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Effects of a Large Dose of Ecdysteroid on Development of Ovary in *Bombyx* Pupae

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Administration of 100 μg 20-hydroxyecdysone into a female *Bombyx* pupa at day 2 retarded its adult emergence by up to 8 days. The hormone treatment caused a significant delay in weight increase of the ovary. However, the ovary weight reached a normal level after the elongated pupal period. The animals thus treated deposited fertilized eggs, which were heavier and larger than normal eggs. These results suggest that the high dose of exogenous hormone perturbed the ovarian development, but this perturbation was compensated by the elongation of developing time of ovaries.

INTRODUCTION

A hormone is usually effective in trace amounts upon its target organs and a high concentration of hormone is usually regarded as harmful to biological processes. However, insect pupae often exibit very high titre of the 'molting' hormone, ecdysteroid. The titre sometimes reaches a level of 10^{-6} M, which is one or two orders of magnitude higher than the 'physiological' concentrations of ecdysteroid estimated from the results of the induction of chromosomal puffs and of morphogenesis of imaginal discs (cf., e.g. Ashburner, 1973; Hanaoka and Ohnishi, 1974; Mandaron, 1973). It has been reported that tanning or ecdysis of integuments cultured *in vitro* requires ecdysteroid at a level of 10^{-6} M (Marks, 1970). However, the role of high titre of the pupal hormone is uncertain at present.

Injection of high doses of various kinds of ecdysteroids is harmful to the silkmoth pupae that are diapausing due to the lack of ecdysteroid, bringing about adults with partial pupal cuticles (metathetely). However, once the normal adult development is commenced by administration of an appropriate dose of the hormone, readministration of large amounts of ecdysteroids seems no more harmful (Williams, 1968). Developing pupae may be resistant to high concentrations of external ecdysteroids. Moreover, injection of 20-hydroxyecdysone (5 μg per animal) at day 4 into developing *Bombyx* pupae caused an increase in accumulation of yolk protein during oogenesis (Takei *et al.*,

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1978). As an attempt to search the mechanisms which make the pupal system resistant to hyperecdysonism and to study the role of the large concentration of pupal ecdysteroid, we injected various doses of 20-hydroxyecdysone into female *Bombyx* pupae and observed the effects. The present paper describes that the pupae which received the hormone at a dose as high as 100 μg per animal at day 2 after larval-pupal ecdysis delay in adult emergence and produce eggs larger and heavier than normal. Application of this excess dose of hormone was also intended to inquire how a developing system responds toward external perturbation.

MATERIALS AND METHODS

Materials

Two hybrid races, N124 x Cl24 and N122 x C115, were used. Ovaries were dissected from pupae on a wax plate immersed in physiological saline, freed of sticking fat bodies and tracheae with forceps, and transferred onto slightly wet filter paper. After several seconds the ovaries were weighed in vials. Some series of samples were then dried thoroughly at 80°C and weighed again. The difference in weight before and after drying was taken as the water content of the ovaries.

Injections of hormones

At day 2 of pupal age, when the titre of endogenous ecdysteroid was at its maximum (Hanaoka and Ohnishi, 1974), 1 to 100 μg per individual of 20-hyroxyecdysone (Rhoto Pharmaceutical Co. Ltd, Osaka) in 10 μl 10% ethanol was injected into female pupae with glass needles. Juvenile hormone (C-18, Eco-Chemical Intermediates, Cambridge) in 10 μl peanut oil was similarly administered. Sometimes the ecdysteroid was injected at day 8 instead of day 2. Controls were given with ethanol or peanut oil only. 'Normal' animals received no operation.

Investigation of weight, number and size of deposited eggs

Freshly emerged moths were copulated for 6 h and allowed to oviposit onto filter paper under cover for several days. After counting the number of deposited eggs, many of them were detached from filter paper at random, weighed and measured for witdth and length by use of a Nikon Shadowgraph at a lo-fold scale. The product of the width and the length was taken to indicate the egg size. The rest of the eggs were cooled at 4°C from day 2 post-oviposition until 3 months in order to break diapause. They hatched after 11 days at 25°C.

RESULTS

The length of time required for newly ecdysed pupae to complete adult development was almost 10 to 12 days for normal pupae, control pupae and those which received 1 μg of 20-hydroxyecdysone at day 2 of pupal stage (Fig.

