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Effect of 20-hydroxyecdysone on diapausing pupae of the fall webworm, *Hyphantria cunea* Drury.

미국흰불나방(*Hyphantria cunea* Drury) 휴면번데기에 대한 탈피호르몬의 영향

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ABSTRACT This study was conducted to investigate the effect of 20-hydroxyecdysone on diapausing pupae of *Hyphantria cunea*. Treatment of 20-HE at the dosage of 5 μg or higher/g live weight terminated diapause in about 50% of *Hyphantria cunea* pupae treated, and some malformed adult emerged at dosages of 10 or 12.5 μg of 20-hydroxyecdysone/g live wt. The maximum concentration of 20-hydroxyecdysone in the whole body of normally developing pupae was about 3.2 μg/g on the 6th day after pupation, and in that of diapausing pupae treated with 20-HE about 4.1 μg/g on the 6th day after the treatment. But diapausing pupae showed a low level(1.7 μg/g) of maximum 20-HE concentration. In diapausing pupae treated with 20-HE, glycongen content was lower than in normally developing pupae, but the changing pattern was similar to that observed in normally developing pupae. Glucose(and/or sorbitol) and trehalose content of 20-HE-injected pupae reached the maximum value on the 6th day after the injection, which were higher than those of normally developing pupae. The amount of soluble haemolymph proteins was lower but the total soluble protein content of haemolymph-removed whole body was higher in 20-HE-injected pupae than that in normally developing pupae.

KEY WORDS *Hyphantria cunea*, 20-HE, Diapause, CHO, Protein

초 록 미국흰불나방 휴면번데기에 5μg/g(생체중) 이상의 탈피호르몬을 처리하면 처리된 용의 약 50%가 우화했으며, 10μg이나 12.5μg/g의 처리구에서는 몇마리의 기형적인 성충이 나타났다. 한편 탈피호르몬의 체내함량을 측정한 결과 정상용은 용화 후 6일째에 약 3.2μg/g의 최대값을 보였으며, 탈피호르몬을 처리한 용에서는 처리 6일째에 4.1μg/g의 최대값을 보였다. 그러나, 휴면 용에서는 낮은 탈피호르몬 함량을 보였는데 측정기간중 얻은 최대치는 1.73μg/g 정도였다. 탈피호르몬을 처리한 휴면 용의 글리코겐함량은 정상용 보다 낮았으나, 성충조직 발육기간 동안의 발육양상은 정상 용의 경우와 비슷하였다. 또한 같은 처리를 받은 휴면 용의 포도당-솔비톨과 트레할로스의 농도는 처리 후 6일째에 최대값을 보였는데, 그 수준은 정상적으로 발육하는 개체의 경우보다 높았다. 탈피호르몬 처리를 받은 휴면 용의 혈림프 내 수용성단백질 함량은 정상 용의 그것보다 낮았으며, 혈림프를 제외한 전체 조직 내에서 정상 용의 경우보다 높았다.

검 색 어 미국흰불나방, 탈피호르몬, 휴면, 탄수화물함량, 단백질함량

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Diapause is a state of developmental arrest and shows a great deal of difference in physiological, biochemical and endocrinological characters when compared to normally developing individuals (Beck 1980). It is induced by the change in concentration of a certain hormone.

In the case of pupal diapause in lepidopterous and dipterous insects, the accepted concept has been that environmental cues suppress the synthesis and/or release of the primary effector (prothoracicotropic hormone, PTTH) for adult development (Denlinger 1985). The failure of PTTH to be synthesized and/or released from the brain results in the consequent failure in the activation of prothoracic glands for synthesis of ecdysone, which is subsequently hydroxylated to active molting hormone, 20-hydroxyecdysone (20-HE) in peripheral tissues (Bollenbacher *et al.* 1977). But in *Heliothis zea*, release of PTTH does not appear to be curtailed by the stimulus which induces diapause but PTTH is actually known to be released shortly after larval-pupal ecdysis even in diapause-destined individuals (Meola and Adkisson 1977). In the case of *Hyphantria cunea*, 50% of diapausing pupae injected with 1 μg 20-HE/individual emerged within 20 days after treatment (Park and Boo 1988) and the oxygen consumption rate increased dramatically on 14th day after 20-HE treatment (Gha and Boo 1993). But it is doubtful whether 20-HE directly affects target tissues or activates the prothoracic gland to synthesize endogeneous molting hormone.

