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20-Hydroxyecdysone Acts in the Male Pupa to Commit Accessory Glands toward Trehalase Production in the Adult Mealworm Beetle (*Tenebrio molitor*)

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During postecdysial adult maturation, the bean-shaped accessory reproductive glands (BAGs) of adult male mealworm beetles produce increasing amounts of trehalase. In order to determine when the BAGs become competent to produce trehalase, we transplanted pupal BAGs into 0-day female adults. After 8 days, trehalase activity had increased in BAGs from 4- and 5-day pupae (at the time of the pupal ecdysteroid peak) but not in those from 1- and 2-day pupae (before the ecdysteroid peak). BAGs from 0- and 2-day pupae were exposed to 20-hydroxyecdysone *in vitro* before implantation into 0-day female adults. Increase in trehalase activity was dose dependent. Both dose (ED_{50}, 5×10^{-6} *M*) and exposure time (>6 hr) of hormone required are greater for commitment than for acceleration of pupal cell cycling (T. Yaginuma, H. Kai, and G. M. Happ, 1988, *Dev. Biol.* **126**, 173–181). Since trehalase activity increased markedly in isolated adult male abdomens, factors from the cephalic and thoracic centers are not required to sustain trehalase production in the adult BAGs.

Ecdysteroids control both growth and differentiation (Riddiford, 1985). By correlation of mitotic events in the insect epidermis with hormonal context *in vivo* (Besson-Laviognet and Delachambre, 1981; Graves and Schubiger, 1982) and by direct application of hormones *in vitro* (Stevens *et al.*, 1980, with Kc cells; Kato and Riddiford, 1987, with epidermis of *Manduca;* Szopa *et al.*, 1985, and Yaginuma *et al.*, 1988, with accessory glands of *Tenebrio*) ecdysteroids were shown to regulate cell cycling. Preliminary evidence suggested that the pupal ecdysteroid peak which accelerates cell cycling in the pupal accessory glands of *Tenebrio* is correlated with a change of competence to make adult-specific proteins (Happ, 1987). In the present paper, we provide a much clearer demonstration that ecdysteroid action in the pupa is necessary for adult-specific protein appearance in the accessory glands of *Tenebrio*.

Male reproductive accessory glands are useful models to study the role that ecdysteroids play in reproductive maturation. In the male mealworm beetle, *Tenebrio molitor,* there are two pairs of accessory glands, the tubular accessory glands (TAGs) and bean-shaped accessory glands (BAGs) (Happ, 1984). Both originate from a mesodermal pouch near the ninth sternite of the last larval instar (Huet, 1966) and grow in size by increasing cell numbers during the 9-day pupal stage (Happ *et al.*, 1985).

During the period of pupal cell division, the many cells which will give rise to the secretory epithelium of the BAG are morphologically similar to one another. At the close of the pupal stage and in the first 2 days after adult ecdysis, the eight morphological cell types characteristic of the adult glands become clearly defined (Dailey *et al.*, 1980; Dailey and Happ, 1983). As the

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definitive adult morphology of the BAG is established, there is rapid synthesis and accumulation of the adult-specific proteins (Happ *et al.*, 1982), including three antigens recognized by monoclonal antibodies (Grimnes and Happ, 1986; Grimnes *et al.*, 1986; Shinbo *et al.*, 1987), and the enzyme trehalase which is detected by its catalytic activity (Yaginuma and Happ, 1988). Trehalase is secreted by the BAGs into the secretory plug, subsequently is incorporated into the superficial layers of the spermatophore, and finally is passed to the female (Yaginuma and Happ, 1988).

Previous qualitative evidence (leucine incorporation into spots on fluorographs (Happ, 1987) and dot blots with monoclonal antibodies (Grimnes and Happ, 1987)) indicated that BAGs become competent to produce adult-specific proteins during the ecdysteroid peak between Pupal Days 3 and 6. In the present study, we employed trehalase activity as a quantitative index of adult differentiation of the BAGs. Our aims were (1) to define the dose–response parameters for 20-hydroxyecdysone which acts in the midpupa to make BAGs competent for adult differentiation, and (2) to investigate the roles of anterior neuroendocrine centers in the expression of the competence to synthesize adult-specific proteins.

MATERIALS AND METHODS

Animals. Larvae of *Tenebrio molitor* were purchased and reared on a diet of Purina Chick Lab Chow at 25°. After pupation, white pupae were sexed and kept at 25° as described previously (Shinbo *et al.*, 1987).

Enzyme preparation. BAGs were dissected from pupae or adults in ice-cold phosphate-buffered saline (PBS) (8.2 m*M* Na_2HPO_4, 1.5 m*M* K_2HPO_4, 140 *M* NaCl, 26 *M* KCl, pH 7.4) and blotted. BAGs were disrupted in 1.5-ml microcentrifuge tube by Teflon pestle (Kontes, Vineland, NJ). This crude homogenate was used as an enzyme source.

Protein content. Protein content was determined by the Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as the standard. An aliquot (70 μl) from the homogenate was mixed directly with 1 *N* NaOH (30 μl) and used for the Lowry assay.

Assay of trehalase activity. Enzyme assays were performed according to the method of Yaginuma and Happ (1988) with slight modifications. Glucose content was determined by the Somogyi–Nelson method (Nelson, 1944). Enzyme activity was expressed as micromole or nanamole of glucose released from trehalase per minute.

