum size about 4 h later. Anytime during the first 2 h of 20HE withdrawal, salivary gland cells were secreting mucoprotein glue into the lumen (Fig. 3 *f*). At 4 h of withdrawal, the lumen was filled with secretion and the number of microvilli was reduced. In the next 2–3 h, secretion was gradually re-

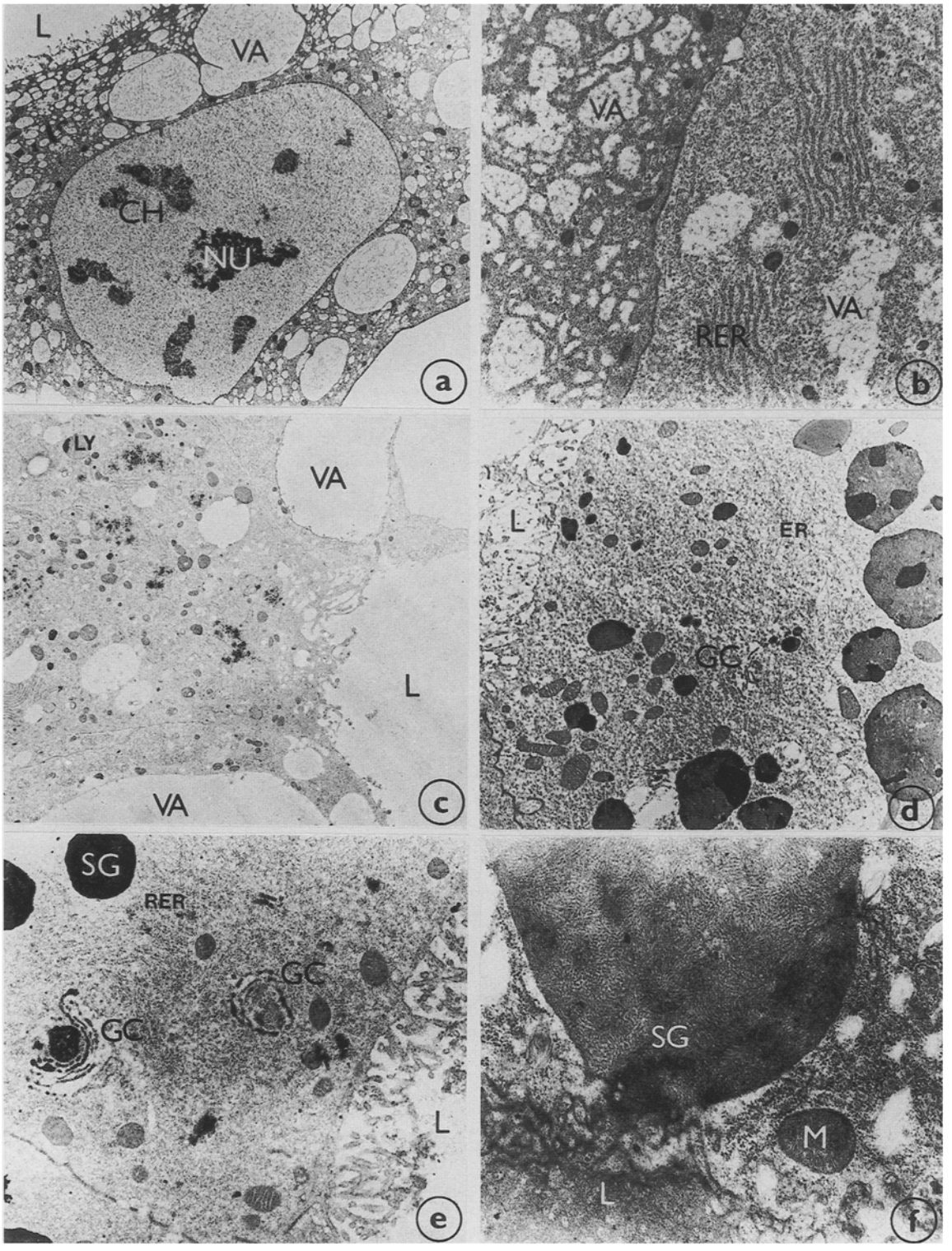

FIG. 3. *a* Strong vacuolization (VA) of the cytoplasm at PS19 induced by readdition of ecdysone to culture medium. Polytene chromosomes (CH) and nucleolus (NU) become diffuse at this stage, indicating their progressive histolysis. L = lumen. Magnification, ×7650. *b*, Difference in degree of cytoplasmic vacuolization between two neighboring corpus cells in PS19/PS20 gland cultured *in vitro* viewed at higher magnification. The left cell is more progressively degenerated than the one on right side which still contain some stacks of rough endoplasmic reticulum (RER) and fewer vacuoles (VA). Magnification, ×18 920. *c*, Status of two neighboring corpus cells in gland cultured for 4 h after reaching PS21 *in vitro*. Both cells display the same high degree of cytoplasmic degeneration. *Note* that vacuoles (VA) are now extremely large and presumably tend to associate with the lumen (L). The cytoplasm also contains lysosome-like bodies (LY). *Arrowheads* indicate junction of cytoplasmic membranes between two cells. Magnification, ×8600. *d*, View of the cytoplasm of PS1 gland cultured for 12 h without the hormone. *Note* diminished Golgi complex (GC), presence of free ribosomes, and diffused areas of endoplasmic reticulum (ER). L = lumen. Magnification, ×11 600. *e*, Prominent Golgi complexes (GC) with rough endoplasmic reticulum (RER) organized in stacks present in PS1 