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Ecdysteroids Accelerate Mitoses in Accessory Glands of Beetle Pupae

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During the 9-day pupal period of *Tenebrio molitor* (the mealworm beetle), the cells of the male accessory glands undergo divisions for 7 days. There are two maxima in the mitotic activity in the glands *in vivo*, one at 1 day and the other at 4 days. The latter peak coincides with the large surge of ecdysterone occurring in the pupal stage. By the use of *in vitro* culture techniques, it has been demonstrated that the first bout of mitosis in both glands proceeds in basal medium, while the second bout of mitosis requires a physiological level of ecdysterone. Ecdysone was less effective than ecdysterone. Sensitivity to ecdysterone did not change significantly between Day 1 and Day 4 of the pupal stage. The results are discussed in relation to the effects of ecdysterone on cell division in mesodermal and ectodermal derivatives.

INTRODUCTION

Steroid hormones control cell cycles in target tissues (Gorski and Gannon, 1976; Yamamoto and Alberts, 1976; Armelin, 1975). In vertebrates, estrogens accelerate mitoses in uterine and mammary epithelia (Bresciani, 1964; Epifanova, 1966), androgens stimulate cell renewal in seminal vesicle and prostate gland (Tuohimaa and Niemo, 1968), and glucocorticoids initiate cell division in density-inhibited fibroblasts (Cunningham *et al.*, 1974) and inhibit proliferation in vascular smooth muscle (Longenecker *et al.*, 1984). In arthropods, it has long been suspected that ecdysteroids influence mitotic cycles. In his classic descriptions of insect metamorphosis, Wigglesworth (1948) showed that mitotic activity in the epidermis rises and falls during molt cycles. More recently, the onset of the mitotic arrest in the epidermis has been correlated with increased titers of ecdysteroids. For example, Besson-Laviognet and Delachambre (1981), working with the sternal epidermis of *Tenebrio molitor*, and two laboratories (Graves and Schubiger, 1982; Fain and Stevens, 1982) working with the imaginal discs of *Drosophila* melanogaster, reported that mitotic arrests in G_2 coincided with an increase in ecdysterone content of the animal. Similar results have been obtained after administration of ecdysterone to cultured cells or tissues. For example, ecdysteroids caused a G_2 arrest in vigorously growing K_c cells (Courgeon, 1972; Stevens *et al.*, 1980) and in a cell line derived from embryos of *D. melanogaster* (Echalier, G. and Ohanessian, A., 1970), and ecdysteroids accelerated mitoses in moth spermiducts (Szöllösi and Landureau, 1977) and locust testes (Dumser, 1980) during culture *in vitro*.

We are interested in the role of ecdysterone in the growth and differentiation of the reproductive accessory glands of *T. molitor*.

In the adult male beetle, there are two pairs of accessory glands—the bean-shaped accessory glands (BAGs), with eight types of secretory cells (Dailey *et al.*, 1981; Dailey and Happ, 1983), and the tubular accessory glands (TAGs), with but one cell type (Gadzama *et al.*, 1977). Both arise from a mesodermal pouch near the ninth sternite of the last larval instar (Huet, 1966) and are morphologically distinct from one another at pupation. During the pupal instar, there is a 10-fold increase in the volume of both pairs of glands that is largely due to cell division (Grimes and Happ, 1980; Happ and Happ, 1982). Mitoses begin soon after pupal ecdysis and continue through the seventh day of the 9-day instar. Counts of dividing cells show that there are two mitotic maxima in the pupa; the first is seen on Days 1-2 and the second on Days 4-5 (Grimes and Happ, 1980; Happ and Happ, 1982; Happ *et al.*, 1985).

The levels of ecdysteroids during pupal development of *T. molitor* have been determined by radioimmunoassay (Delbecque *et al.*, 1978). Cell divisions in the BAG/TAG complex continue through the major pupal ecdysteroid peak, which occurs on Days 4-5. In the present paper, we report the successful maintenance *in vitro* of the BAG/TAG complex from pupal *T. molitor* and we demonstrate that ecdysterone stimulates mitoses *in vitro* within the secretory epithelium of these glands.

MATERIALS AND METHODS

Animals. Stock cultures of *T. molitor* L. were obtained from commercial sources and maintained in the labo-

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ratory at 25°C on a diet of Purina Chick lab chow supplemented with potato. As last larval instar mealworms pupated, they were picked and the sexes were separated. By this method, precisely aged male pupae were obtained for the experiments. The pupal stage lasted for 9 days in the conditions provided.

Scanning electron microscopy. Glands were dissected out in phosphate-buffered saline (PBS) at pH 7.4 and immediately fixed in 3% glutaraldehyde in 0.1 *M* phosphate buffer at pH 7.3 for 24–48 hr at room temperature. Postfixation was carried out in 1% osmium tetroxide in the same phosphate buffer, at 4°C for 3–4 hr. The specimens were then dehydrated in graded alcohols, critical point-dried, and sputter-coated with gold-palladium. They were examined in a JEOL JSM-35U microscope at 15 kV.

Culture media. Two media were used in our experiments. The first culture medium is based on that of Landureau S-20 (Landureau and Grellet, 1972), in which the concentrations of calcium chloride, magnesium sulfate, and fetal calf serum were reduced. The second medium was a combination of the salts of Lenoir-Rousseaux medium (Lenoir-Rousseaux, 1981) and the amino acids and vitamins of Landureau S-20, again with a reduction of serum levels and the elimination of peptone. Table 1 shows the differences between the two media. All media were prepared using reagent-grade chemicals, and were sterilized by filtration through 0.22-μm filters prior to use.

Cell counts. A modification of the method used by Selman and Kafatos (1975) was used to estimate cell numbers. Briefly, the glands were dissected in PBS and mixed with 20% sucrose and HEK-1 m*M* Mg^{2+} buffer. They were rinsed and then transferred into fresh buffer in a Dounce tissue grinder, and homogenized. The homogenate was then mixed with 8% Tween 40 followed by 0.77% Azure blue. After centrifugation at 1720*g* for 3 min the resulting pellet was resuspended in sodium deoxycholate and spun again at 1720*g*. This second pellet was resuspended in HEK-EDTA with 0.1% BSA at pH 7.46, and aliquots were counted in a hemocytometer.

Culture methods. All procedures were carried out in a laminar-flow hood using standard aseptic techniques. Some glands were cultured in small culture dishes while others were kept in hanging drops; all were incubated at 25°C.

Culture dishes. Pupae were surface-sterilized with 70% ethanol, rinsed twice with sterile distilled water, and blotted dry on sterile tissue paper. Glands were dissected out in basal medium containing 1% fetal calf serum in order to reduce adherence to dishes and forceps. The glands were transferred to fresh medium containing 500 units of a streptomycin sulfate/penicillin mixture for 1.25 hr. After a thorough rinse in fresh medium, the glands were transferred to Falcon organ culture dishes containing 300 μl of the final medium and a water-saturated atmosphere with four gland complexes per dish. The medium was routinely changed at 5-day intervals unless specified otherwise.