Glycogen is a major energy source during the diapausing period and its content is usually higher in diapausing pupae than in normally developing ones. Some of it is converted into simple carbohydrates, which are distributed in insect haemolymph to increase its osmotic pressure. For instance, in diapausing pupae of *Pieris brassicae*, sorbitol, glucose, mannose, glycerol, mannitol and inositol increased several folds in contents between 5 and 55 days after pupation at 20℃ and diapausing pupae receiving ecdysone showed a significant decrease in contents of glucose, mannose, sorbitol and mannitol (Pullin 1989).

Many studies also reported chances in contents of protein in diapausing insects (Lefevere *et al.* 1989, Chippendale and Kilbbly 1969, Brown 1980, Dortland 1978). Chippendale and Kilbby (1969) reported that in *P. brassicae*, about 4.9 mg protein was lost from the haemolymph in an individual between the prepupal stage and pupation. while during the same period the fat body gained about 5.5 mg protein. However, no changes were detected in either haemolymph or fat body proteins during pupal diapause. Fat body proteins were histolysed and absorbed in the differentiating adult tissues. The highest rates of synthesis of vitellogenin and two diapause proteins by the fat body occurred during the pre-diapause period of *Leptinotarsa decemlineata* (Dortland 1987). And, a soluble haemolymph protein which was of a minor significance in non-diapausing larvae was contained in diapausing *Cyndia pomonella* larvae (Brown 1980).

In this study, we compared 20-hydroxyecdysone concentration and contents of carbohydrates and proteins between normally developing and diapausing pupae treated with 20-HE to examine the effect of 20-HE on diapause-destined pupae of *Hyphantria cunea*.

METERIALS AND METHODS

Inscets

The fall wobworm, *Hyphantria cunea* Drury, larvae were collected from field in Suwon area

and reared in an insectary on an artificial diet (*Morus alba* leaf powder mixed with some elements (Dongbang Yurayng Corp.), 100g+propionic acid, 1.2 ml+distilled water, 280 ml). Larvae hatched from eggs were exposed to diapause-inducing condition (13L/11D, 25 ± 1℃) and diapause-free condition (16L/8D, 25 ± 1 ℃) (Choi and Boo 1987a).

Body weight and haemolymph protein content in the 7th instar larvae

After the last molt, 30-40 individuals of 7th instar larvae were selected at random and weighted with a chemical balance (Chyo Julpiter SD-160, Chyo Balance Corp.) at 24hr intervals until pupation. Haemolymph was collected through the punctured prolegs of 7th instar larvae and the protein content was measured by the Lowry mthod (Lowry *et al.* 1951).

Effect of 20-hydroxyecdysone (20-HE) on diapausing pupae

For assaying the effect of 20-HE on termination of diapause, each 20 diapausing pupae were injected with different dosages (1, 3, 5, 7.5, 10 and 12.5 μg 20-HE/g live weigh) of 20-HE on the 15th day after pupation. One or two μl volume of each dosage was carefully delivered with a 25 μl Hamilton syringe. As a control, 20 diapausing pupae were injected with 2~3 μl of distilled water. Injection of 20-HE was carried out through the membraneous cuticle, between the 1st and 2nd abdominal segments, which was swabbed with 0.5% solution of sodium hypochlorite. All instruments and glasswares were washed with and kept in 7.5% ethanol before and during the injection procedure.

Extraction and analysis 20-hydroxyecdysone

Extraction of 20-HE from *H. cunea* pupae (diapause-bound, normally developing and 7.5 μg 20-HE/g live wt.-injected pupae) was done as described by Lafont *et al.* (1982).

Ten pupae, 5 males and 5 females, were homogenized with 4ml of extraction solution (chlorofrom: distilled water=1:1 (v/v)) in a glass homogenizer. The homogenizer was washed once with 2ml extraction solution and the washing was added to the original homogenate. The combined homogenate was vortexed and centrifuged at 4,000 rpm for 15 min. and the water phase was collected into another tube. The sediment was once more undergone the previous procedure with 4 ml extraction solution and the second water phase was added to original water extract. And the third extraction was carried out with 1 ml of distilled water in a manner of just standing for 5 min. The water extracts were absorbed to disposable SEP-PAK C_{18} cartridge by means of a peristaltic pump at a flow rate of 2 ml/min. The SEP-PAK C_{18} cartridge was washed with 25% methanol, which was analyzed by HPLC (WATERS millipore Model 518, μ Bondapak™ C_{18} (3.9×300mm) column). Solvent (20% v/v acetonitrile in water) was eluted isocratically at a flow rate of 1 ml/min. An detection was achieved at 254 nm.