gland maintained in culture medium with low concentration of 20HE (1×10^{-10} *M*) for 12 h. Golgi are evidently engaged in active elaboration of small electron-dense granules which are precursors of large secretory granules (SG). L = lumen. Magnification, ×11 600. *f*, Early stage of exocytosis of secretory granule (SG) into the lumen (L) in the gland in which late larval puffs have been prematurely induced by hormone withdrawal after initial 4 h of culture. M = mitochondrion. Magnification, ×21 370.

leased from the lumen as described above. In other words, premature induction of puffs typical of the late larval period could not speed up the process of glue secretion or accelerate premature appearance of structures typical for very late larval and early prepupal stages. When glands were allowed to complete secretion and then processed for electron microscopy, the lumens were free of mucoprotein secretion and again contained flocculent material. By these criteria, glands after hormone withdrawal were indistinguishable from glands at the comparable times of culture in the continuous presence of 20HE.

Discussion

Data presented in this paper showed that the sequential development of major morphological characteristics observed in cultured salivary glands was very similar, if not identical, to that described for glands explanted from late last instar larvae and prepupae of *D. melanogaster* (Lane et al., 1972; von Gaudecker, 1972) *D. hydei* (Poels et al., 1971), and *D. pseudoobscura* (Harrod and Kastritsis, 1972a, 1972b). We suggest that the sequence of puffs induced by 20HE in salivary glands cultured *in vitro* is physiologically significant, as seen by the morphological alterations of their cells.