TABLE 1
FORMULATION OF MODIFIED LANDUREA S-20 AND LENOIR-ROUSSEAUX 59/S-20 FOR THE CULTURE OF ACCESSORY GLANDS OF *T. molitor*

Components	S-20 (m*M*)	LR59/S-20 (m*M*)
Inorganic salts		
$CaCl_2$	1.80	$CaCl_2 \cdot 2H_2O$ 1.36
H_3PO_3	11.00	—
KCl	14.00	2.68
$MgCl_2 \cdot 6H_2O$	—	0.98
$MgSO_4 \cdot 7H_2O$	0.80	0.81
$MnSO_4 \cdot H_2O$	0.19	—
NaCl	145.00	154.00
Amino acids (as per Landureau and Grellet, 1972)		
Water-soluble vitamins (as per Landureau and Grellet, 1972)		
Other		
Fetal calf serum	1%	—
Glucose	4.0 g/liter	8.0
Horse serum	—	1%
Phenol red	10 mg/liter	10 mg/liter
$NaHCO_3$	—	3.65
Trehalose	—	3.2 g/liter
pH	7.0	6.85
osmolarity	400 mOsm	440 mOsm

Hanging drops. After surface-sterilization of the pupae, the glands were dissected out and rinsed well in fresh medium. They were then placed singly in hanging drops of approximately 40 μl volume, formed on 35-mm plastic Petri dishes. The medium was changed at 4-day intervals. All cultures were incubated at 25°C.

Mitotic index in vivo. Pupae were injected with 3 μl 1% colchicine in double-distilled water between the last two ventral sclerites, using a Hamilton syringe. After 2 hr at 25°C the gland complexes were dissected out and immediately fixed in ethanol:acetic acid (3:1) at room temperature. Upon transfer to 22% lactic acid, the BAGs and TAGs were dissected from each other. Each gland was placed on a microscope slide, excess lactic acid was blotted away, and the tissue was flooded with acetoorcein stain. A squash preparation was made by covering the tissue with a coverslip and applying moderate pressure for a few seconds. Excess stain was blotted away and the coverslips were sealed with molten petroleum jelly. They were allowed to stain overnight, and then counted.

Mitotic index in vitro. Glands that were in culture were incubated for 2 hr with 5×10^{-7} *M* colchicine

added to the culture medium. They were then treated as above.

Materials. Amino acids, antibiotics, colchicine, and vitamins were from Sigma Chemical Company; salts were all reagent grade; acetoorcein stain was from Chroma-Gesselcha (Schmid, GMBH Co.); ecdysterone from Calbiochem Ltd. or Sigma Chemical; ecdysone (E-9004) from Sigma Chemical (98% ecdysone, 0.3% ecdysterone by HPLC); and TC horse serum from Difco.

RESULTS

Growth and Changes in Shape Occur during Pupal Development

Scanning electron micrographs of BAG/TAG complexes illustrate the increases in size and the significant changes in shape which have been reported during pupal development (Happ *et al.*, 1982; Happ and Happ, 1982). In 0-day pupae, the glands resemble a pair of mittens; the BAGs are the hands and the TAGs are the thumbs pointing inward (Fig. 1). The surface of the complex appears smooth in these preparations. During the first 3 days of pupal life, the TAGs elongate and the BAGs begin to curl inwards at their tips (Figs. 1, 2). At Day 4, the lengthening TAGs have grown upwards between the BAGs and reach through to the dorsal surface of the complex. At Day 5, increased tracheation becomes visible on the surface of the glands and they begin to be coated with fat body cells, which can be removed only by very careful dissection (Fig. 3). During the final 4 days of instar, tracheation continues to develop and the TAGs lengthen (Fig. 4).

Glands Survive for Weeks in Vitro

Both the culture medium of Lenoir-Rousseaux and the S-20 formulation of Landureau (see Table 1) proved equally suitable for organ survival during short-term incubations (24 hr) and also for long-term cultures (such as the 9 days of the pupal stage or even for periods of 7–9 weeks). In cultures lasting several days or longer, the TAGs and the BAGs became somewhat flattened; the TAGs appeared more ribbon-like and the BAGs more angular than during development *in situ.* Integrity of the tissues was improved by addition of the S-20 amino acids and vitamins to the basic Lenoir-Rousseaux medium. Both media allowed some growth and significant levels of mitotic activity in liquid cultures (Petri plates) and in hanging drop cultures. For convenience, most of the experiments were carried out in liquid cultures using Landureau's medium.

In both types of media, the levels of sera and of protein additives had to be reduced to low levels (1% or less) in order to avoid cell migration from the explants. It was possible to support satisfactory growth in completely defined S-20 (without the addition of fetal calf serum) and to accelerate mitotic activity in such defined media by the addition of ecdysterone (see below). However, the glands in serum-free medium tended to stick to the plastic dishes and thus to suffer mechanical damage during manipulation. This problem was especially severe in glands explanted from young pupae. In order to avoid these injuries due to manipulation, 1% fetal calf serum was routinely used in our cultures.

