Cultured Integument of *Chilo suppressalis* as a Bioassay System for Juvenile Hormones

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Cultured integument from diapausing larvae of *Chilo suppressalis* Walker reproduced larval-pupal ecdysis after two successive treatments with 20-hydroxyecdysone, firstly with 0.1 $\mu\text{g/ml}$ for 24 hr, and then with 1.0 $\mu\text{g/ml}$ for 24 hr. When a juvenile hormone was added to the culture medium at the time of the first ecdysone treatment, the pupal type cuticle formation was inhibited, the extent depending upon its activity. The rate of the pupal type cuticle formation relative to that of the control, when plotted against the concentration of the juvenile hormone, showed a typical dose-response relationship. The median inhibitory concentration of some juvenile hormones was determined as JH II: 0.04 ng/ml, JH III: 1.9 ng/ml and methoprene: 0.079 ng/ml. Cultured integument from the *Chilo* diapausing larvae was shown to be one of the most sensitive *in vitro* assay systems for juvenoids.

Various bioassay methods have been developed for juvenile hormone (JH) activity. In most of them, employing the whole or a part of an insect body, the JH activity is determined as the inhibitory potency against the larval-pupal metamorphosis, including pigmentation.^{1~4)} One of the disadvantages of these methods is that they require a precise synchronization of the stages and ages of test insects. In addition, the observed activity reflects not only the intrinsic JH activity, but also includes, in general, factors due to penetration, metabolic degradation and effects on the synthesis of biomaterials such as chitin and protein. To avoid factors other than the intrinsic activity, attempts have been made to develop *in vitro* assay systems using cultured imaginal discs, salivary glands, and cell lines derived from ovarioles and embryos.⁵⁾ However, most of these *in vitro* systems still do not show clear responses specific to JH even in such high concentration ranges as 10~100 $\mu\text{g/ml}$. For an *in vitro* assay specific to JH activity, a culture system must be developed in which a tissue responds to ecdysteroids with morphogenic changes. Mitsui and Riddiford reproduced *in vitro* the formation of pupal type cuticle by

successively culturing epidermis of the 5th instar larvae of *Manduca sexta* in a medium containing 1 $\mu\text{g/ml}$, and then 5 $\mu\text{g/ml}$ of 20-hydroxyecdysone (20-HED).⁶⁾ They showed that the presence of 3 $\mu\text{g/ml}$ of JH I in the culture medium at the first 20-HED treatment inhibited the formation of pupal type cuticle, that is, larval type cuticle was formed.

Agui has developed a culture system for the integumental tissue prepared from larvae of the rice stem borer, *Chilo suppressalis* Walker, as an assay system for ecdysteroids.⁷⁾ Recently, we found that the effect of chitin synthesis inhibitors can be estimated by the inhibition of new cuticle formation on the cultured integument from *Chilo* larvae.^{8,9)} On the integumental tissues from early last instar larvae, the larval-pupal ecdysis was induced by two successive treatments with 20-HED under certain conditions.¹⁰⁾ The presence of 3 ng/ml of JH II at the time of the first 20-HED treatment completely inhibited the larval-pupal transformation. Thus, the system was expected to be refinable as a highly sensitive *in vitro* assay system for JH's and their analogs.

Since a precise synchronization of the larval stages is required for the use of early last instar

larvae, we have been examining the use of diapausing larvae for the JH assay. In fact, we found that the pupal transformation was induced even on integumental tissues from diapausing larvae, although less extensively, under conditions similar to those applied to the last instar larval integument.¹⁰⁾ Under a short-day photoperiod, the *Chilo* larvae are maintained in the diapause stage for more than 6 months. Most biological events in the diapausing larvae are much steadier than those in the last instar non-diapause larvae. It was expected, therefore, that the physiology would be much more uniform among individual larvae so that the precise synchronized rearing is unnecessary. We here report the result of our examination in some detail and discuss characteristics of the bioassay procedure using the diapausing larval integument.