Determination of carbohydrates in pupae

Ten pupae, 5 males and 5 females, were homogenized with 3 ml of 50% (v/v) ethanol in a glass homogenizer, which was washed three times each with 1 ml ethanol (50%), and each washings was added to the original homogenate. After centrifugation at 4,000 rpm for 15 min., the supernatant was separated from the sediment. The sediment was again mixed with 2 ml of 50% ethanol twice and the second and the

third supernatants were added to the first supernatant. The combined supernatant was concentrated under nitrogen gas flow. After the volume was measured with micropipette, the concentrated extract was filtrated with a membrane filter (pore size: 0.45 ㎛, Gelman Inc.) for analysing sugars and sugar alcohols by HPLC (Nucleosyl 10-NH_2 column, PYE UNICAM PU 4023 refractive index detecter, PYE UNICAM PU 4010 pump, Philips). Acetonitrile-water mixture (80:20, v/v) was eluted isocratically at a flow rate of 1 ml/min.

The sediment was mixed with 10 ml of 10% (w/v) trichloroacetic acid (TCA) and incubated at 100℃ for 15 min. And then centrifuged at 4,000 rpm for 15 min. The supernatant was separated from the sediment, which was undergone through the same procedure and the second supernatant was added to the first supernatant. The content of glucose hydrolyzed from glycogen in the supernatant was determined by anthrone/surphuric acid method (Seifter *et al.* 1950, Travely and Harrison 1952) using the spectrophotometer (PV 8600 Series UV/VIS, single beam spectrophotometer, Philips) at 620 nm.

Determination of protein contents in pupae

Haemolymph sample were collected into the phenylthiourea-containing tubes from normal, 20-HE-injected and 18-day-old diapausing male pupae, which were punctured in the head region. The punctured-pupal were centrifuged at a low speed below 500 rpm(0 ± 1℃). And the collected haemolymph were again centrifuged at 4,000 rpm for 10 min. to remove haemocytes and then the supernatant was used to determine the protein contents by the Lowry method (Lowry *et al.* 1951). Haemolymph-removed bodies of pupae were again centrifuged at 2,000 for 10 min. And the carcasses were homogenized in 20 ㎕ simple Ringer solution with a glass rod after washing with 100 ㎕ of simple Ringer solution. The homogenate was centrifuged at 4,000 rpm and the supernatant was used for determination of soluble protein content.

RESULTS

Dialy changes in body weight and haemolymph protein content of the 7th instar larvae

The body weight of the 7th instar larvae, reared under diapause-free condition, increased during the feeding period lasting for 2 days after the last molt and then sharply declined from wandering stage to pupation time (Fig 1). In the case of the 7th instar larvae, reared at diapause-inducing condition, the body weight were heavier than that reared in dipause-free condition during the feeding period and the

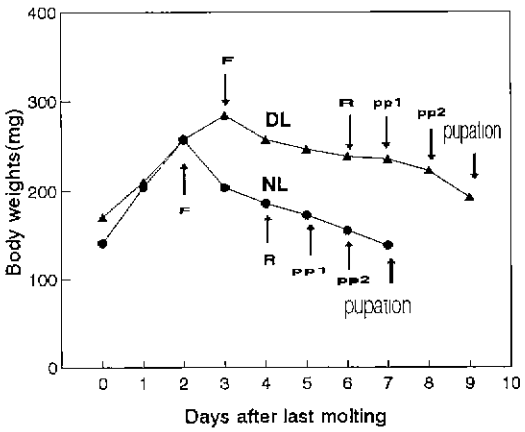

Fig. 1. Changes in body weight of the diapause-bound (DL) and normally developing (NL) last (7th) instar larva of *H. cunea*.
F: End of feeding (beginning of wandering)
R: Resting day
pp1: The day to begin spinning
pp2: The larval body is retracted to show a pupal body from.