Preparation of isolated abdomens. Male animals were anesthetized on ice and cut in half just in front of the mesothoracic legs. The wound was closed with melted paraffin wax and the animals were kept on wet filter paper at 25°.

Anatomical observations. BAGs were isolated in ice-cold PBS and the length and width of one gland of the pair was measured with an ocular micrometer. Size of the BAGs was expressed as length × depth. The presence of the semisolid secretory plug, a precursor of the spermatophore, was checked by dissection.

Transplantation of BAGS. TAG/BAG complexes were dissected in Landureau S-20 culture medium according to the method of Yaginuma *et al.* (1988). A microcapillary (50-μl microcap glass tube, Drummond Scientific) with a sharpened tip was fitted onto a 23-gauge needle by special glue (Bond Aron Alpha, Toa Inc., Tokyo, Japan). This needle was attached to a 1-ml disposable syringe. The TAG/BAG complex was sucked carefully in the microcapillary and injected into the abdomens of adult animals which had been anesthetized on ice. The wound was sealed with melted paraffin wax. Thereafter, these animals were reared on a diet at 25°.

In vitro culture. All procedures were carried out according to the method of Yaginuma *et al.* (1988).

Reagents. 20-Hydroxyecdysone (Calbiochem) was dissolved in 10% isopropanol (stock solution) and the concentration was checked at 240 nm (E_{240} = 12,670). Hydroxyurea (Sigma) was dissolved in distilled water.

Statistical analyses. The data were analyzed by one-way analysis of variance followed by Duncan's new multiple range test ($P < 0.01$) or by Student's *t* test or the Cochran–Cox test.

RESULTS

Competence of BAGs to Make Trehalase

To determine when the developing BAGs acquire the competence to produce trehalase, we transplanted glands from pupae of varying ages into adult hosts. TAG/BAG complexes were dissected in Landureau's S-20 medium and transplanted into adult animals that had ecdysed within the previous 3 hr. Eight days later, implanted BAGs

were retrieved by dissection and the trehalase activity was assayed. In pilot experiments when 5-day pupal BAGs were implanted into male or female hosts, trehalase activity increased strongly, irrespective of the sex of the host (data not shown). Thereafter, we used only female hosts so that we could not mistake the implanted glands for the BAGs of the hosts.

BAGs from pupal Days 1 through 5 were implanted into 0-day adult female hosts and allowed to develop for 8 days. When the implants were derived from 1- and 2-day pupae, there was no increase in protein content during *in vivo* culture. However, when BAGs from 4- and 5-day pupae were implanted, protein content increased. For implants from 4- and 5-day pupae, the average protein content exceeded that of BAGs from normal 0-day adults (Fig. 1). Trehalase was produced in the implants from 4- and 5-day pupae but not in younger glands (Fig. 2A). On the basis of enzyme activity, we conclude that the competence of BAGs to produce trehalase changed as after the third pupal day (Fig. 2B). This change in competence coincides with the pupal peak of ecdysteroid.

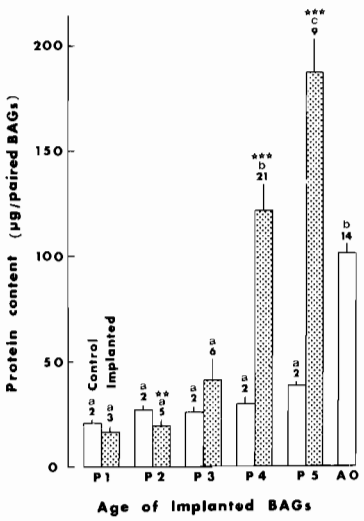

FIG. 1. Protein content of implanted BAGs. Glands from 1- to 5-day male pupae were transplanted into 0-day female adults and allowed to develop for 8 days. Control values show protein content in BAGs before implantation. For control determinations, ten and four pairs of BAGs were pooled for pupa and adult, respectively. For implantation experiments, five pairs of BAGs were used for 1- to 3-day pupae and one pair of BAGs was used for the 4- to 5-day pupa. The number above each column indicates the number of replicates. Vertical bars on the column show ±SEM. P1: 1-day pupa; A0: 0-day adult. Results were compared using the Duncan's new multiple range test. Values without a common superscript $^{(a-c)}$ are significantly different ($P < 0.01$). Asterisks indicate the significance of differences between control and implanted BAGs for the same age ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$), based on a comparison by Student's t test or the Cochran–Cox test.

Effect of 20-Hydroxyecdysone in Vitro on Competence to Produce Trehalase

In *T. molitor,* the ecdysteroid titer rises during the middle of the pupal stage (Delachambre *et al.,* 1980). By correlation, we suspected that ecdysteroids acting between pupal Days 3 and 5 might effect the change in competence for trehalase production.

To investigate directly whether ecdysteroids change the competence of young pupal BAGs, we removed glands from 0- and 2-day pupae and maintained them for 24 hr in Landureau's S-20 medium containing 20-hydroxyecdysone (10^{-5} *M*). At the end of the incubation, the BAGs were washed five times in hormone-free medium (3 ml) and then implanted into 0-day female adults. Eight days later, protein content and trehalase activity were determined in the implants (Fig. 3). Protein and trehalase activity increased markedly in BAGs treated with 20-hydroxyecdysone (Fig. 3).