MATERIALS AND METHODS

Animals. The rice stem borer diapausing larvae were reared aseptically on an artificial diet at 25°C under short day photoperiods (8 hr light and 16 hr dark).^{11,12)} About 30 days after hatching, the last larval ecdysis occurred. At this stage, the growth of larvae was almost equivalent with that of the early last instar non-diapause larvae. Diapausing larvae kept taking the diet normally until about 50 days old with occasional stationary ecdysis, then they reduced the uptake of the diet as well as the frequency of stationary ecdysis. Larvae just before and after the stationary ecdysis were carefully avoided when selecting animals for use.

Hormones. 20-Hydroxyecdysone (20-HED, Rhoto Pharmaceutical Co.), juvenile hormones II and III (JH II,

JH III, purchased as a hexane solution from Calbiochem-Behring Corp.) and methoprene (Otsuka Pharmaceutical Co.) were dissolved in ethanol and stored at -20°C. 1-(2,3-Methylenedioxyphenyl)pentene was synthesized from piperonyl bromide and butyraldehyde by the Wittig reaction and purified by silica-gel column chromatography to give a yellowish syrup. Each hormone was added to a chemically defined Grace's medium containing 0.1 µg/ml of 20-HED as an ethanol solution and the whole was sonicated. Glassware coated with silicone was used. The final concentration of ethanol was adjusted to 0.5%.

Assay procedures.¹⁰⁾ The integument was removed from the abdominal segments of the dorsal part of the larvae and cut into fragments (3 × 4 mm²) in Ringer-Tyrode's solution. The fragments were incubated with 0.9 ml of chemically defined Grace's medium containing 0.1 µg/ml of 20-HED, with and without the addition of various concentrations of each JH compounds, in plastic culture wells (1.5 cm in diameter and 1 cm deep, Linbro Disposotray) for 24 hr. Five to eight pieces were placed in each culture well where they floated on the culture medium so as to expose their surface to air. The pieces from a single larva were distributed in separate groups in separate culture wells so that every group was matched. Then, they were transferred to the medium containing 1.0 µg/ml of 20-HED and incubated for 24 hr. They were further cultured

FIG. 1. Schematic Summary of the Assay Procedure.

Culture in Grace's medium containing ■ 0.1 µg/ml of 20-HED and each test compound, ▨ 0.1 µg/ml of 20-HED, and □ 1.0 µg/ml of 20-HED. □ shows the culture in Grace's medium, which was changed every two or three days.

Larval type

Pupal type

FIG. 2. Surface Patterns of Newly Formed Larval- and Pupal-type Cuticle.

TABLE I. SCORING SYSTEM FOR THE ESTIMATION OF PUPAL CUTICLE FORMATION

Score	Extent of pupal pattern on the surface of new cuticle
0	No pupal pattern
1	Less than ca. 25%
2	ca. 25~50%
3	ca. 50~75%
4	More than ca. 75%
5	Pupal pattern spread all over the surface

in hormone-free medium for 7 days (Fig. 1). The medium was changed every 2 or 3 days. With this procedure, integumental tissue was exposed to the test compounds for the first 24 hr.

*Types of new cuticle.*¹⁰⁾ On the 9th day of culture, the old cuticular shell was removed from the tissue. Then, under a dissecting microscope, the epidermal cells were scraped from the fragment with forceps which left a film of newly formed cuticle. The new cuticle was spread on a glass slide and dried, then it was embedded in Enteran New (Merck Co.) for microscopy. Under the microscope, the pupal cuticle was easily distinguished from the larval cuticle by its surface pattern. The larval cuticle is translucent and elastic, with irregular wrinkles on its surface. The pupal cuticle is brittle and tanned, its surface showing a repeated pattern of small rectangular blocks (Fig. 2).