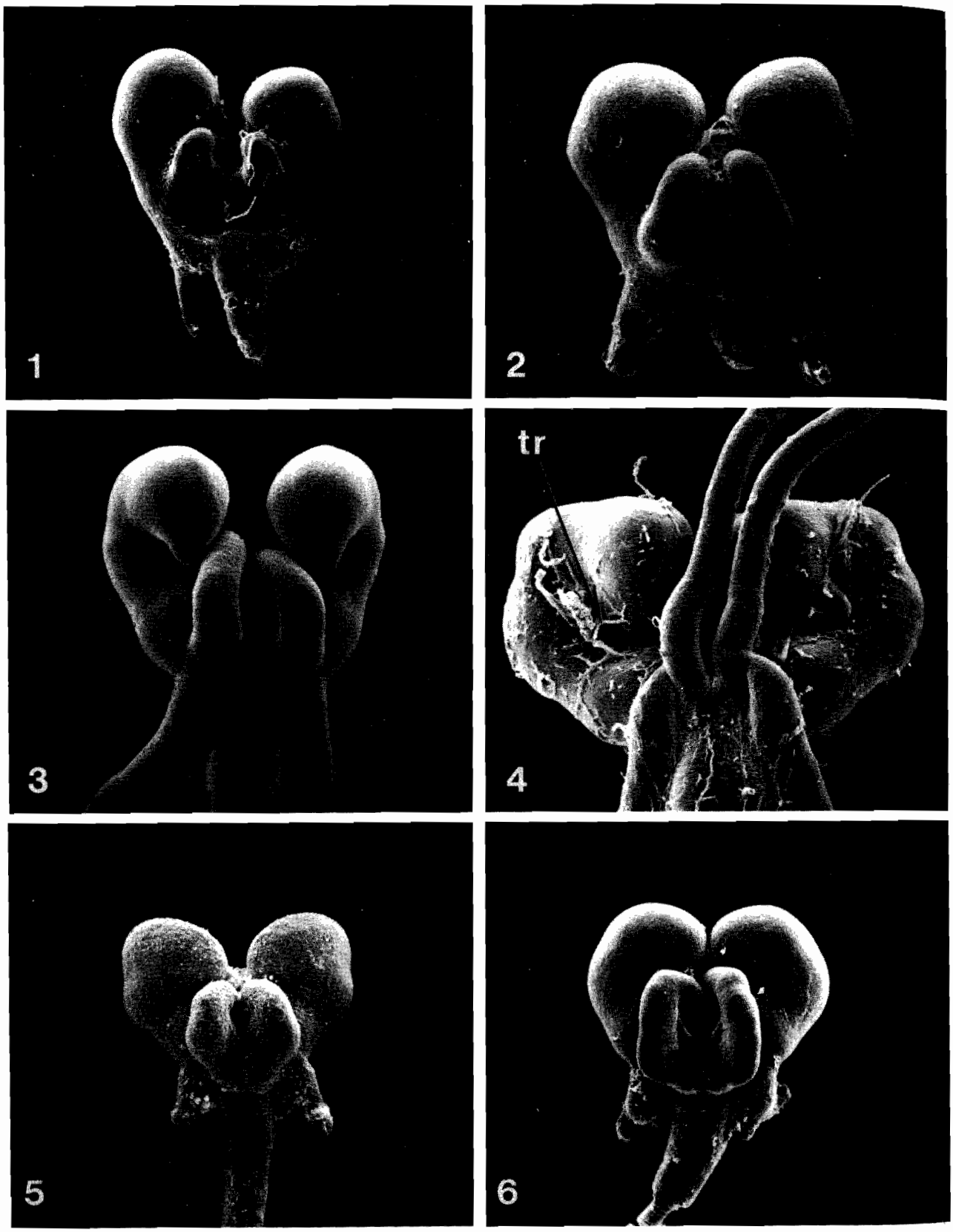
Ecdysterone Stimulates Growth in Vitro

In comparison to many other insects, the absolute concentration of ecdysterone during the development of *T. molitor* is quite high; it reaches a concentration of 8×10^{-6} *M* at the apex of the major pupal peak (Delbecque *et al.*, 1978). In our initial experiments, we maintained the glands *in vitro* for a period equivalent to the pupal instar. BAG/TAG complexes were explanted at pupal ecdysis and maintained in basal medium for 4 days. At that time, ecdysterone (final concentration of 10^{-5} *M*, which approximates peak pupal levels) was added and the glands were maintained in the presence of ecdysterone for 5 more days. After this interval *in vitro*, the glands were fixed for scanning electron microscopy. As shown by comparison of Fig. 5 (basal medium) and Fig. 6 (ecdysterone-supplemented medium), the hormone-treated glands were significantly larger than the controls. The increase in the length of the TAGs, which had grown up between the BAGs, was especially noticeable (Fig. 6). When control or ecdysterone-treated glands were reexposed on the ninth day to fresh medium containing 10^{-5} *M* ecdysterone, there was no further growth.

Ecdysterone Accelerates Mitotic Activity in Vitro

We counted the nuclei from glands cultured with and without ecdysterone. After the first 4 days in basal medium, cell numbers in the TAGs increased significantly although growth did not equal that which occurs *in vivo* (Fig. 7A). For the BAGs, there was a net loss of cells during those 4 days in basal medium (Fig. 7B). When we examined squashes of the glands after 48 hr or more in basal medium, a considerable number of nuclei on the 0-day explants were pycnotic. However, following addition of ecdysterone at 4 days, cell numbers increased in both the TAGs and the BAGs, but not in the controls (Fig. 7).

To trace the daily changes in mitotic rates, we measured mitotic indices in cultured TAGs and BAGs. During normal development *in situ,* there are mitotic peaks at 1 1/2 and 4–5 days (Happ *et al.*, 1985). When we cultured 0-day glands *in vitro* in basal medium, mitoses continued for 1–2 days and then declined (Figs.



FIGS. 1–6. Scanning electron micrographs of the male accessory glands from pupal *T. molitor*. All magnifications 63×.
FIG. 1. Ventral view of the bean-shaped glands (B) and the tubular accessory glands (T) in the 0-hr pupa.
FIG. 2. Ventral view of the accessory glands in the 1-day pupa showing the paired seminal vesicles (S) and the ejaculatory duct (E).

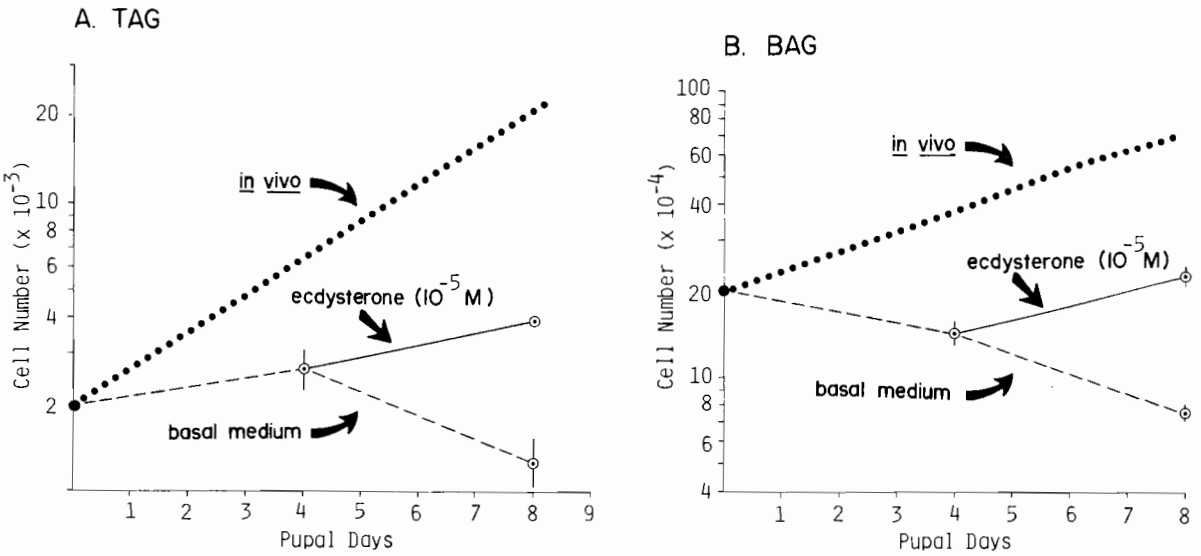


FIG. 7. Increases in cell numbers in TAGs and BAGs cultured for the pupal interval *in vitro*. In some cultures, ecdysterone was added at 4 days. The dotted line shows the increase in cell numbers *in vivo* (from Happ *et al.*, 1985).