We exposed 0-day pupal BAGs to concentrations of 20-hydroxyecdysone varying from 10^{-8} to 10^{-4} *M*. Glands were incubated for 24 hr, washed, implanted into 0-day female hosts, and allowed to develop for 8 days. Protein content and trehalase activity increased in a dose-dependent manner (Fig. 4). On the basis of the specific

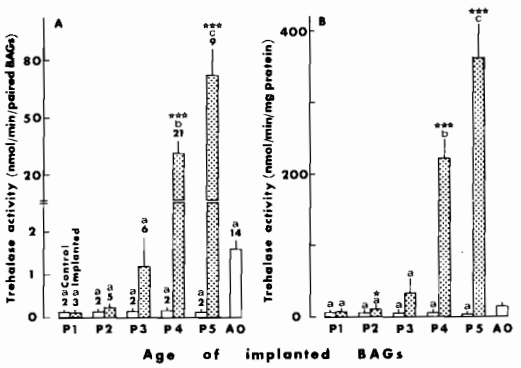

FIG. 2. Trehalase activity as a function of age of implanted BAG. (A) Trehalase activity per pair of BAGs. (B) Trehalase activity per milligram of protein in BAGs. Control values are trehalase activity before implantation. BAGs were implanted into 0-day female adults, allowed to develop for 8 days in their hosts, and then recovered for determinations of trehalase activity. For control determinations, ten and four pairs of BAGs were pooled for pupae and adults, respectively. For implantation experiments, five pairs of BAGs were used for 1- to 3-day pupae. One pair of BAGs were used for 4- and 5-day pupae. Number above each column represents the number of replicates. Vertical bars on the column show ±SEM. P1: 1-day pupa; A0: 0-day adult. Results were compared using the Duncan's new multiple range test. Values without a common superscript $^{(a-c)}$ are significantly different ($P < 0.01$). Asterisks indicate the significance of differences between control and implanted BAGs for the same age ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$), based on a comparison by Student's t test or the Cochran–Cox test.

activity of trehalase, the ED_{50} was 5×10^{-6} M for 20-hydroxyecdysone.

To determine the minimum time required for hormone action *in vitro,* 0-day pupal BAGs were incubated with 20-hydroxyecdysone (10^{-5} M) for various times, and after washout, the glands were implanted into 0-day adults. In BAGs incubated for 6 hr with the hormone, no significant increase in protein content or trehalase activity was found. However, in BAGs incubated with the hormone for 24 hr, increases in protein content and trehalase activity were clearly observed (Table 1).

In order to investigate the role of DNA synthesis in the change in competence, we incubated 0-day pupal BAGs for 24 hr in medium containing 20-hydroxyecdysone (10^{-5} M) and varying concentrations of hydroxyurea. Hydroxyurea at concentrations of 5×10^{-3} M arrests cell cycling in BAGs at early S phase (Yaginuma *et al.*, 1988). After washout of the inhibitor and hormone, the glands were implanted in 0-day adults. Eight days later the protein content and trehalase activity were determined (Table 2). The commitment to trehalase production occurred in the presence of hydroxyurea.

Effect of Ligation

According to the previous results, commitment occurs in the midpupa and yet normal differentiation does not begin until adult ecdysis. Several experiments were carried out to investigate the role of cephalic and anterior thoracic centers on the onset of adult differentiation.

To investigate the role of neuroendocrine centers in the late pupa, male abdomens were ligatured between adult apolysis (at pupal Day 5) and adult ecdysis (at pupal Day 9). When abdomens of 8-day pupae were ligatured, the trehalase activity in the BAGs continued to increase (Table 3). Since no delay in trehalase production occurred in the BAGs after ligature of the pupae, it appears that the cephalic and thoracic centers are not required for the onset of terminal differentiation of the BAGs which is associated with ecdysis. Furthermore, since no acceleration of trehalase production took place in ligatured pupae, it appears that the anterior centers are not responsible for the lag between hormone action at Days 4–5 and its consequences at ecdysis.

We varied the age of the host in order to determine whether factors acting at ecdysis triggered trehalase synthesis. No difference was found when 5-day pupal BAGs were implanted into newly ecdysed or day-old female adults (Table 4).