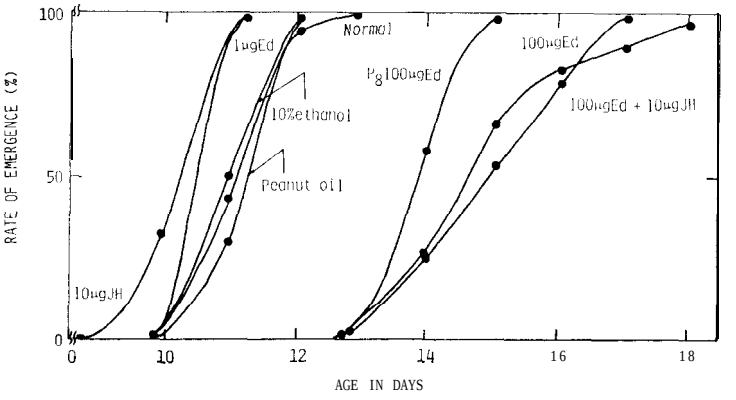

Fig. 1. Effects of 20-hydroxyecdysone (Ed) and C-18 juvenile hormone (JH) on the date of emergence. The abscissa shows the days after larval-pupal ecdysis. The hormone was injected usually at day 2 or sometimes at day 8 (P_8). The dose (per pupa) of the hormone was specified beside each trace. For each dose 23 pupae were used. For 'normal', see Methods. Data with N 122×C 115 are seen. N 124x C 124 gave similar results.

1). This was also the case for 10 μg dose of the same hormone (not shown). When 100 μg of the ecdysteroid was given at day 2, the first imago appeared at day 13 and the last imago appeared at day 18; thus adult emergence was up to 8 days later than normal. Day-8 administration of 100 μg of the ecdysteroid also retarded emergence but gave a smaller interval than day-2 administration. Juvenile hormone (JH) treatment (10 μg) at day 2 quickend emergence by one day. This effect of JH was diminished by 100 μg 20-hydroxyecdysone injected simultaneously. When 1 mg of 20-hydroxyecysone was administered, the pupae died within a few days.

Fig. 2 shows the time course of changes in wet and dry weight of ovaries. The hormone treatment did not seem to affect the weight at least until day 6. At day 8, slight but statistically significant retardation in increase of the wet and dry weight was observed in the case of 100 μg ecdysteroid. The weight at this day apparently depended upon the hormone dose. This was more clearly seen when the dry weight of the ovary was replotted against the hormone dose (Fig. 3).

The number of eggs deposited after mating (Fig. 4) seemed to be reduced due to 100 μg ecdysteroid, although the difference was not significant. Moreover, ecdysteroid injection made the deviation of egg number smaller than normal (Fig. 4).

As shown in Fig. 5, the average weight of freshly deposited eggs seemed to be increased by administration of 100 μg ecdysteroid at day 2 or 8, but apparently decreased by JH injection at day 2.

The animals treated with 100 μg hormone at day 2 gave a wide distribution of the 'size' (see Methods) of deposited eggs compared to normal and other cases (Fig. 6). It is remarkable that many large-sized eggs were pro-

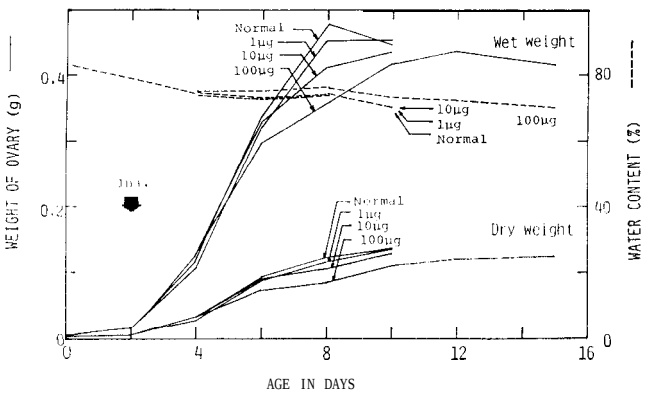

Fig. 2. Effects of 20-hydroxyecdysone on the weight of ovary during pupal stage. For abscissa, see Fig. 1. The hormone was injected into pupae (N 124×C 124) at day 2 at the dose (per pupa) specified in the figure. For each dose 15 pupae were used. Results are expressed in mean ovarian weight per pupa. For 'normal', see Methods. At day 8, the wet or dry weight for the 100-μg hormone sample was significantly smaller than the other samples ($p < 0.05$).