8A and B). A sharp increase in the mitotic index followed addition of ecdysterone at 4 days, whereas in the control cultures there was very little cell division throughout the remainder of the experiment.

The nuclear and mitotic counts *in vitro* suggest that the mitoses continue for 1-3 days in the absence of ecdysterone, but that mitoses do not persist through the mid-pupal age unless ecdysterone is added.

The Mitotic Response to Ecdysterone Begins Early in the Pupal Stage

We investigated the mitotic response in progressively older glands following application of ecdysterone *in vitro*. Glands were removed from pupae and maintained *in vitro* for 1 day, with or without peak concentrations of ecdysterone (10^{-5} *M*). For the 0 pupal day explants, the 23-hr exposure to ecdysterone strongly increased the mitotic rate in the TAGs from 3 to 8% and in the BAGs from 2 to 7%. The basal mitotic rate and that in ecdysterone-stimulated cultures both dropped proportionately to a lower level on Day 3. Cell division increased somewhat in the control explants from Days 4 and 5 and, at that age, ecdysterone *in vitro* enhanced mitoses twofold. In glands explanted from late pupae, mitotic rates were very low in both control and hormone-stimulated cultures (Fig. 9).

Ecdysterone Concentrations near Physiological Peak Levels Are Needed for Mitotic Stimulation in Vitro

Glands were exposed to a variety of ecdysterone concentrations to determine whether the mitotic response seen *in vitro* required physiological levels of hormone. The radioimmunoassay results of Delbecque *et al.* (1978) provided benchmark data. At ecdysis to the pupa, titers of ecdysterone were about 10^{-7} *M* and the mid-pupal peak reached almost 10^{-5} *M*.

In the first dose-response series, the mitotic response of 0- or 4-day pupal glands was measured after 23 hr of culture (Fig. 10). When 0-day pupal explants have aged 1 day *in vitro*, the basal mitotic index is 2-3%, which is about 70% of the mitotic rate at the same age *in vivo*. Hormone dosages which were physiological for that age (10^{-7} *M*) did not enhance the mitotic activity *in vitro*. However, 10^{-5} *M* ecdysterone, a concentration which is hyperphysiological for 0-day pupae, increased the mitotic rates in 0-day BAGs and TAGs to levels well above those usually seen *in vivo*. For the 0-day explants, a hyperphysiological dose produced a hyperphysiological response (Fig. 10).

When the 4-day explants were cultured for 1 day, the mitotic rate in basal media was 1.5-2%, less than half of that expected from the same age *in vivo*. As hormone doses were increased, no significant increases

FIG. 3. Ventral view of the 4-day gland complex.

FIG. 4. Gland complex at 6 days. Note that the glands are now covered with tracheae (tr).

FIG. 5. Gland complex explanted from 0-hr pupa and cultured in basal S-20 for 9 days.

FIG. 6. Gland complex explanted from 0-hr pupa and cultured *in vitro* for 9 days. Glands were cultured in basal medium (S-20) for the first 4 days and then in S-20 supplemented with ecdysterone (10^{-5} *M*) for days 5-9.

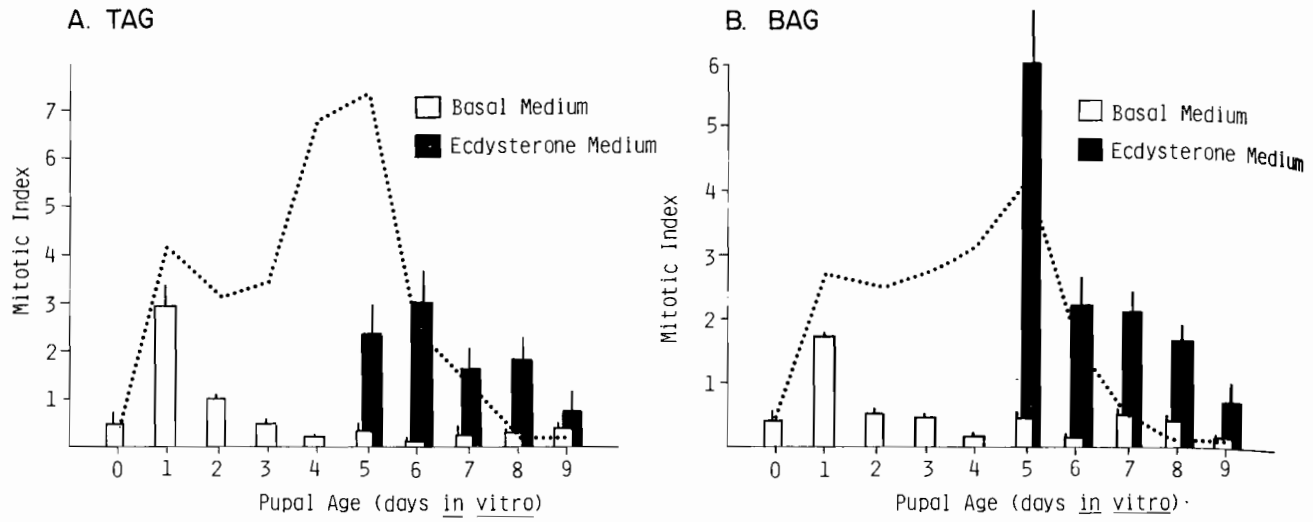


FIG. 8. The mitotic indices of pupal accessory glands *in vitro*. Glands were explanted at pupal ecdysis and cultured for 4 days in basal medium. Ecdysterone (10^{-5} *M* final concentration) was added at 4 days to one group of glands; controls were maintained in basal medium. The mitotic indices are based on counts of at least six different gland pairs; 1000 cells per gland were counted. The dotted line shows the normal mitotic indices *in vivo*.

in mitotic rate were observed until hormone levels exceeded 10^{-6} *M*. With 10^{-5} *M* (an approximately physiological concentration of hormone at 4–5 pupal days), there was maximal mitotic stimulation to a level that resembled the physiological rate. For 4-day explants, physiological hormone doses were required for physiological levels of mitoses (Fig. 10).

Did 0-day explants remain physiologically responsive during long-term culture *in vitro?* The second dose-response series was designed to answer this question. After 0-day explants had been cultured *in vitro* for 4 days in basal medium, the glands were maintained for one more day in basal medium or in either of two doses of ecdysterone (10^{-6} *M*, 10^{-5} *M*). The results (Fig. 11) show that, after 4 days *in vitro*, the BAGs and the TAGs remained competent to respond mitotically to physiological levels of ecdysterone.