In comparison to data obtained from *in vivo* studies, the application of culturing technique allowed us to draw a few interesting conclusions about the relationship between puffing and salivary gland morphology. In the time when early puffs (2B5, 23E, 63F, 74EF, and 75B) reached their maximum size and a series of new puffs (62E, 71CD, and 78D) was induced (Ashburner, 1972a, 1972b; 1974; Ashburner and Richards, 1976), glands became actively engaged in the secretion of glycoprotein granules into the lumen. However, the activity of puffs at 62E, 71CD, and 78D did not seem to be necessary for secretion, as revealed by experiments when 20HE was removed from the culture medium, when a group of late larval puffs (21F, 22C, 29F, 63E, and 82F) was prematurely induced at a time when 62E, 71CD, and 78D should have been active. Under the conditions of the premature appearance of late larval puffs, the early late larval puffs 62E, 71CD, and 78D were either not induced or quickly regressed if they began activation (Ashburner and Richards, 1976; Walker and Ashburner, 1981). By using transcription and protein synthesis inhibitors, Poels (1971) and Boyd and Ashburner (1977) found that for stimulation of secretion into the lumen, the first 3–3.5 h of 20HE action was necessary. This period coincides with the activity of early puffs 2B5, 23E, 63F, 74EF, and 75B. It is tempting to speculate that products of these loci may be responsible for stimulation of granule movement and secretion. Recent studies on expression of *BR-C, E74, E75,* and *E63*, genes cloned from 2B5, 74EF, 75B, and 63F puffs show that their transcription culminates within 3–4 h after 20HE administration or after release of endogenous ecdysteroids (Andres et al., 1993; Karim et al., 1993; Andres and Thummel, 1995). The former three genes encode transcription factors (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991) and could possibly control processes associated with cessation of synthesis of secretory components, whereas *E63* codes for a calmodulin-like Ca^{2+} binding protein which might be involved in the subsequent exocytosis of secretory products. To prove this will require more experimentation, although work of Kiss et al. (1988) and Vijay et al. (1988) provides some evidence that initiation of the synthesis of secretory mucoproteins and formation of secretory granules is abolished or reduced in mutants of the 2B5 locus, a step preceding secretion.

On the other hand, premature induction of late larval puffs by hormone withdrawal did not accelerate the developmental processes at the fine structural level. In the period when puffs 21F, 22C, 29F, 63E, and 82F reach their maximum activities *in vivo* (PS9–PS12) or in glands cultured under the continuous presence of the hormone not only all glue secretion is finished at this time, but also all glue has been released from the lumen and nuclear membranes evidently show a reduced number of nuclear pores. None of these morphological features could be seen in glands with puffs prematurely induced at 21F, 22C, 29F, 63E, and 82F. In contrast, these glands showed that they are rather functionally equivalent to PS5–PS8 because they are actively engaged in glue exocytosis into the lumen, although the number of nuclear pores is not yet reduced. Poels (1970) working with *D. hydei* and Stocker and Kastritsis (1972) with *D. pseudoobscura* described an example of uncoupling puparium formation from its typical puffing pattern under experimental conditions when injection of 20HE into very young last instar larvae induced the characteristic puffing response, although animals did not pupariate. In *D. lebanonensis* this asynchrony is even more apparent because initiation of metamorphosis is under the control of circadian rhythm or circadian rhythm plus ecdysone rather than ecdysone alone (Eeken, 1977). Thus, our observations suggest two conclusions. First, the initial pulse of 20HE during the first 3–3.5 h is sufficient to start fine structural changes of salivary glands related to the larval puffing cycle, and the hormone does not seem to be necessary during whole cycle. Second, products of late larval puffs either are not involved in morphological changes of salivary gland cells at the end of larval period or such changes will not become apparent before previous ultrastructural alterations are completed. Also it is logical to assume that visible alterations in cell morphology will take more time to complete than cross talk or direct interaction between genetic loci mediated via transcription factors which can be manifested in premature puff induction upon 20HE washout.

Development of ultrastructural alterations in cultured salivary glands will stop with the continued presence of 20HE a few h after mucoprotein secretion is released from the lumen. This clearly corresponds to the arrest of the puffing pattern at PS13/PS14 observed by Ashburner (1972b). One cannot rule out the possibility that this "arrest" period can serve as a checkpoint for synchronization between cells moving at different developmental speeds as well as for developmental changes inside individual cells. Similar checkpoints in insect development were proposed for epidermal cells by Sláma (1975, 1980) in relation to periods of ecdysteroid peaks. The end of the arrest period in cultured *Drosophila* glands is achieved by complete washout of 20HE which must last at least 3 h. The manner in which salivary gland cells responded to the hormone and its washout in our experiments precisely parallels the behavior of prepupal puffs described by Richards (1976a, 1976b).