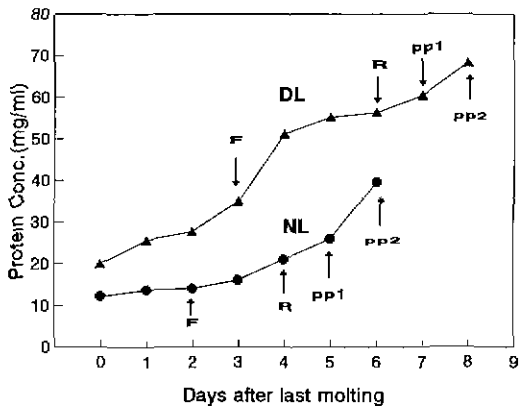

Fig. 2. Increase in contents of total soluble haemolymph proteins from the diapause-bonnd (DL) and normally developing (NL) last (7th) instar larva of *H. cunea*.
F: End of feeding (beginning of wandering)
R: Resting day
pp1: The day to begin spinning
pp2: The larval body is retracted to show a pupal body from.

maximum body weight was shown on the 3rd day after the last molt. With the initiation of wandering phase, it also decreased, but at a much slower rate than that of normal larvae until pupation (Fig 1).

Concentration of proteins in haemolymph of normally developing larvae showed almost no change until the 2nd day after the last larval molt and then increased from the wandering stage slowly in the beginning but at a much rapid rate later (Fig. 2). Haemolymph protein concentration of diapause-bound larvae was higher than that of normally developing larvae even on the first day of the 7th instar and increased sharply from the 2nd day (24hr before the end of feeding) to the 1 day after beginning of the wandering stage. Then there was a little increase at the spinning stage (Fig. 2).

Effect of 20-hydroxyecdysone on termination of pupal diapause

Table 1 represents the result that was obtained during 15 days after injection of 20-HE to 15-day-old diapausing pupae. Injection of small dosages (1 or 3 μg/g) caused a very low rate of adult emergence and higher dosages at or greater than 5 μg/g terminated diapause in 50%~60% of treated pupae. But some adults emerged with grossly malformed wing(s) at the dosages of 10 or 12.5 μg/g. While normally developing pupae generally emerged to adult on the 12th day after pupation, diapausing pupae treated with 20-HE emerged during several days from the 8th to 15th day after the treatment with the dosage of 5 μg/g but from the 10th to 13th day (except for two individuals at the 8th day) at the dosage of 7.5 μg/g (Fig. 3). Larger dosages (10 or 12.5 μg/g) showed a daily adult emergence pattern confined within a narrower range and the 11th day had more adults emerged than any other day. Therefore, the dosaged of 7.5 μg/g was selected as the level of 20-HE to observe the effect of molting hormone on termination of diapause in subsequent experiments.

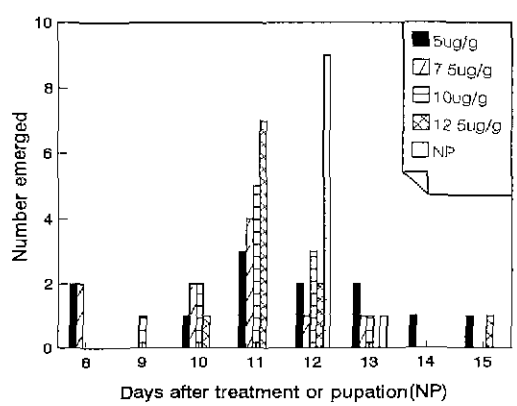

Fig. 3. Daily pattern of adult emergence in diapausing *H. cunea* pupae treated with 20-HE on the 15th after pupation (NP denotes the normally developing pupae without any treatment).