Adult Development of BAGs in Abdomens Isolated at Ecdysis

To determine whether factor(s) originat-

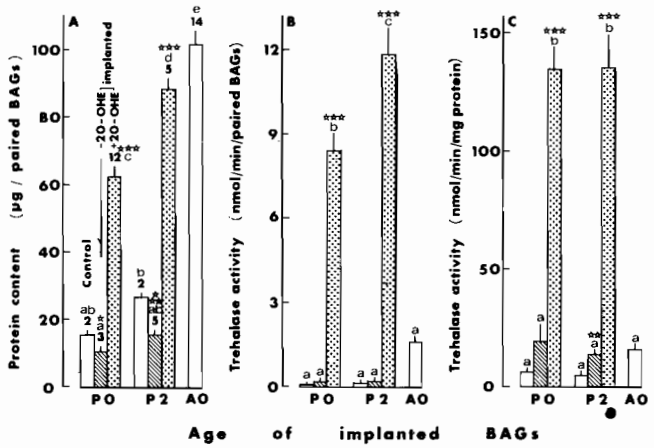

FIG. 3. Effect of 20-hydroxyecdysone on the ability of early pupal BAGs to increase their protein content and trehalase activity after implantation. (A) Protein content per pair of BAGs. (B) Trehalase activity per pair of BAGs. (C) Trehalase activity per milligram of protein of BAGs. Control values show protein content and trehalase activity in BAGs before *in vitro* culture. After dissection, accessory glands were incubated in medium with 20-hydroxyecdysone (10^{-5} *M*) or in hormone-free medium for 24 hr. After washout of the hormone, the BAGs were transplanted into 0-day female adults. BAGs were isolated from the hosts 8 days later for determination of protein content or trehalase activity. For controls (no *in vitro* culture), incubations in basal medium, and incubations with 20-hydroxyecdysone, ten-, six-, and two-paired BAGs were pooled, respectively. The number above each column shows the number of replicates. Vertical bars show ±SEM. P0: 0-day pupa: A0: 0-day adult. Results were compared using the Duncan's new multiple range test. Values without a common superscript $^{(a-e)}$ are significantly different ($P < 0.01$). Asterisks indicate the significance of differences between control BAGs (before culture) and implanted ones which had been cultured *in vitro* either with (−20-OHE) or without (−20-OHE) 20-hydroxyecdysone. Significance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$), was judged by Student's *t* test or the Cochran–Cox test.

ing from the brain, corpora cardiaca, corpora allata, or prothoracic gland were required for the postecdysial adult development of BAGs, teneral male adults were ligatured at the mesothorax and the anterior segments were cut off and discarded. The development of the BAGs in these isolated abdomens was compared with that in fed and starved intact animals.

Starvation or removal of the anterior centers at the time of adult ecdysis reduced the postecdysial growth of the BAGs. During development of BAGs in fed animals, the wet weight, volume, and protein content increased for the first 8 days of adult life. BAGs in starved animals grew well for the first 6 days but lagged behind fed animals at 8 days (Fig. 5A). For BAGs developing in isolated abdomens, the wet weight, volume, and overall protein content were markedly lower than those for BAGs of intact, fed animals.

Although smaller in size than controls, the BAGs in ligatured abdomens showed morphological and biochemical evidence of postecdysial differentiation. Formation of a secretory plug was seen in almost all of the BAGs from intact animals and in over half of those from isolated abdomens (Table 5).

BAGs in isolated abdomens produced trehalase. Although the total activities of trehalase in the glands from starved animals and isolated abdomens were somewhat lower than those in fed controls (Fig. 5B), the specific activities of trehalase in controls and in isolated abdomens were indistinguishable by Duncan's multiple range test at 2 and 4 days (Fig. 5C). At older ages, the specific activity of trehalase in isolated abdomens lagged somewhat behind the fed

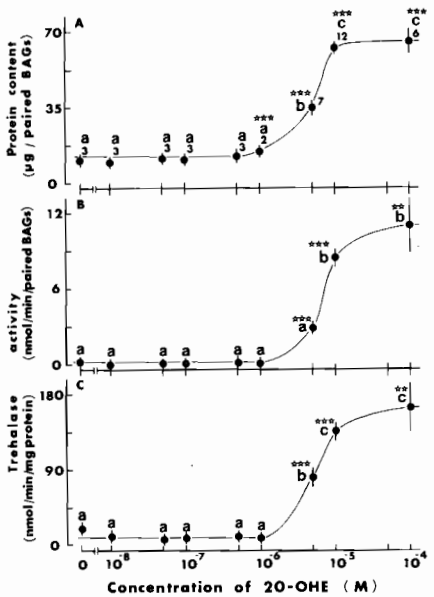

FIG. 4. Dose–response curve for the commitment by 20-hydroxyecdysone. (A) Protein content per pair of BAGs. (B) Trehalase activity per pair of BAGs. (C) Trehalase activity per milligram of protein of BAGs. Accessory glands were dissected from male 0-day pupae and incubated with varying concentrations of 20-hydroxyecdysone for 24 hr. The hormone was washed out, and then the BAGs were transplanted into 0-day female adults for 8 days of development. After dissection of the BAGs from the female hosts, protein content and trehalase activity were determined. Seven pairs of BAGs were pooled for hormone concentrations of 0 to 10^{-6} *M* and three pairs of BAGs were pooled for concentrations of 5×10^{-6} to 10^{-4} *M*. The number above each point shows the number of replicates. The vertical bars show the means ±SEM. Results were compared using the Duncan's new multiple range test. Values without a common superscript $^{(a-c)}$ are significantly different ($P < 0.01$). Asterisks indicate the significance of differences between control and implanted BAGs for the same age ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$), based on a comparison by Student's *t* test or the Cochran–Cox test.

controls. These results show that the brain, corpora cardiaca, corpora allata, and anterior thoracic centers were not necessary in postecdysial adults at the onset of trehalase synthesis and the time of the formation of a plug.