Ecdysterone Increases Mitotic Rates in Secretory Cells More Than in Muscle Cells

The secretory cells of each gland are surrounded by a thin muscle coat. Did the mitotic and cell count data include a mixture of muscle and secretory cells?

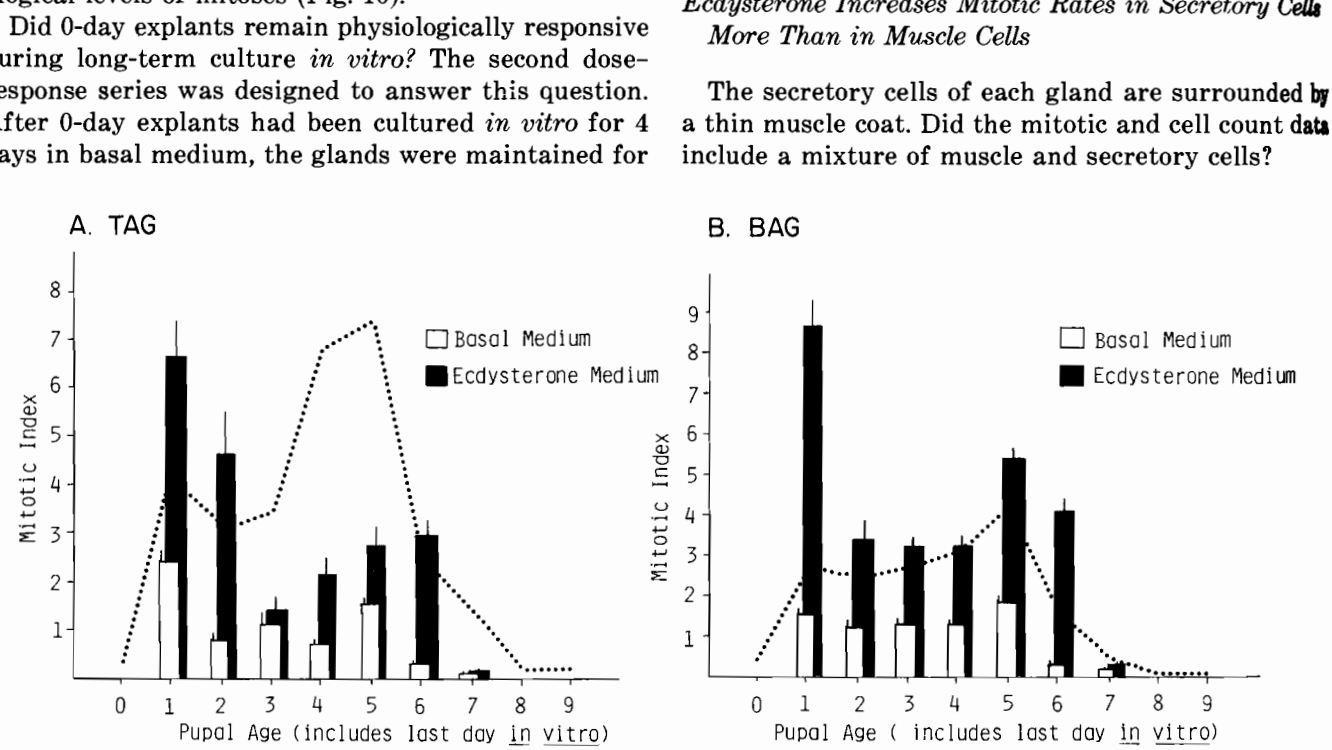


FIG. 9. Mitotic indices in glands which have been explanted from pupae of various ages and then cultured for 23 hr *in vitro* in basal medium or in ecdysterone-supplemented medium (10^{-5} *M* final concentration). The dotted lines show the mitotic rates *in vivo*. Standard error is indicated for each explant.

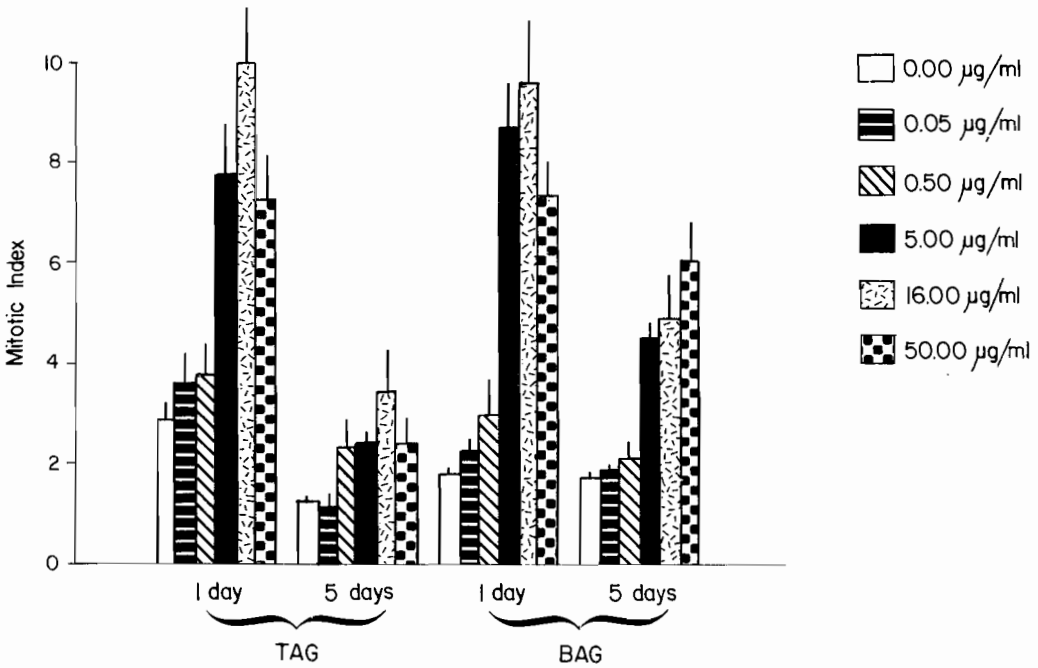


FIG. 10. Dose-response data for TAGs and BAGs which have been explanted at 0 or 4 days and then exposed to varying concentrations of ecdysterone for 23 hr *in vitro*. Standard error is indicated for each.

To distinguish unequivocally between ecdysterone effects on muscle nuclei and on secretory nuclei, we determined mitotic indices in sectioned tissue where the integrity of each tissue type was preserved. 0- and 4-day pupal glands were cultured for 24 hr *in vitro*, with or without ecdysterone, and then treated with colchicine for 2 hr. As shown in Fig. 12, cell division in the secretory epithelium was significantly stimulated by ecdysterone while the mitotic index in the muscle coat never exceeded 1%, a level difficult to distinguish from background.