Table 1. Effect of 20-hydroxyecdysone (20-HE) injection on the termination of 15-day-old diapausing *H. cunea* pupae

20-HE Conc. (μg/g)	No. Treated	No. Emerged	No. Died	No. Malformed Adult	No. with no Response
12.5	20	11	6	5	3
10	20	12	6	3	2
7.5	20	10	9	1	1
5	20	12	7	1	1
3	20	6	4	0	10
1	20	1	5	0	14
control	20	0	0	0	20

Concentration of 20-hydroxyecdysone in pupae

Ecdysone and 20-hydroxyecdysone were clearly separated by HPLC. 20-HE peak was observed at 9 min. after injection of 40 μl sample (Sigma) into HPLC and α-ecdysone peak at 14 min. after injection. More than 90% of ecdysteroids absorbed to Sep-Pak C_{18} were eluted in the 60% methanol washing. But α-ecdysone peak was not observed in extract of whole body homogenate of *H. cunea* pupae. Standard curves were obtained by plotting peak hights against various concentration of synthetic 20-HE.

The concentration of 20-HE from the whole body of normally developing pupae (NP) increased from a low level to a peak of 3.2 μg/g on the 6th day and then declined to a low level prior to eclosion (Fig. 4). In contrast, diapause-bound pupae (DP) showed a low level of peak (1.7 μg/g) at the 6th day and then continuously declined to an undetectable level with some fluctuations. In pupae (IP) receiving 7.5 μg 20-HE/g, most of 20-HE seemed to be degraded within 10 hours but two peaks appeared later at the 3rd (2.6 μg/g) and the 6th day (4.1 μg/g) after injection.

Determination of carbohydrate contents

Normally developing, diapausing and 20-HE-injected pupae had the same kinds of carbohydrates and there were same unknown peaks of carbohydrates. In the condition of this analysis system, glucose and sorbitol were not seperated clearly. Glycogen concentration measured by the anthron method was almost the same between non-diapausing (NP) and diapausing pupae (DP) right after pupation (Fig. 5). Then, there was a continuous decrease in glycogen content along the adult development in non-diapausing pupae and the largest decrease was seen between the 5th and the 6th day (to about 35% of its initial content). In diapausing pupae,

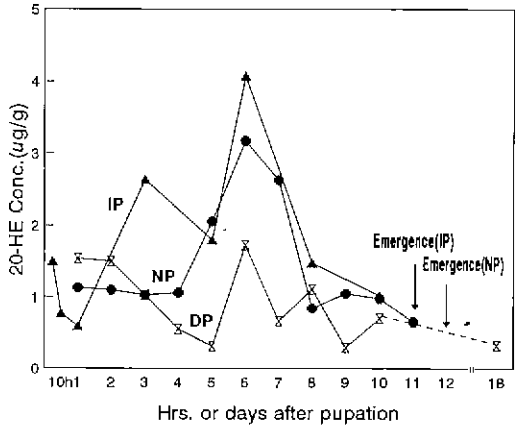

Fig. 4. Changes in 20-hydroxyecdysone (20-HE) titre from normally developing (NP) and diapausing *H. cunea* pupae with (IP) or without (DP) 20-HE treatment.

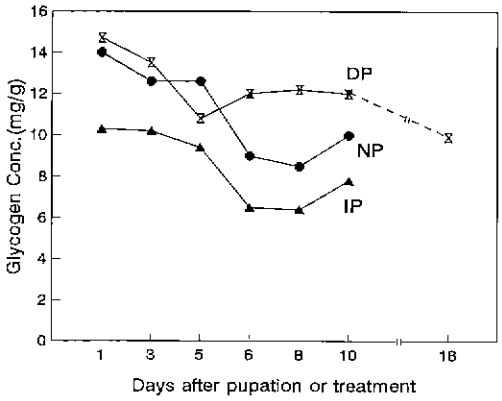

Fig. 5. Changes in glycogen content from normally developing (NP) and diapausing *H. cunea* pupae with (IP) or without (DP) 20-HE treatment.

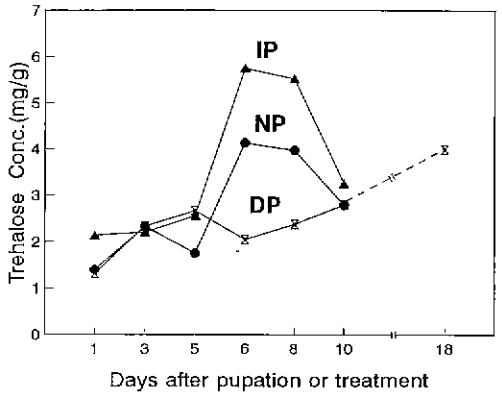

Fig. 6. Changes in trehalose content from normally developing (NP) and diapausing *H. cunea* pupae with (IP) or without (DP) 20-HE treatment.