DISCUSSION

Metamorphosis is associated with commitment to a new developmental program. In many insects the commitment may precede its expression by some time. In the epidermis of larval *Manduca sexta,* Riddiford has elegantly demonstrated that the cells become committed to the production of pupal cuticle on Day 3 while the synthesis of the cuticle proteins does not begin until Day 6 (1978, 1981, 1985). Similarly in *T. molitor,* the critical period for the adult molt is in the last larval instar (Stellwaag-Kittler, 1954; Delbecque, 1976) and yet the adult cuticle deposition does not begin until 5–7 days later in the midpupa.

Acceleration of Cell Cycling and the Control of Commitment

The trehalase produced by the BAGs begins to accumulate at the end of the pupal stage (Yaginuma and Happ, 1988) as do many other adult-specific proteins (Happ *et al.,* 1982; Grimnes and Happ, 1986; Grimnes *et al.,* 1986; Shinbo *et al.,* 1987). The present study shows that the competence of the glands to produce trehalase depends on events several days earlier, in the midpupa. When we transplanted pupal glands of varying ages into adult hosts, the ability of pupal BAGs to synthesize trehalase appeared to change on the third pupal day (Figs. 2 and 3). A similar result was obtained with leucine incorporation into proteins separated on two-dimensional gels (Happ, 1987) and with dot blots using the monoclonal antibodies PL 3.4, PL 6.3, and PL 21.1 (Grimnes and Happ, 1987).

Because ovaries are known sources of ecdysteroids in many insects (Hagedorn, 1985) and ovaries are present in all female hosts used in the present study, it is important to consider the use of female hosts for implanting male BAGs. Three facts suggest that ovarian factors were not responsible for the changes of competence to synthesize adult-specific proteins: (1) According to the data of Briers and De Loof (1983), ecdysteroids in adult males and females are

TABLE 1
EFFECT OF DURATION OF EXPOSURE TO 20-OHE ON TREHALASE ACTIVITY OF 0-DAY PUPAL BEAN-SHAPED ACCESSORY GLANDS (BAGs) IMPLANTED INTO 0-DAY FEMALE ADULTS

Exposed duration (hr)	n	Protein content (μg/BAGs)	Trehalase activity (nmol/min/BAGs)	Trehalase activity (nmol/min/mg protein)
0	3	10.5 ± 0.9^{a}	0.21 ± 0.08^{a}	19.5 ± 6.6^{a}
2	2	12.0 ± 1.5^{a}	0.16 ± 0.02^{a}	13.8 ± 3.7^{a}
6	2	10.0 ± 0.4^{a}	0.13 ± 0.01^{a}	13.1 ± 0.7^{a}
24	12	$62.2 \pm 2.8^{b,***}$	$8.40 \pm 0.68^{b,***}$	$134.6 \pm 8.7^{b,***}$

Note. Accessory glands were dissected from 0-day male pupae and incubated in S-20 medium with 20-hydroxyecdysone (10^{-5} *M*) for various times. After washout, BAGs were transplanted into 0-day female adults. Eight days later BAGs were isolated from the hosts. Six pairs of BAGs were pooled for the 0- to 6-hr incubations with hormone and two pairs of BAGs were pooled for the 24-hr incubation with hormone. Means ± SEM are shown. *n*, Number of replicates. Results were compared using the Duncan's new multiple range test. Values without a common superscript $^{a-b}$ are significantly different ($P < 0.01$). Asterisks indicate the significance of differences between control and implanted BAGs for the same age ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$), based on a comparison by Student's *t* test or the Cochran–Cox test.

at very low levels (below 50 ng/g tissue (roughly 1×10^{-7} if 1 g equals 1 ml) for ecdysone and 15 ng/g tissue roughly 3×10^{-8} for 20-hydroxyecdysone). (2) As described previously under Results, after 5-day pupal BAGs had been implanted into male or female hosts, trehalase activity increased irrespective of the sex of the host. (3) Finally, the only younger implants which showed strong increases of trehalase in female hosts were those which had been exposed to 20-hydroxyecdysone *in vitro* (Fig. 3). Brain-derived hormones, ecdysteroids, and juvenile hormones control the development of reproductive organs in both female and male insects (Giradie, 1983; Hagedorn, 1985; Koeppe *et al.*, 1985). In the present study, we show that BAGs become competent to produce adult-specific proteins at the time of the pupal ecdyster-

TABLE 2
EFFECT OF HYDROXYUREA DURING EXPOSURE TO 20-OHE ON TREHALASE ACTIVITY OF 0-DAY PUPAL BEAN-SHAPED ACCESSORY GLANDS (BAGs) IMPLANTED INTO 0-DAY FEMALE ADULTS

20-OHE (*M*)	Hydroxyurea (*M*)	n	Protein content (μg/BAGs)	Trehalase activity (nmol/min/BAGs)	Trehalase activity (nmol/min/mg Protein)
10^{-5}	0	12	62.2 ± 2.8^{a}	8.40 ± 0.68^{a}	134.6 ± 8.7^{a}
10^{-5}	5×10^{-4}	4	34.6 ± 2.9^{b}	2.20 ± 0.17^{b}	67.5 ± 6.8^{b}
10^{-5}	5×10^{-3}	4	25.0 ± 0.3^{c}	1.56 ± 0.48^{b}	61.9 ± 18.6^{b}
10^{-5}	10^{-2}	4	23.3 ± 0.9^{c}	$1.13 \pm 0.19^{b,c}$	$48.4 \pm 7.5^{b,c}$
0	0	3	10.5 ± 0.9^{d}	0.21 ± 0.08^{c}	19.5 ± 6.6^{c}