Ecdysone Accelerates Mitoses But to a Lesser Extent Than Does Ecdysterone

Glands of 0- and 4-day pupae were exposed to ecdysone *in vitro* and the mitotic index was determined. The results are compared with our earlier ecdysterone experiments in Fig. 13. Ecdysone enhanced mitotic activity but to a lesser degree than did ecdysterone.

DISCUSSION

Ecdysterone and Cell Cycles in the BAG and the TAG

Our earlier studies on the mitotic activity in male accessory glands of *T. molitor* suggested that there were two bouts of cell division in the TAG (Happ and Happ, 1982) and the BAG (Grimes and Happ, 1980). Cell counts from glands developing *in situ* show that the TAG cells divide four times during the pupal stage and that the BAG cells divide twice (Happ *et al.*, 1984). In the present study of development *in vitro*, we showed that a first bout of mitosis proceeds without added ecdysterone while the mitoses at 4 days are ecdysterone-dependent. This effect appears to be specific for ecdysterone since ecdysone did not restore mitoses to the same level as ecdysterone.

In vitro, the cells of the BAG and the TAG divided at about 70% of their rate *in situ*. These reduced rates of mitosis may account for the small size achieved after growth *in vitro*. TAG in particular grew to a final volume of only one-fourth of its normal size. The mitotic rate fell off sharply within 48 hr of explantation and could not be fully restored to physiological levels at Days 4–5 even when high levels of ecdysterone were added. Although the basic nutritional requirements of the cells were fulfilled in the cultures, some unknown factor may yet be missing. One candidate is oxygen or its delivery system. During the latter portion of the pupal stage *in vivo*, tracheal trunks become associated with the glands (Fig. 4) and tracheoles penetrate into the secretory epithelium (Grimes and Happ, 1980; Happ and Happ, 1982).

The absolute daily concentration of ecdysterone and even the precisely patterned rise and fall of hormone could be critical for optimal growth. In another series of experiments, we tried to mimic the fluctuations in ecdysteroids reported *in vivo* (Delbecque *et al.*, 1978). We explanted the glands at pupation and adjusted the hormone concentration whenever appropriate at daily

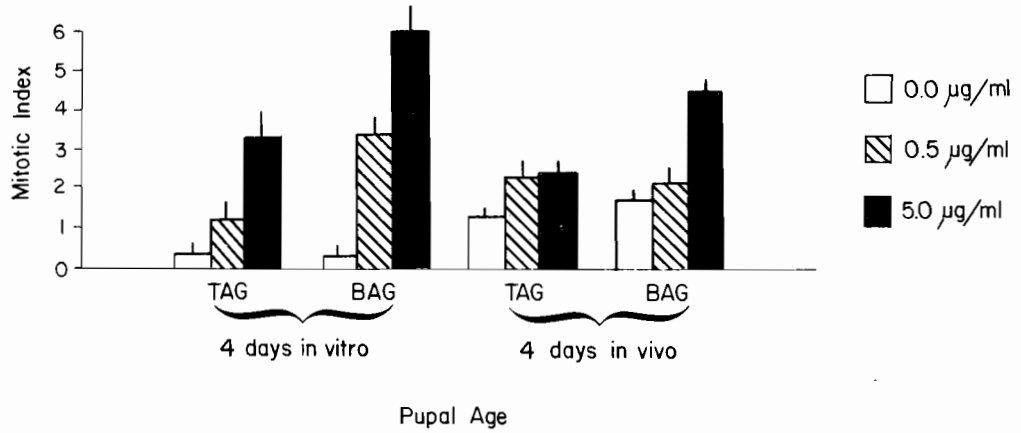


FIG. 11. Mitotic responses to three doses of ecdysterone are compared for glands which have matured for 4 days *in vivo* with those which have been maintained for 4 days *in vitro* in basal medium. Both groups were exposed to ecdysterone (10^{-5} *M*) *in vitro* for 23 hr. Their age at the end of the experiment is 5 days after pupal ecdysis. Standard error is indicated for each.

intervals. The mitotic index still fell in the days after explantation and did not begin to rise detectably when ecdysterone concentrations of 10^{-6} *M* were applied at Day 3. Somewhat to our surprise, the physiological peak dose (10^{-5} *M*) did not elicit the second bout of mitoses in these cultures. We believe that in this experiment, the glands were perturbed by shock of frequent changes of media.