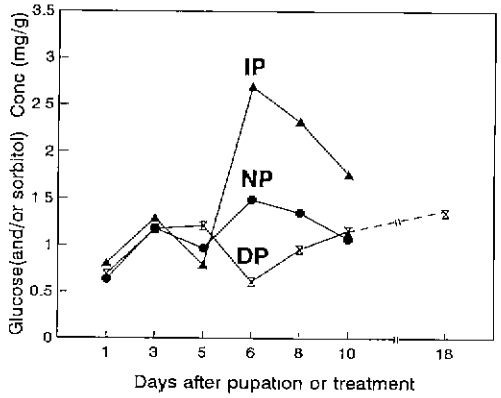

Fig. 7 Changes in glucose (and/or sorbitol) content from nomally developing (NP) and diapausing *H. cunea* pupae with (IP) or without (DP) 20-HE treatment.

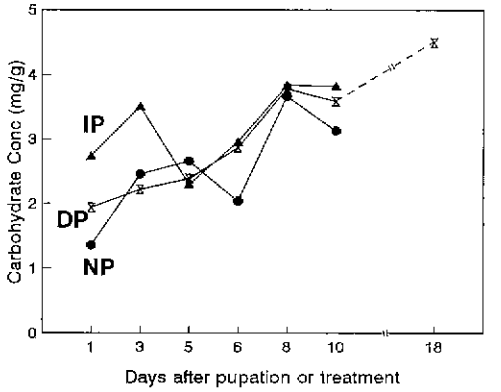

Fig. 8. Changes in other carbohydrates (fructose, inositol, xylose, glycerol) contents from normally developing (NP) and diapausing *H. cunea* pupae with (IP) or without (DP) 20-HE treatment.

although about 25% of initial glycogen content declined by the 5th day, it increased on the 6th day again (about 5% of its initial content) and this high level was maintained until the 10th day (Fig. 5). But glycogen in diapausing pupae showed a slightly lower level at the 18th day. In diapausing pupae (IP) receiving 7.5 μg 20-HE /g live wt, the initially mesured concentration of glycogen was lower than that in any other pupae because this samples were from 15-day-old diapausing pupae. But its pattern of change in glycogen concentration was similar to that of normally developing pupae (Fig. 5).

Trehalose concentration of normally developing pupae was about 1.4 mg/g on the 1st day after pupation. The maximum value (about 4 mg/g) was seen on the 6th day (when the glycogen content rapidly declined-see Fig. 5) and the level was maintained until the 8th day. But the concentration started to decline to reach the level of about 2.8 mg/g on the 10th day (Fig. 6). In diapausing pupae, trehalose content gen-

erally increased until the 18th day, the last day of measurement, to show about 4 mg/g in contrast to the change in glycogen content (Fig. 5). Diapausing pupae receiving 20-HE had the higher maximum value (about 5.9 mg/g) than that of normally developing pupae at the 6th day after 20-HE injection. But the pattern of change was similar to that of normally developing pupae. The glucose (and/or sorbitol) content in normally developing, diapausing and 20-HE-injected pupae was lower than that of trehalose. But the pattern of change in glucose (and/or sorbitol) concentration was similar to that of trehalose, except for one fact that there was no considerable increase in diapausing pupae to the 18th day (Fig. 7). Total quantity in the rest of carbohydrates (fructose, inositol, xylose, glycerol) in 20-HE-injected pupae was generally higher than that in any other types of pupae during the 10 days (Fig. 8). Especially, 20-HE-injected pupae showed remarkably higher values in other carbohydrates content than normally developing or diapausing pupae during the first three days in adult development. But it fell down sharply on the 5th day. Normally developing and diapausing pupae showed a gradual rise during this period. After this period the content increased to reach the maximum value on the 10th day, just before eclosion or kept increasing in the case of diapausing pupae.