Note. Accessory glands were dissected from 0-day male pupae, and incubated with 20-hydroxyecdysone (10^{-5} *M*) and varying concentrations of hydroxyurea in S-20 medium. After washout of hormone and inhibitor, the BAGs were implanted in 0-day female adults. Eight days later BAGs were isolated from the hosts and assayed for protein content and trehalase activity. For control determinations after incubation in basal medium followed by implantation, ten pairs of BAGs were pooled. After incubation in culture media containing hormone, two pairs of BAGs were pooled. After incubation with inhibitor plus hormone followed by implantation, five pairs of BAGs were pooled. Means ± SEM are shown. *n*, Number of replicates. Results were compared using the Duncan's new multiple range test. Values within each column without a common superscript $^{a-d}$ are significantly different.

TABLE 3
TREHALASE ACTIVITY OF MALE BEAN-SHAPED ACCESSORY GLANDS (BAGS) IN ISOLATED ABDOMENS LIGATED IN 8-DAY PUPAE

Ligated stage	Examined stage	n	Protein content (μg/BAGs)	Trehalase activity (nmol/min/BAGs)	Trehalase activity (nmol/min/mg protein)
Untreated	8-day pupa	3	78.4 ± 3.8^{a}	0.57 ± 0.10^{a}	7.3 ± 1.5^{a}
Controls	0-day adult	14	101.4 ± 3.7^{b}	1.59 ± 0.15^{a}	15.9 ± 1.5^{a}
	1-day adult	3	160.3 ± 8.7^{c}	23.11 ± 2.15^{c}	144.2 ± 22.1^{c}
8-day pupa	0-day adult	3	102.5 ± 5.4^{b}	1.63 ± 0.34^{a}	16.1 ± 3.5^{a}
8-day pupa	1-day adult	3	$93.4 \pm 4.0^{a,b,**}$	$7.90 \pm 1.55^{b,**}$	83.5 ± 13.3^{b}

Note. Isolated male abdomens were prepared by cutting just ahead of the mesothoracic legs of pupae. Two pairs of BAGs were pooled and used for the determination of protein content and trehalase activity. Each value shows a mean ± SEM. n, Number of replicates. Results were compared using the Duncan's new multiple range test. Values without a common superscript $^{a-c}$ are significantly different ($P < 0.01$). Asterisks indicate the significance of differences between untreated control and pupae ligated at 8 days ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$), based on a comparison by Student's t test or the Cochran–Cox test.

oid peak which begins at Day 3 and reaches its maximum on Days 4–5 (Delachambre *et al.*, 1980). When BAGs from 0- and 2-day pupae were exposed *in vitro* to 20-hydroxyecdysone, they became competent to produce trehalase. The effect is dose dependent with an ED_{50} of 5×10^{-6} *M*. This ED_{50} is near to the ecdysteroid levels (8×10^{-6} *M*) characteristic of the midpupal peak *in vivo* (Delachambre *et al.*, 1980). Ecdysteroid application *in vitro* has been shown to change the commitment in the epidermis of *M. sexta* where the ED_{50} was 13 times higher than the maximum titer in the hemolymph (Riddiford, 1978). For the commitment of the epidermis of *M. sexta*, the ET_{50} (time for 50% response) is 17 hr (Riddiford, 1978), a duration of the same general order as that for the BAGs of *T. molitor* (between 6 and 24 hr, Table 1).

There are two consequences of ecdysteroid action on BAGs in the midpupa: acceleration of the cell cycle (Yaginuma *et al.*, 1988) and acquisition of the competence to make adult-specific proteins (Happ, 1987; Grimnes and Happ, 1987; the present study). Is there a link between these two effects of ecdysteroid?

The cell-cycle effect and the competence effect can be compared on the basis of the dose–response and time–response data. In 0-day pupal BAGs, ED_{50} for the hormone-stimulated flow of cells from G_2 phase into G_1 phase is 5×10^{-7} *M* 20-hydroxyecdy-

TABLE 4
EFFECT OF AGE OF HOST ON TREHALASE ACTIVITY OF 5-DAY PUPAL BEAN-SHAPED ACCESSORY GLANDS (BAGS) IMPLANTED INTO FEMALE ADULTS

Age of host adult	n	Protein content (μg/BAGs)	Trehalase activity (nmol/min/BAGs)	Trehalase activity (nmol/min/mg protein)
0–3 hr	4	117.0 ± 25.9	51.4 ± 20.02	383.4 ± 87.1
1 day	5	157.7 ± 21.7	54.61 ± 9.95	346.3 ± 20.0

Note. Accessory glands were dissected from 5-day male pupae, washed in culture media, and then transplanted into female adults. Eight days later, glands were isolated from the hosts and one pair of BAGs were used for the determination of protein content and trehalase activity. Each value shows the mean ± SEM. n, Number of replicates. Results were compared using Student's t test of the Cochran–Cox test. There were no significant differences between 0–3 hr and 1 day within each column.