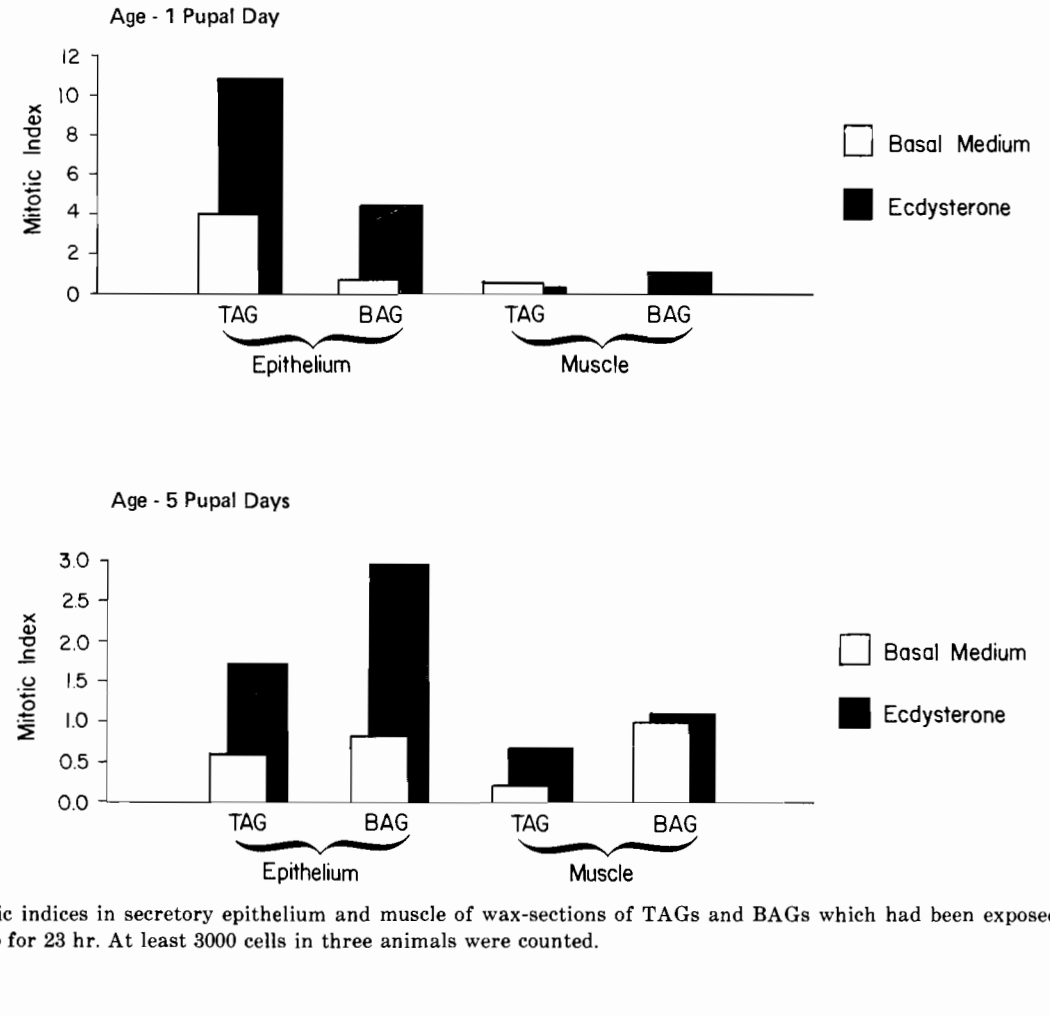


FIG. 12. Mitotic indices in secretory epithelium and muscle of wax-sections of TAGs and BAGs which had been exposed to ecdysterone (10^{-5} *M*) *in vitro* for 23 hr. At least 3000 cells in three animals were counted.

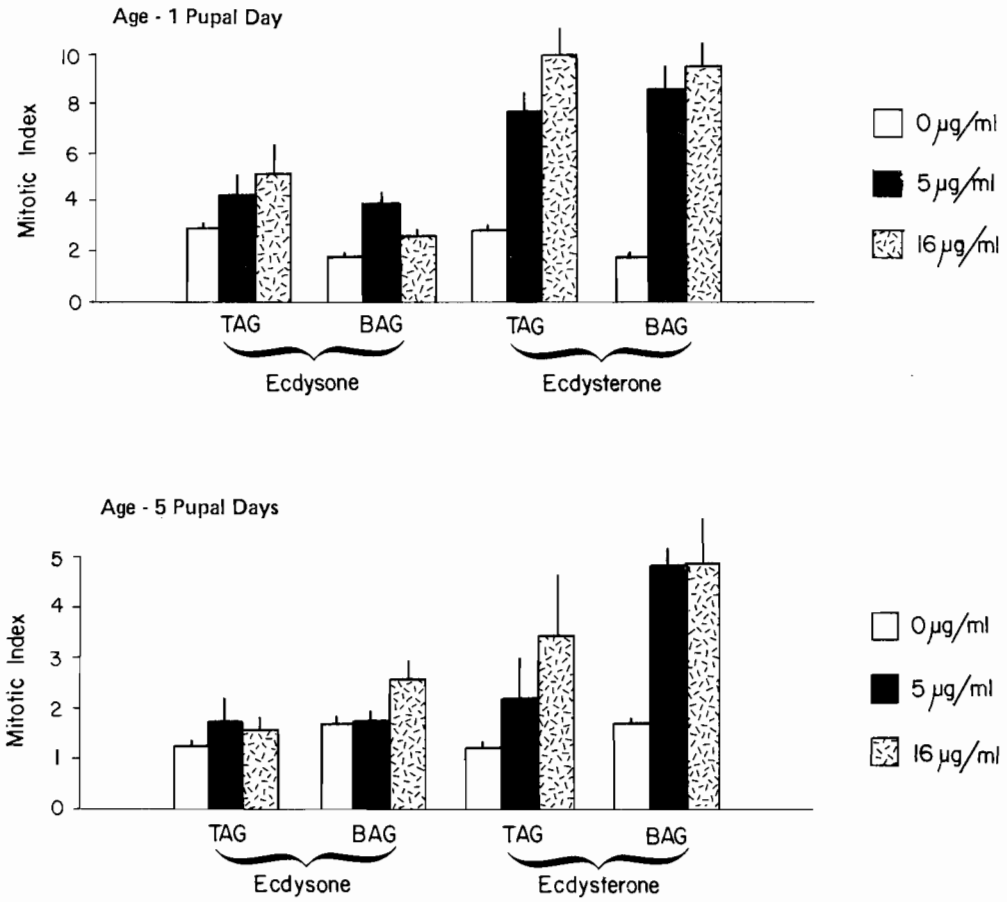


FIG. 13. Comparison of the mitotic response to two concentrations of ecdysterone and ecdysone. Glands were cultured for 23 hr *in vitro* in basal or in hormone-supplemented media. Standard error is indicated for each.

Over Days 0–5 of the pupal stage, the dose–response relationships for ecdysterone remain unchanged. There does not seem to be a significant switch toward greater sensitivity to ecdysterone in mid-pupal life. The very young pupal glands, which do not encounter high titers of ecdysterone *in vivo*, can nonetheless respond strongly to hormone when they are exposed to it. The threshold for minimal detectable mitotic response and the dose of ecdysterone required for maximal mitotic stimulation were not significantly different when we compared the 0- and 4-day explants (Fig. 9).

Within the interval of high mitotic activity, the mitotic index in ecdysterone-supplemented media varied with the age of the tissue. There appeared to be a low point in the response to ecdysterone at 3 days (Fig. 7) which coincided with a minimum for mitotic activity *in vivo* (Happ *et al.*, 1985). However, we cannot dismiss the possibility that this dip in the observed stimulation of mitosis is merely an artifact, explained by the plausible assumption that the cells are not distributed randomly throughout the cycle but show partial synchrony, especially during the early days of the pupa. On this assumption, the dip in mitotic rate could merely reflect the fact that most of the cells are concentrated in some phase other than M at the time of the 3-day sample.

Do the same cells participate throughout the mitotic interval? Or are there two distinct lineages, one of which undergoes mitosis in the early pupa while the second fails to divide until ecdysterone titers rise? We believe that our data are consistent with only one cell population which continues to divide through pupal Days 0–6. If a significant fraction of the cells were to remain quiescent until the surge of ecdysterone, there would not be enough cell division to account for the observed increase in cell numbers (Fig. 7). The cell cycle in the TAG lasts about 48 hr and cell numbers increase 14-fold in less than 8 days (Happ *et al.*, 1984). Almost all of the cells must be a part of the growth fraction throughout the 8-day period to account for this 14-fold increase in cell number or else part of the cell population must undergo more than four division cycles. Furthermore, there is a tendency for relative mitotic rates in both basal and ecdysterone-supple-

mented media to remain proportional to one another. For example, mitoses in both media are high at 1 and 5 pupal days and low at 3 pupal days (Fig. 9). This proportionality is illustrated by plotting the control (basal) mitotic rates against those in ecdysterone-stimulated cultures (Fig. 14). Such a synchronous shift in mitotic rates suggests that there is but a single population of dividing cells.