Changes in pupal protein contents

Haemolymph of normally developing male pupae (NP) contained a low level of protein at the beginning. After 1st day, it increased and reached the maximum value (about 134 mg/ml at the 6th day (Fig. 9). In the case of 20-HE-injected pupae (IP), protein concentration increased at the beginning to reach a more or less

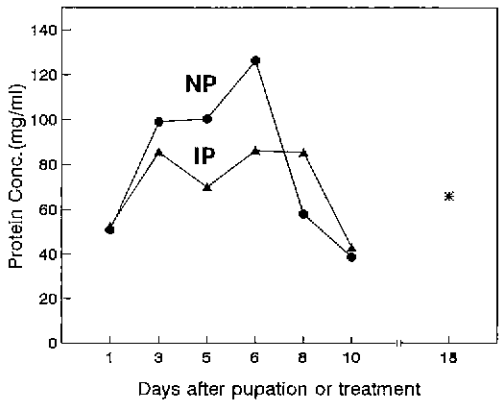

Fig. 9. Changes in total soluble haemolymph proteins from normally developing (NP), and 20-HE treated diapausing male pupae (IP) of *H. cunea*.

*: Protein content of diapausing pupae without 20-HE treatment, which was measured only once, 18th day after pupation.

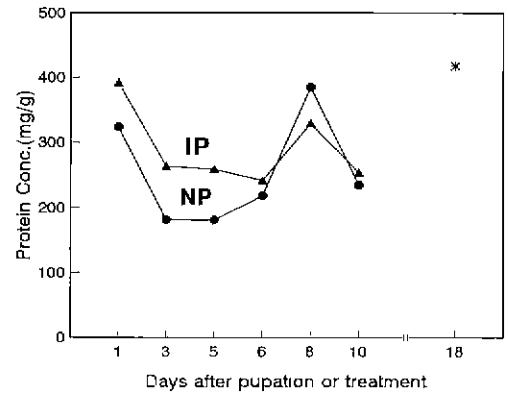

Fig. 10. Changes in total soluble proteins from haemolymph-removed body of normally developing (NP) and 20-HE treated diapausing male pupae (IP) of *H. cunea*.

*: Protein content of diapausing pupae without 20-HE treatment, which was measured only once, 18th day after pupation.

plateau level of about 80 mg/ml which was maintained for 5 days before dropping again. Diapausing pupae at the 18th day showed a level of about 65 mg/mg (Fig. 9). The change in total soluble protein content from haemolymph-removed body in 20-HE-injected

and normally developing pupae is shown in Fig. 10. The concentration of total soluble proteins generally declined with a small increase on the 8th day in both types of pupae. Diapausing pupae at the 18th day showed a higher concentration (about 440 mg/g) in soluble proteins than the other two types of pupae.

DISCUSSION

In the 7th instar larvae of *H. cunea*, diapause-destined larvae showed about one day longer feeding period and heavier body weight than nondiapause-destined larvae (Fig. 1). It is thought that the heavier body weights of diapause-destined inviduals are related to the accumulation of energy source which would be consumed during the diapause development. In fact, the concentration of haemolymph protein in diapause-destined larvae was higher than that in nondiapause-destined larvae of *H. cunea* (Fig. 2). This kind of difference in prediapause period is probably caused by the difference in functioning of endocrine systems. In such a respect, Loeb (1982) has reported that haemolymph ecdysteroid titre in the last third of the last instar was approximately five times higher in non-diapause than in diapause-bound larvae of *Heliothis virescens*.

The effect of 20-hydroxyecdysone on the termination of pupal diapause of *H. cunea* was studied in terms of the rates and daily patterns of adult emergence from diapausing pupae treated with six dosages of 20-HE. The dosage needed for 50~60% adult eclosion of diapausing pupae was about 5~7.5 μg/g. At the lower levels (1 or 3 μg/g), a few diapausing pupae eclosed, and some others eclosed in about 40 days after 20-HE injection. But the time required for adult eclosion was not dependent on the dosage of 20-HE injected. At the dosage of 5 μg/g, the time needed was about 11.5 days and at 7, 10 or 12.5 μg/g it was about 11.1 days. However, the number of adults emerged or malformed-adults increased in proportion to the dosage (Table 1, Fig. 3).