Scoring and rating of the pupal cuticle formation. Each of the new cuticle preparations was scored according to the extent of development of the pupal pattern (Table I). The rate of pupal cuticle formation was defined as shown in Eq. (1) for the integumental fragments simultaneously cultured in each set of experiments.

$$\text{Pupal rate (\%)} = \frac{\sum_{k=0}^5 (k \times N_k)}{5 \times \sum N_k} \times 100 \quad (1)$$

where k is the score and N_k is the number of fragments having the score k . The pupal rate relative to that of the control was used for to estimate the JH effect at each concentration of the test compounds.

RESULTS

Diapause stage and new cuticle formation

With the "control" procedure (Fig. 1), the responses of the integumental tissues prepared from various ages of diapausing larvae (30~111 days old) were examined. The two successive treatments with 20-HED induced new cuticle formation normally on almost all

TABLE II. PUPAL RATE OF THE INTEGUMENTAL FRAGMENTS PREPARED FROM 36 DAY-OLD DIAPAUSING LARVAE^a

Experiment number	Number of fragments							Total number of fragments	Pupal rate (%)
	Score								
	0	1	2	3	4	5			
1	1	7	2	0	1	0		11	27
2	5	5	0	0	0	0		10	10
3	0	3	3	3	0	2		11	51
4	0	4	0	2	4	1		11	56
5	0	0	3	6	2	0		11	58
6	0	2	3	4	2	0		11	51
7	0	0	2	4	3	1		11	66
8	0	1	1	5	3	0		10	60
9	0	0	5	3	2	0		10	54
10	0	0	4	2	6	3		15	71
11	0	0	5	2	8	2		17	68

^a Integumental fragments were treated with 20-HED (0.1 $\mu\text{g/ml}$) for 24 hr, then 1.0 $\mu\text{g/ml}$ for 24 hr. On the 9th day of the culture, the types of newly formed cuticle were scored.

fragments regardless of the age of diapause from which they are prepared (97.2% out of the total number of 823 fragments). The rate of the pupal type in the new cuticular preparations was evaluated according to Eq. (1). Table II shows the results of 11 runs of the experiment for the integumental fragments prepared from 36 day-old diapausing larvae. The pupal rate is plotted against the age of the diapause from which the tissues were prepared (Fig. 3). Each point in Fig. 3 represents the pupal rate determined with at least 10 fragments. Although the value varies from one run to another, tissue fragments from the 30~50 day-old larvae formed the pupal type cuticle at the rate of 10~71%, whereas the rate for those from 50~111 day-old larvae was not more than 20%. Thus, we selected diapausing larvae of an age between 30 and 50 days as donors.

Segment position and pupal type cuticle formation

To prepare integumental fragment, the larval integument was cut along each intersegmental membrane so as to obtain one fragment from one segment. Nine fragments (from

FIG. 3. The Relationship between the Age of Diapausing Larvae after Hatching and Pupal Cuticle Formation.

the 2nd to 10th segments) were usually available from one larva. Tissue fragments from 10~15 diapausing larvae (36 days old) were grouped according to the segment position and each group of tissues was cultured under the conditions of the "control" (Fig. 1). Figure 4 shows the pupal rate of tissues from each segment. Integument tissues from the 2nd to 9th segments formed the pupal type cuticle at almost unchanged rate of 51~67%. Thus, the tissue fragments from the 2nd to the 9th segments of 30~50 day-old diapausing larvae were selected for use.

Dose of JH and the relative pupal rate

With the "assay" procedure (Fig. 1), the effect of JH and its analogs was examined. For instance, various concentrations of JH II, 0.005~1.0 ng/ml, were added to the culture medium only at the first treatment of 20-HED. On the 9th day of the culture, the pupal rate of newly formed cuticle was calculated (Table III). The pupal rate relative to that of the control decreased with the increase in concentration of each JH compound. The plot of the relative rate value against the log of the concentration shows a typical sigmoidal relationship for each compound (Fig. 5). The log value of the reciprocal of the median inhibitory concentration against pupal cuticle formation, pI_{50} , was calculated by the probit method.¹³⁾ The reproducibility of the pI_{50} value is apparent from the example for JH II. As

FIG. 4. Position of Segments and Pupal Type Cuticle Formation.