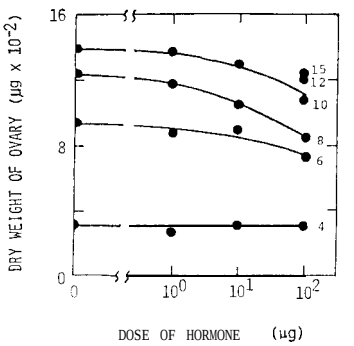

Fig. 3. Dry weight of ovary as a function of the dose of 20-hydroxyecdysone (Ed) injected at day 2. The numerical numbers 4-15 are the age of pupae when the ovaries were weighed. Dose zero means control. Data were replotted from Fig. 2.

duced by 100 μg hormone given at day 2; this makes the mean size of the eggs significantly larger compared to control (Fig. 7). A shoulder toward the large size was seen in the size distribution pattern when 100 μg hormone was given at day 8 (Fig. 6). JH reduced the size of eggs significantly (Figs. 6 and 7).

The eggs deposited from the hormone treated animals were hatchable after break of diapause (see Methods). The average weight per eggshell remnant after hatching did not seem to be affected severely by the hormone treatment (Table 1). In the case of 100 μg ecdysteroid the value was 83% of

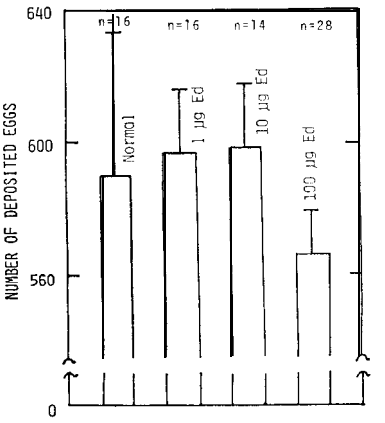

Fig. *4.* Effects of 20-hydroxyecdysone (Ed) on the number of deposited eggs. Ed was injected into pupae (N 124×C 124) at day 2 at the dose (per pupa) indicated in the figure. For each dose 14 to 28 pupae were used as indicated on top. Each bar represents the mean number of deposited eggs per pupa with a 95% confidence interval. For 'normal', see Methods.

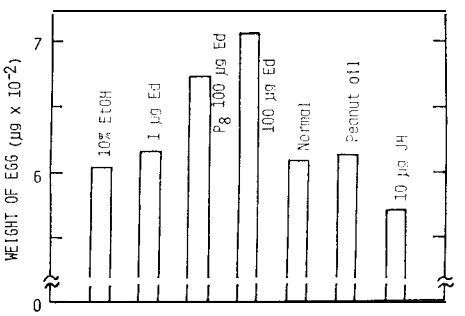

Fig. 5. Effects of 20-hydroxyecdysone (Ed) and C-18 juvenile hormone (JH) on the weight of deposited eggs. The hormone was injected into pupae (N 122×C 115) at day 2 at the dose (per pupa) indicated in the figure. For each measurement 500 eggs were taken randomly out of a pool from 23 moths. They were weighed at once and the average weight per egg was obtained by division. For other details, see Figs. 1 and 2.

control and 92% of normal. The eggshell layer is partly ingested by the larva during hatching, and weighing the eggshell leftovers is an indirect measure for chorion proteins. We infer that the final amounts of accumulated chorion proteins in the hormone treated pupae were at levels comparable to normal (cf. also the footnote in Discussion). On the other hand, the weight of hatched larvae was affected by 100 µg ecdysteroid given at day 2 of pupal stage (Table 1).

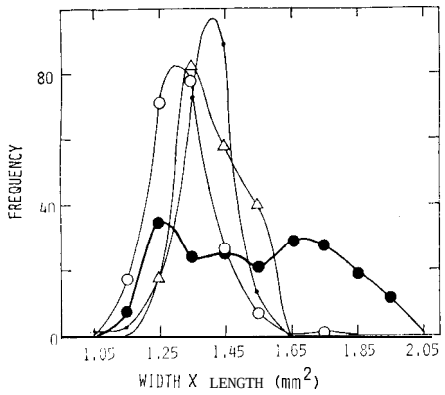

Fig. 6. Effects of 20-hydroxyecdysone (Ed) and C-18 juvenile hormone (JH) on the size distribution of deposited eggs. Pupae (N 122 x C 115) received hormone as indicated below at day 2 (except for P_8) as mentioned in Fig. 1. Deposited eggs *were* measured for width×length (see Methods). For each analysis 200 eggs taken randomly out of a pool of eggs deposited by 20 moths were used. •, 10% ethanol (EtOH), peanut oil, normal, or 1 μg Ed; ●, 100 μg Ed; △, P_8 100 μg Ed; ○, 10 μg JH.