Pupal diapause of *H. cunea* is apparently caused by the absence of the molting hormone. The homogenate of whole body showed a higher level of 20-HE in non-diapausing than in diapausing pupae. In non-diapausing pupae, the 20-HE peak appeared on the 6th day and it seemed to trigger differentiation of adult tissues. Gha and Boo (1993) reported that the rate of respiration in *H. cunea* pupae rose sharply at the 6th day after pupation accompanied by the differentitation of adult tissues. However, not all Lepidoptera exhihibit this pattern of molting hormone peak during pupal-adult development. In particular, a second peak of ecdysone occurs in the haemolymph of developing pupae of *Pieris brassicae* (Lafont *et al.* 1975). Though the peak of 1.7 μg/g in diapausing *H. cunea* pupae seemed not to be related to the differentiation of adult tissues, it is not a negligible level for insects. However, the significance of that peak remains to be explained.

For *H. cunea* pupae receiving 7.5 μg 20-HE/g live weight, almost all of injected 20-HE disappeared within 10hrs and the concentration of 20-HE fell to a low level within a day. But, the peaks of 20-HE appeared again on the 3rd and the 6th day, and this means that the two peaks must be originated from its own endocrin organ. Therefore, 20-HE injected to diapausing pupae did not directly trigger adult development but it must have activated the prothoracic gland directly or indirectly. Marks (1972) reported that 20-HE treatment to the brain of *Leucophaea*

maderae caused the median neurosecretory cells to release their products in vitro. In contrast to *H. cunea*, ther was a just small 20-HE peak from 20-HE-injected pupae of *Mamestra configurata* at the 10th day when normally developing pupae showed a large peak (Bodnaryk 1985). Agui and Hiruma (1977) said that the relatively large dosage of 20-HE given to diapausing pupae of *Mamestra brassicae* may interfere with feedback regulation of ecdystroid biosynthesis, acting as a positive feedback regulator for synthesis and release of PTTH in the brain leading to diapause termination. On the other hand, in *P. brassicae*, with injected 20-HE inhibiting further synthesis of endogeneous ecdystroids (Beydon and Lafont 1983).

Despite the fact that diapausing pupae receiving 20-HE showed a higher 20-HE titre than normal pupae, that fact can not be treated as a direct evidence for the difference in pupal-adult development for both types of pupae. In such a logical respect, change in metabolite contents may provide some information for detecting the developmental difference between 20-HE-injected and normally developing pupae.

Among the several types of carbohydrate, glycogen, trehalose and glucose were predominant in *H. cunea* pupae. The content of glycogen decreased sharply during the 5th to 6th day when 20-HE titre reached the maximum value in normally developing pupae of *H. cunea* (Figs. 4, 5). The reduced quantity of glycogen content might have been utilized for energy production or converted into other types of simple carbohydrates such as trehalose, glucose, etc. during this period. Concentrations of trehalose and glucose were actually maximal on the 6th day and sustained at a high level until the 8th day (Figs. 6, 7). These facts mean that 20-HE triggered the adult tissue development which continued until the 8th day. Then the pigmentation of compound eyes appeared on the 8th day after pupation (Gha and Boo 1993).

Choi and Boo (1987b) reported that diapausing pupae of *H. cunea* have almost equal amounts of trehalose and sorbitol. But, in this study, trehalose content was about three times higher than that of glucose (and/or sorbitol) (Figs. 6, 7). Therefore, diapausing *H. cunea* pupae would demand trehalose to increase the osmotic pressure of heamolymph for coldhardness. After injection of 20-HE to diapausing pupae, trehalose and glucose (and/or sorbitol) contents showed a dramatic increase, which indicates that the metabolism for the development of adult tissues was prompted by the 20-HE treatment.

The content of soluble haemolymph proteins in 20-HE-injected pupae did not change significantly after the 3rd day (Fig. 9) but normally developing pupae showed a large increase on the 6th day (Fig. 10) when the glucose and trehalose content also increased sharply in both types of pupae. On the other hand, in haemolymph-removed whole body tissues, the soluble protein content was higher in 20-HE-injected pupae than in normal pupae and showed an increase on the 8th day in both types of pupae. Diapausing pupae at the 18th day showed a higher content of soluble proteins than any other types of pupae in the haemolymph-removed tissues but a lower level in haemolymph. These facts suggest that in 20-HE-injected pupae, proteins stored in tissues were used for adult development during the pupal-adult development, but in normally developing pupae, more portein seemed to be transferred through haemolymph from one tissue to another than in diapausing pupae. However, it remains to be investigated whether such an interpretation is

orrect and, if so, what significance it implies.

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