FIG. 5. Relationship between the Dose and Inhibitory Effect of JH II on Pupal Cuticle Formation Using 46 Day-old Diapausing Larvae.

This plot is based on the data of experiment number 4 shown in Table III.

shown in Table III, the pI_{50} values determined from 8 repeated runs are remarkably constant, in spite of the fluctuation of the pupal rate value of the control experiments from 27 to 56%. The χ^2 test for each determination shows no evidence of heterogeneity of departure from the probit regression lines within each set of experiments using tissues derived from each group of larvae. The mean of eight pI_{50} values

TABLE III. PUPAL RATE OF NEWLY FORMED CUTICLE IN THE PRESENCE OF JH II
AND pI_{50} VALUES OF REPEATED RUNS

Experiment number	Age of larvae ^a	Pupal rate (%) ^b												pI ₅₀ ^c (g/ml)	
		Concentration of JH II (ng/ml)													
		0.0	0.005	0.01	0.025	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.6	1.0	
1	40	27 (10)	26 (10)	13 (6)	20 (6)	12 (10)	8 (10)				7 (12)			10.3±0.5	
2	40	32 (10)	28 (10)	16 (6)	20 (10)	20 (5)	10 (10)				4 (6)			10.4±0.5	
3	42	49 (13)					35 (13)	22 (13)	20 (14)		21 (14)		14 (14)	8 (15)	9.7±0.3
4	46	56 (14)	54 (7)	51 (10)		31 (14)	23 (13)	17 (12)	11 (14)	7 (13)					10.2±0.2
5	46	35 (10)		26 (11)	20 (10)	10 (10)	10 (10)			8 (10)				6 (10)	10.5±0.7
6	46	45 (10)		34 (10)	32 (10)	16 (5)	14 (10)				4 (8)				10.4±0.5
7	49	36 (10)			10 (10)	9 (11)	7 (11)			6 (11)				5 (10)	11.2±0.9
8	49	33 (10)			27 (10)	12 (9)	7 (10)			2 (10)				4 (10)	10.5±0.5

^a Days after hatching.

^b Figures in parenthesis are the number of fragments used for the determination of pupal rate.

^c Figures with ± are the 95% confidence interval. The average pI_{50} value of eight runs was 10.4 ± 0.4 .

is 10.4 ± 0.4 ($I_{50} = 0.04$ ng/ml). pI_{50} values from six repeated runs out of eight are located within the 95% confidence interval, 10.1~10.7. Similar reproducibility was also observed for methoprene; the mean pI_{50} value of seven runs was 10.1 ± 0.2 ($I_{50} = 0.079$ ng/ml). This system was also useful for the assay of weak JH compounds, JH III: $I_{50} = 1.9$ ng/ml; 1-(2,3-methylenedioxyphenyl)pentene: $I_{50} = 12.3$ ng/ml.

DISCUSSION

The diapause of the *Chilo* larvae was shown to be maintained by a high titer of JH in hemolymph.¹⁴ According to Yagi,¹⁵ the JH titer is as high as 3,700 galleria unit/ml of hemolymph (equivalent to 18.5 ng JH I/ml) on the 2nd day after the final larval ecdysis, then gradually decreases to ca. 500 G.U./ml (2.5 ng JH I/ml) on the 16th day. Larval, intermediate, or pupal ecdysis was induced by treatment of the diapausing larvae with 20-HED, depending upon the JH titer in the hemolymph.