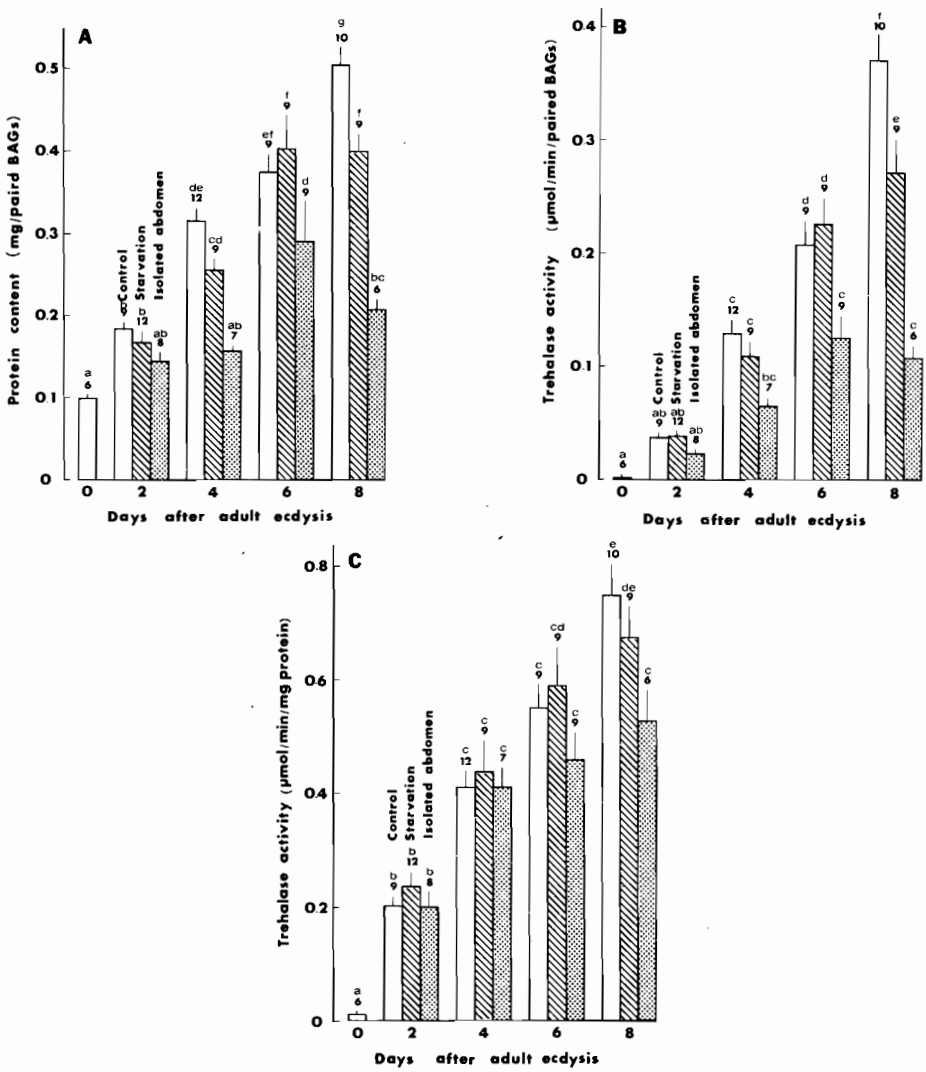

FIG. 5. Effect of removal of anterior centers on protein content and trehalase activity in BAGs during postecdysial adult development. (A) Change in protein content per pair of BAGs. (B) Change in trehalase activity per pair of BAGs. (C) Change in trehalase activity per milligram of protein of BAGs. One group of males was starved just after adult ecdysis and another group of males was used for preparation of isolated abdomens. Isolated male abdomens were prepared by cutting just ahead of the mesothoracic legs of newly ecdysed adults. For 0-day adults, four pairs of BAGs were pooled. For 2- to 8-day adults, one pair of BAGs were used. The number above each column represents the number of replicates. Vertical bars on the column indicate a value of ±SEM. Results were compared using the Duncan's new multiple range test so that values without a common superscript $^{(a-g)}$ are significantly different ($P < 0.01$).

sone (Yaginuma *et al.*, 1988), an order of magnitude lower than 5×10^{-6} *M*, the ED_{50} for the change in competence (present paper). Furthermore, cell cycling is accelerated sharply with hormone exposures of only 30 min whereas the change in competence requires over 6 hr of exposure to ecdysteroid. The hormone dose and time of exposure required *in vitro* for the two effects differ markedly. Comparison of these

TABLE 5
ADULT DEVELOPMENT OF MALE BEAN-SHAPED ACCESSORY GLANDS (BAGs) IN ISOLATED ABDOMENS OF ADULT *Tenebrio molitor*

Treated stage (day)	Examined stage (day)	Examined/Treated animals	Wet weight of paired BAGs (mg)[b]	Volume of BAG (mm^2)[c]	Formation of plug
Untreated	0[d]	10/10	1.08	$0.176 \pm 0.044^{a'}$	0/10
Untreated	2	10/10	1.70	$0.288 \pm 0.018^{b,*}$	0/10
Untreated	4	09/09	2.21	$0.362 \pm 0.038^{b',c',**}$	9/9
Untreated	8	30/30	3.33	$0.446 \pm 0.033^{c',***}$	28/30
Starved[e]	8	21/21	2.60	$0.406 \pm 0.020^{b',c',***}$	19/21
0[d]	8	09/41	1.72	$0.292 \pm 0.032^{b',*}$	5/9
1	8	09/29	2.07	$0.317 \pm 0.016^{b',*}$	6/9
2	8	01/14	—[f]	0.284	0/1
3	8	02/14	2.03	$0.298 \pm 0.039^{b'}$	2/2
4	8	04/14	2.25	$0.357 \pm 0.043^{b',c',*}$	3/4