In many cell populations, the control of growth seems to be brought about by factors in the media which act at a specific "restriction point" in G_1 (Pardee, 1973). There is increasing recent evidence for a control point in G_2 as well (Prescott, 1976; Dethlefsen *et al.*, 1980). In acting to accelerate mitoses in the TAG and the BAG, ecdysterone could remove a specific block at some restriction point in G_2. Alternatively, ecdysterone might be adjusting the probability at which cells shift from an indeterminate resting state to a determinate cycling state (Smith and Martin, 1973). Such a mechanism of steroid hormone action has been invoked to explain the way that testosterone acts to accelerate cell cycling in a mouse mammary tumor (Shinogi 115) (Robinson *et al.*, 1976).

Either the detection system (sensitivity to hormone) or the cellular response (mitosis) must be significantly altered between 6 and 9 days of pupal age. Cell division stops after the sixth pupal day *in vivo* (Grimes and Happ, 1980; Happ *et al.*, 1982, 1985). When glands were removed from 6-day pupae and cultured *in vitro*, neither ecdysterone-treated nor control cultures showed significant mitotic rates (Fig. 11). Yet, after glands explanted from 0-day pupae have been maintained *in vitro* for 4 days in basal medium and then exposed to ecdysterone, the cells continue to divide past the seventh day (Figs. 7). The prolonged responsiveness to ecdysterone *in vitro* may be due to retardation of cell division (and therefore perhaps aging?) in culture. It is also possible that there is some unknown signal, present in the animal and absent from our culture system, which blocks cell division after the sixth day. We have no information about the mechanisms that control the terminal arrest which leaves most cells blocked in G_2 in the TAGs and the BAGs (Happ *et al.*, 1984).

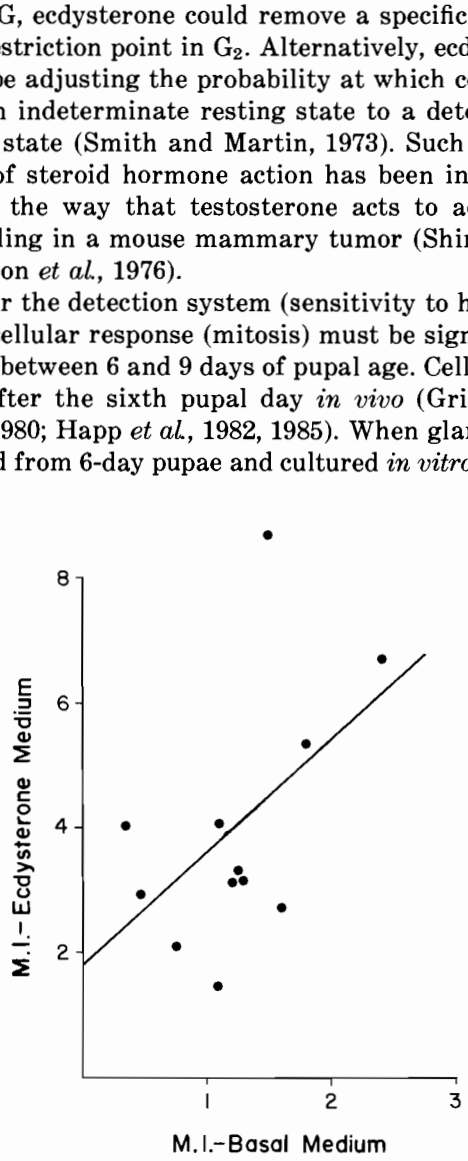


FIG. 14. Comparison of the mitotic index in ecdysterone-supplemented medium (10^{-5} *M*) versus the paired control culture in basal medium. Data from Fig. 9. (Regression equation $y = 1.79 + 1.83x$, $r = 0.53$).

Ecdysterone and Cell Cycles in Insects

In the present paper, we have shown that ecdysterone accelerates mitoses in the BAGs and the TAGs of *T. molitor*. The hormone acts in an opposite manner on the epidermal cells of this animal. Caveney and Blennerhasset (1980) reported no mitoses when the epidermis of mealworms was exposed to ecdysterone *in vitro*. Lenoir-Rousseaux (1978, 1981) found that ecdysterone promotes cuticle deposition but not cell divisions during leg regeneration *in vitro*. In an earlier study with the spermathecal accessory gland, an epidermal derivative in female *T. molitor*, we saw no mitoses after administration of ecdysterone *in vitro* (Szopa and Happ, 1982). Finally, in a detailed study of the cell cycle of the epidermal cells of larval and pupal *T. molitor*, Besson-Laviognet and Delachambre (1981) found that ecdysterone peaks were precisely correlated with G_2 arrests. By mechanisms yet to be investigated, the epidermal cells were released into mitosis at later times when ecdysterone levels were low (Besson-Laviognet and Delachambre 1981).

In other insects, a similar pattern is seen—ecdysterone is a "blocker" (arresting cells at some point in their cycle) in some tissues and an "opener" (removing a block) in others. Ecdysterone inhibits cell division in regenerating legs of cockroaches (Marks *et al.*, 1967; Marks, 1970). In the imaginal discs of *D. melanogaster* (Fain and Stevens, 1982; Graves and Schubiger, 1982), and also in the K_c cell line (Maroy *et al.*, 1980; Stevens *et al.*, 1980), and the S cell line (Vitek *et al.*, 1984) from the same species, ecdysterone causes arrest in G_2. The K_c cell line is presumed to be an ectodermal derivative (Moir and Roberts, 1976; Debec, 1974; Schneider, 1972). In the spermiduct of moths (Szöllösi and Landureau, 1977) and in germ cells (Schmidt and Williams, 1953; Nishiitstutsugi-Uwo, 1961; Takeuchi, 1960; Williams and Kambysellis, 1969; Yagi *et al.*, 1969; Takeda, 1972; Dumser and Davey, 1975; Dumser, 1980), ecdysterone

accelerates cell divisions. In the testes of locusts, the data suggest that ecdysterone is acting to remove a G_2 block (Dumser, 1980).

From the preceding examples, a common pattern emerges. It can be summarized as follows: Ecdysterone is a blocker (often a G_2 blocker) when it acts at high levels on ectodermal derivatives like the epidermal cell. On the other hand, ecdysterone at high titers is an opener (perhaps a G_2 opener) on mesodermal derivatives like the TAG and the BAG. Is this hypothesis generally applicable? Except for *D. melanogaster* (ectoderm), locust testes (mesoderm) and *T. molitor* (ectoderm and mesoderm), very little is known about cell cycle parameters or when ecdysterone acts during the cell cycle, and almost nothing is known of the kinetics of hormone action in G_2 for any eucaryotic cell. Both descriptive and mechanistic studies are needed to test the generality of this hypothesis in other insect systems.

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