The apparent case of asynchrony was observed in the degree of degeneration between neighboring cells at the end of the prepupal puffing cycle (Fig. 3 *f*). This phenomenon was described also in glands explanted from prepupae (Lane et al., 1972; von Gaudecker, 1972). There can be several factors involved in cell- to-cell variability in response to the hormone, but two of them seem to play an important role. Ashburner (1973) found that individual PS1 salivary glands at the critical concentration of 20HE appear to react in a mosaic fashion; some nuclei progress beyond the early puffs, whereas others do not. Thus, a random sample of nuclei from such glands has contained uninduced late puffs and late puffs in various stages of

induction up to their maximum. We suppose that a similar mechanism underlies the differences in the degree of degeneration in individual gland cells. Although we worked well above the critical concentration of 20HE, there still may be cells in prepupal glands which do respond later or slower than most others. In addition, this factor can be combined with various levels of polyteny attained by most distal corpus cells and proximal corpus or transitional cells (Rodman, 1967; Rudkin, 1972). The nuclei of posterior cells can attain a level of polyteny 2–3 times higher than can nuclei in anterior cells, and thus, the response of posterior cells to the hormone can be more advanced than in anterior cells.

Salivary glands cultured in medium are not able to break down completely. This situation may be caused by factors missing from the hemolymph. Hydrolytic enzymes which are important for initiation of destruction are mostly made by fat bodies and are released into the hemolymph (Van Pelt-Verkuil, 1979a, 1979b). Initially degenerated basal lamina is then attacked and the tissue phagocytosed by hemocytes, as was observed *in vivo* by von Gaudecker (1972) and us (not shown). As indicated more than 20 yr ago by Ashburner (1972b), it would appear that the correct conditions for *in vitro* induction of the complete developmental pathway by ecdysones center around the choice of medium. Since then, not much was done about this, and it is obvious that coculturing salivary glands with pieces of fat body and a defined amount of hemolymph chemically treated to prevent its melanization (Farkaš, 1988; Farkaš and Mat'ha, 1989) can help to solve this problem. Besides this, certain consistent differences between the puffing cycle induced *in vitro* and the *in vivo* pattern have been described (Ashburner, 1967, 1969, 1972a, 1972b). For example, *in vivo* a puff at 58BC is characteristic of PS4–PS7, but this puff is never induced *in vitro*. Even though our observations do not indicate that these differences are significant in the normal course of ultrastructural changes studied, devising more suitable medium seems to be a reasonable direction for future research.

One of the few differences between the glands *in vivo* and those cultured *in vitro* is the release of secreted glue. *In vivo* it is expectorated strictly at the time of puparium formation, i.e., at PS10/PS11. We speculate that expectoration of glue, which affixes the puparium to the substrate (Fraenkel and Brookes, 1953), is probably mediated by hemolymph pressure pulsations of pupariating larva, originating from peristaltic movements during retraction as described for two cyclorrhaphous dipterans, *Sarcophaga bullata* and *S. crassipalpis* (Žd'árek et al., 1979; Žd'árek, 1985), and recently also in *D. melanogaster* (Sláma, personal communication). This makes expectoration rapid. In culture, the glands have ducts cut open and thus secretion can be continuously released into the culture medium shortly after it enters the duct.