Note. [a]These isolated adult male abdomens were prepared by cutting just ahead of the mesothoracic legs. [b]Pairs of BAGs were pooled and then weighed. [c]Size of BAGs was expressed as the length (mm) × the depth (mm) of one BAG. [d]Males within 0–1 hr after adult ecdysis. [e]Males starved just after adult ecdysis. [f]Not determined. Each value shows the mean ± SEM. Results were compared using the Duncan's new multiple range test. Samples of gland volumes without a common superscript $^{a-c}$ are significantly different ($P < 0.01$). Asterisks indicate the significance of differences between control and implanted BAGs for the same age ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$), based on a comparison by Student's t test or the Cochran–Cox test.

results *in vitro* with the pupal development of the BAGs *in vivo* (Happ *et al.*, 1985; Happ, 1987) and with hormone levels *in vivo* (Delachambre *et al.*, 1980), suggests that acceleration of cell cycling is brought about as ecdysteroid titers begin to rise on pupal Day 3 while the change in competence requires the higher hormone levels characteristic of pupal Days 4 and 5.

In many systems, changes in the pattern of gene expression have been reported to require a cell division (Holtzer *et al.*, 1972; Wielgus *et al.*, 1979; Wolbert and Kubbies, 1983). However, a "quantal cell cycle" does not appear to be necessary in all cases (Kumaran, 1978; Chiu and Blau, 1984). 20-Hydroxyecdysone acted to change the competence of the BAGs in the presence of hydroxyurea at a concentration of 5×10^{-3} *M* (Table 2). Such a concentration of hydroxyurea blocks cell cycling near the G_1–S boundary (Yaginuma *et al.*, 1988). Our data are consistent with commitment without DNA synthesis in the pupal BAG.

Regulation of Postecdysial Adult Development of BAGs

Once committed at Pupal Day 4–5, the BAGs grow in size over pupal Days 6–8 and then begin to make adult-specific proteins. Does the onset of synthesis of adult-specific proteins depend upon additional humoral triggers? Does the peak differentiation of the BAGs depend upon continuing modulation by neuroendocrine and other humoral factors?

Synthesis of trehalase follows the ecdysteroid decline. After the ecdysteroid titer falls on pupal Days 7–8, it does not rise significantly in the late pupa (Delachambre *et al.*, 1980) or in the first 10 days of adult life (Briers and De Loof, 1983). Could the decline in ecdysteroid promote differentiation? In certain insects, a high titer of ecdysteroid delays the adult ecdysis (Schwartz and Truman, 1983). After administration of hormone *in vitro,* some cuticle proteins of *Drosophila melanogaster* accu-

mulate in response to ecdysteroid decline (Doctor *et al.*, 1985). We do not have an unambiguous way to test this possibility for the BAGs. The onset of differentiation does occur within Landureau's S-20, but the dramatic postecdysial adult accumulation has not yet been seen *in vitro* (Grimnes and Happ, 1987, J. J. Lenoir-Rousseaux, unpublished). In preliminary experiments we found that after injection of 20-hydroxyecdysone into 0-day male adults, the increase in trehalase activity of BAGs was inhibited. However, the physiological significance of this apparent inhibition is not clear since the effect was not dose dependent.

Juvenile hormone controls the development of accessory glands in some adult insects, for example in *Leptinotarsa decemlineata* (De Loof and Legasse, 1972) and *Periplaneta americana* (Blaine and Dixon, 1973). For the male accessory glands of *P. americana,* it has recently been shown that juvenile hormone controls trehalase activity (Ogiso and Takahashi, 1984). Trehalase activity of BAGs increased markedly in the isolated male abdomens, but it fell short of that achieved in intact adults (Fig. 3). The reduced differentiation could reflect the absence of the corpora allata. However, when juvenile hormone was applied to the surface of the isolated abdomens, it failed to stimulate BAG growth and differentiation (Yaginuma, unpublished).

The onset of differentiation associated with ecdysis (Table 3) and a postecdysial increase in trehalase activity (Fig. 5, Table 5) seem independent of cephalic or thoracic centers. However, the BAGs within isolated male abdomens did not achieve total trehalase activity characteristic of 8-day intact adults, and the hemolymph volume appeared reduced. We cannot exclude the possibility that factors from anterior centers act as metabolic hormones which indirectly enhance the growth and differentiation of the BAGs by increasing the availability of precursors for protein synthesis.

The experiments reported in this paper clearly establish the role of ecdysteroids in the change of competence in BAGs that occurs in the midpupa. It is significant that the commitment effect is distinct from the cell-cycle effect (Yaginuma *et al.*, 1988). The expression of this commitment after ecdysis may be gland autonomous.

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