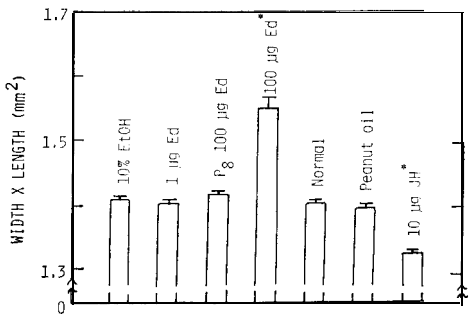

Fig. 7. Effects of 20-hydroxyecdysone (Ed) and C-18 juvenile hormone (JH) on the mean size per egg. Data were arranged from Fig. 6. *, significant at $p < 0.01$ by the Kolmogorov-Smirnov test.

DISCUSSION

Our work shows that the developing silkworm pupae are resistant to large doses of ecdysteroid. Administration of 10 μg of 20-hydroxyecdysone, probably lo-fold excess of the endogenous amount of ecdysteroid, did not disturb the time schedule of adult development. Only the animals met with 100-fold excess of the hormone exhibited changes in period of emergence, but they seemed unchanged morphologically. The resistance of pupae to the exogenous hormone was also manifest in the reproduction system maturing during pupal stage. The eggs deposited from the animals thus treated with the 100-fold excess hormone could hatch, implying that the oogenesis and fertilization

Table 1. Effects of 20-hydroxyecdysone (Ed) and C-18 juvenile hormone (JH) on the weight of eggshells and larvae after hatching. Pupae (N 122 xC124) were treated with above substances at day 2 (or 8) as mentioned in Fig. 1. For each measurement 500 hatched individuals and eggshell leftovers were taken randomly out of a pool of eggs deposited by 20 moths. They were air-dried and weighed. The average weight per shell or larva was calculated by division.

Treatment	Average weight of		Hatched eggs per total
	eggshell	larva	
	(μg)		(%)
Normal	62.9	147	94. 4
10% ethanol	69.5	149	91.0
1 μg Ed	62.1	118	60. 2
100 μg Ed	57.7	183	82. 8
P_8 **100** μg Ed	67. 9	119	83.6
Peanut oil	68. 7	110	53.2
10 μg JH	67. 1	99	74.8

occurred normally. However, the 100-μg hormone treatment lowered the increase in wet and dry weight of ovary during oogenesis. Interestingly, this lowering was finally offset by the extended period of oogenesis and this extension was warranted by the delay of adult emergence. The oocyte maturation might possibly be coordinated with eclosion, both being dependent upon ecdysteroid.

The eggs from the animals treated with the 100-fold excess hormone showed hypertrophy in weight and size of eggs. The weight increase could not be ascribed to the chorion proteins (see Table 1), and thus to oocyte materials. In fact, newly hatched larvae derived from the animals that had been treated with 20-hydroxyecdysone at day 2 of pupal age were found to be heavier than control by more than 20% (Table 1).[1] Yolk formation is stimulated by the exogenous ecdysteroid according to Takei *et* al. (1978) and Ono ***et al.*** (1975), and this, together with the elongation of oogenesis period, might cause the excess accumulation of yolk substances, which in turn explains the occurrence of hypertrophic larvae. It is possible to suppose that the high titre of ecdysteriod at the early pupal stage has a role in determining the extent of oocyte development. This inferrence is further supported by the fact that juvenile hormone, a possible antagonist of ecdysteroid, reduced the egg size and the period of oocyte maturation (Figs. 6 and 1). Based upon these arguments, we suggest that the ovary recovers from the artificial hyperecdysonism by a simple way, an elongation of developmental time. This might be a mode of response of developing systems against perturbation.

The action of excess ecdysteroid presumably involves the following processes. (1) Promotion of some mechanisms responsible for catabolic processes of ecdysteroid. (2) Lowering the hormonal efficiency by the reduction of

[1] Larger larvae must have ingested more of eggshells than normally sized larvae during hatching and measurements of eggshell weight after hatching in the former case may suffer from a more intense underestimation.

their affinities for receptors. (3) Slow transmission of hormonal information to post-transcriptional levels, e.g. initiation processes of peptide synthesis (cf. Liao ***et al., 1975).*** Reseach work which should permit approach to any of the above possibilities will be of interest in relation to the mode of action of the steroid hormone.

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