There is a second and quite important difference between salivary glands *in vivo* and *in vitro*. Prepupal salivary glands during normal development, between PS12 and PS17, initiate one or more new secretory programs (Mitchell et al., 1977; Sarmiento and Mitchell, 1982), although type and composition of this secretion remains unclear. We never observed such activity in cultured glands, indicating some limits of the *in vitro* conditions in completely reprogramming their activities. This would not be the first such example. Woodard et al. (1994) have shown that there is a lack of correspondence between level of *βFTZ-F1* transcript and size of its corresponding mid prepupal puff 75CD in the presence of 20HE and cycloheximide. This observation is considered to be an artifact caused by culture conditions. But there is also another plausible explanation. Mitchell et al. (1977) have not proved that the new secretory program(s) in prepupae are closely related to hormone- dependent puffs. It was shown by studying *l(2)gl*, an ecdysteroid-deficient mutant, that the appearance of some series of puffs in late larvae and early to mid-prepupae depends on time of developmental events rather than on hormonal milieu (Korochkina and Nazarova, 1977). Therefore, some minor puffs usually present in prepupae, but not prominent enough to be used as stage-specific markers, could be absent under *in vitro* conditions, and their absence may be the reason that the prepupal secretory cycle was not initiated.

Absence of ecdysone or 20HE in the medium with explanted PS1 glands for longer periods resulted in reduced areas of rough endoplasmic reticulum and Golgi complex, and subsequently in decreased formation of mucoprotein secretory granules. The increased numbers of free ribosomes in the cytoplasm resemble those observed in very young 3rd instar salivary glands (Lane et al., 1972). In other words, in the absence of ecdysones, salivary glands lose their typical stage-specific ultrastructural features. Ashburner (1972b) found that after 12–24 h of preincubation of PS1 salivary glands in hormone-free medium, the early ecdysone-inducible puffs, 74EF and 75B, still showed characteristically rapid response but they did not attain the size seen after shorter or no preincubation periods, and the propagation of ecdysone-induced puffing pattern was not normal. The puffing pattern of salivary glands cultured for periods over 24 h turned out not to be homologous to any pattern during normal development (Nagel and Rensing, 1971). This indicates that the presentation, or at least maintenance of PS1 stage, may require some factor in addition to the components present in the culture medium. If PS1 glands were exposed to low concentrations of ecdysones (1×10^{-10} *M* for 20HE and 1×10^{-8} *M* for ecdysone) in the medium, they did not respond by induction of early puffs and stimulation of secretion but maintained the status of PS1 glands at least at the ultrastructural level, indicating that the missing factor could be a hormone. This result was unexpected but actually it supports several previous and recent observations. Transplantation of PS1 glands for up to 7 d into abdomens of 3–5-d-old females did not affect the synthesis of mucoproteins and formation of secretory granules which, upon explantation from abdomens followed by culture with 20HE, responded by normal puffing and exocytosis (Zhimulev and Kolesnikov, 1975; Richards, 1976d). All that need be said here is that females of that age display low level of ecdysteroids as determined by analytical methods (Bownes et al., 1984; Smith and Bownes, 1985; Grau and Lafont, 1994). Studies on ecdysteroid-deficient mutant $l(1)su(f)^{ts67g}$ showed that when production or release of the hormone is blocked in the 3rd instar larvae, the synthesis of secretory mucoproteins in salivary glands is abolished (Hansson et al., 1981; Hansson and Lambertsson, 1983, 1989). Those larvae are not able to pupariate, but both the synthesis of mucoproteins and the initiation of pupariation can be rescued by exogenous 20HE (Crowley et al., 1984). In another ecdysteroid deficient mutant, *l(2)gl*, the interecdysial as well as ecdysone-specific puffing patterns are missing or defective but mucoproteins are synthesized. This phenotype is also partially rescuable by injection of 20HE into larvae or by culturing glands with 20HE (Zhimulev and Kolesnikov, 1975; Korochkina and Nazarova, 1977).

Several ecdysone-regulated genes residing in puff sites have already been isolated and cloned. Many of them code for transcription factors which can be found at numerous chromosomal loci where they regulate the activity of a target gene (Urness and Thummel, 1990;

Hill et al., 1993). Certainly, so far there is no direct evidence for a strong link between a particular puff gene or group of puffs and described morphological changes. We hope that the role of individual genes nested in puff sites or their coordinate action on functional changes in cell morphology can be revealed in the near future by exploring both the effects of ectopic expression of cloned ecdysone-regulated genes in transformed flies, and the effects of mutations covering puff regions.

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