In our previous study using non-diapause larvae as donors, the pupal type cuticle formation was induced on almost all of the tissues from early final instar larvae by two successive 20-HED treatments.¹⁰ With the diapausing larval tissues prepared from the 30~50 days old larvae, however, the rate of the pupal type was 10~71% and fluctuated from one run to another (Table II). The low rate and the fluctuation could be attributable in part to a residual effect of the JH titer remaining in the tissue fragments. The tissue fragments, however, from the diapausing larvae of the later stage (50~111 days old) scored pupal rates of not more than 20%. Thus, the pupal rate in the present study seems not necessarily related with the JH titer of the donor animals.

Agui observed that the rate of apolysis of cultured integument induced by 20-HED at the concentration of 0.3 μ g/ml varies with respect to the age of diapausing *Chilo* larvae.¹⁶ According to his observation, tissue fragments from 30~45 day-old diapausing larvae showed the rate as high as those from non-

diapause larvae. The rate of apolysis decreased with the duration of diapause to almost one-third (60~80 day-old diapausing larvae). We observed that the maximum thickness of new cuticle induced by 20-HED on the cultured integument from diapausing larvae was almost constant regardless of the duration of diapause (30~130 days old).⁸ Thus, the lower pupal rate for epidermal fragments from the later stage (50~110 days old) of diapausing larvae could mostly be attributable to the loss of sensitivity of tissue to 20-HED at the early processes of metamorphosis.

In the course of the present study, we found that the fluctuation in pupal rate (as shown in Tables II and III) was related to the body color of the donor animals. We divided the 49 day-old diapausing larvae into two groups: light yellow and dark yellow. The integument fragments derived from the dark yellow larvae showed a much lower pupal rate (25%) than those from the light yellow larvae (73%). The fluctuation in pupal type cuticle formation may be due to the heterogeneity of the diapausing larvae with respect to body color. Yagi reported that the body color of diapausing *Chilo* larvae became darker with the duration of diapause.¹⁷ All the larvae older than 60 days after hatching were dark in color. He also observed for the ligated larvae that the darkness was induced by topical applications of JH I. A high JH titer could be expected in the bodies of the dark yellow larvae. The lower pupal rate of the tissue from the dark yellow larvae, however, cannot be ascribed to the JH titer, since the pupal rate of newly formed cuticle is not related to the JH titer of the donor animal as discussed above. Lower pupal rates are likely due to the lack of some essential physiology in the dark yellow integumental tissue required for pupal type cuticle formation.

In spite of the fluctuations in the pupal rate of the control runs, the relative pupal rate value determined from each set of runs simultaneously performed, including the control, is remarkably constant among sets for each of the JH compounds. The sensitivity of the

TABLE IV. COMPARISON OF VARIOUS ASSAY METHODS OF JUVENILE HORMONE ACTIVITY

Animal (unit)	I_{50} ^a		
	JH II	JH III	Methoprene
<i>Aedes aegypti</i> ^b (ppm)	0.26	0.35	0.00017
<i>Galleria mellonella</i> ^a (μ g/pupa)	0.13	12	5.7
<i>Musca domestica</i> ^b (μ g/prepupa)	> 100	> 100	0.0035
<i>Manduca sexta</i> ^c (ng/larvae)	Not tested	2.0	Not tested
<i>Chilo suppressalis</i>			
Non-diapause larvae integument tissue (ng/ml medium)	0.24 ^d	Not tested	ca. 0.25 ^e
Diapausing larvae integument tissue ^f (ng/ml medium)	0.04	1.9	0.079

^a Median inhibitory concentration or dose.

^b Reference 2.

^c Reference 1.

^d Reference 10.

^e S. Imoto, unpublished study.

^f This study.

cultured integument from the *Chilo* diapausing larvae is very high. When the I_{50} values are compared with values derived from other systems (Table IV), our bioassay system seems to be the most sensitive of those so far developed. Compared with the non-diapause larval integument, the sensitivity to JH II and methoprene is raised about 3~6 times in this procedure.

In summary, the cultured integumental system of the *Chilo* diapausing larvae is a quantitative and highly sensitive *in vitro* assay system for JH compounds. Improvement of the lower rate of pupal cuticle formation in the control runs will be sought in future work.

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