Adult Development Induced by the Injection of Non-Hormonal Agents into Brainless Pupae of Silkworms

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Numerous solutions of non-hormonal agents have been injected into debrained test pupae of *Bombyx* and *Samia*. *Bombyx* (N122×C115) pupae were debrained shortly after pupation and injection was performed within 4–5 weeks after operation. Though intact brainless pupae remained largely unchanged, the pupae injected with non-hormonal solutions underwent adult development with a high rate for 2–4 weeks after the injection. When deionized water or buffers were injected, the emergence rate averaged about 17%. A higher rate was found in pupae injected with a series of light metal solutions or with amino acids. Injection of solutions containing heavy metals led in remarkably high percentage of the test *Bombyx* to imagos. The highest rate, 67% (average), was found after injection of ferrous or ferric ions.

Removal of *Samia* brain was performed between 12–48 hr after pupation and the resulting diapausing pupae were injected some time between 2–6 months after the operation. The brainless pupae of *Samia*, unlike *Bombyx* pupae, scarcely developed into adult form after the injections. However, after injection of solutions containing ferrous or ferric ions, even *Samia* pupae developed into adults with a percentage rate of five or six.

INTRODUCTION

The Eri-silkworm, *Samia cynthia ricini* (Saturniid.) has long been used as our experimental animal. The critical period of brain hormone (BH) for imaginal differentiation is at around 72 hr after pupation at 24–25°C (NISHITSUTSUJI-UWO, 1958). If the brain is removed between 12–48 hr after pupation, the resulting diapausing pupa does not in principle develop within 500 days unless brain or prothoracotropic hormone (BH) is supplied. Hence, debrained pupae of Eri-silkworms are a most reliable test animal for BH assay.

On the other hand, KOBAYASHI and his collaborators have extensively used debrained pupae of the particular strains of *Bombyx mori* as a test animal for BH assay. They obtained so called 'dauer pupae' by brain removal shortly after pupation (KOBAYASHI, 1955, 1957; KOBAYASHI and Yamashita, 1958).

To check the species-non-specific character of BH and to attempt to resolve inconsistencies among the results of purification done by different insect endocrinologists (see, NISHITSUTSUJI-UWO, 1972), it was necessary to use KOBAYASHI's test animal, *Bombyx*, as well as our test animal, *Samia*. The use of *Bombyx* was further advantageous because they are commercially available and BH assay requires a very large number of test animals.

By following the directions of Dr. KOBAYASHI, dauer pupae were successfully obtained in large number, and 4–5 weeks after removal of their brains, the dormant pupae received BH injection. The following unexpected phenomena occurred: 1) Emergence from female pupae was much more frequent than from male pupae, even when only water was injected. 2) Emergence from injected pupae, both male and female, was more frequent than from those not injected, even when the injection solution contained no BH. 3) Control injection solutions containing certain ions and amino acids induced adult development in brainless *Bombyx* pupae at a remarkably high rate. 4) The above phenomena were never observed in the brainless *Samia* pupae. 5) After injection of samples containing BH, results in the *Bombyx*-test those in the *Samia*-test were often contradictory.

To understand these points it was necessary to know the character of dormancy in the debrained pupa. Extensive testing of the effects of injection of solutions containing no BH was therefore performed on *Bombyx* as well as on *Samia*. An interesting phenomenon was reported by WILLIAMS (1967) that when *cecropia* pupae are chilled at 5°C for a long period, the titer of endogenous BH becomes so high that the injection of aqueous solutions of zinc acetate, copper sulfate, cobalt chloride and ferrous sulfate can cause a substantial number of brainless pupae to initiate adult development. We, therefore, paid special attention to the effects of solutions containing metal ions, especially heavy-metal ions, among solutions of other non-hormonal agents, and in this paper we deal with the effects of their injection on the adult development of brainless diapausing pupae.

MATERIALS AND METHODS

'Dauer pupa' of Bombyx: A racial hybrid between Japanese No. 122 and Chinese No. 115 was used following the recommendation of KOBAYASHI, who kindly supplied eggs of this hybrid. The worms must be reared under the best conditions otherwise many debrained pupae develop into adults. Just after the completion of spinning, animals were taken out of their cocoons and observed every 10 min. Within 10 min after pupation, pupae were transferred to a cold room at 1–2°C and kept for 8–16 hr. The brain was removed while anaesthetized by the cold and the wound was sealed with melted paraffin. Pupae operated upon were kept at 25°C. Individuals which developed into adults within 4 weeks were discarded and the remaining pupae (so called 'dauer pupae' by KOBAYASHI) were used as test animals under the following conditions: (1) Injection was performed within 4 to 5 weeks after removal of the brain, because observation must be continued for 4 weeks after injection and even non-injected pupae frequently develop into adults after 7 weeks (ISHIZAKI, 1972). (2) Females and bled pupae were excluded from the experiment because of their tendency to undergo adult development more than male or unbled pupae.

Brainless diapausing pupae of Samia: Eri-silkworm, *Samia cynthia ricini* is a saturniid silkworm and has no 'diapause' stage. It can be reared all the year round, either on fresh leaves or on an artificial diet (FUKUDA *et al.*, 1961). Removal of the brain was carried out within 12–32 hr after pupation under anaesthesia by chilling and the wound was sealed with paraffin. Debrained pupae kept at 25°C were used some time between 2–6 months after the operation. There was no difference based on sex, but bled pupae and pupae which shrank during the long storage were excluded because of

their tendency to undergo adult development for some reason which is not clear.

Injection: Pupae were anaesthetized by keeping them at around 0°C for 4–12 hr, then 40 μ l of each test-solution was injected into the assay pupae at the dorso-lateral site of the 4th abdominal segment. Five test-pupae of *Samia* and 10 test-pupae of *Bombyx* were routinely used for the assay of each experimental lot. Each test solution was injected by assaying in a series of either 3 or 4 fold dilution with water. Bioassays were usually made on both *Samia* and *Bombyx* simultaneously. Many solutions were repeatedly tested using test animals reared in different seasons. Animals before and after injection were kept at 25°C.

Observations were continued every other day for 4 weeks (sometimes 6 weeks) after injection. Specimens which died or emerged within 10–12 days were excluded from the available numbers, as were those which developed into adults later than 4 weeks.

When developing pupae were found in the non-injected control group after 3 weeks ratio of over 10%, all data were discarded. This situation was occasionally found in the *Bombyx*-test.

In the present experiment, most of brainless moths did not emerge from the exuviae by themselves. Therefore, the term emergence used in this paper means the completeness of adult formation.

RESULTS

Some examples of bioassay on Bombyx

Table 1 shows an example of a bioassay performed on July 14, 1971, 5 weeks after brain removal. Within 2–4 weeks after injection, only one debrained *Bombyx* of the control group underwent a change to adult form. When a partially purified BH¹ solution was injected, the change to adult form was completed with a typical dose response between 17–20 days (mainly in 18, 19 days) after injection. With injection of 0.05 M acetic acid, adult formation (average 27%) was retarded for a few days. With 0.05 M KCl and 0.05 M CaCl₂, 40–30% (average) of imago appeared. There seemed to be no relationship between concentration of ions and emergence rate. Injections of 0.05 M MgSO₄ and 0.05 M FeCl₃ induced adult formation at an extremely high rate. As seen in Table 1, the time required for emergence in these two series was not longer than that after BH injection.

Table 2 shows another example of a bioassay, performed on November 5, 1971, 4 weeks after brain removal. In this table, the results obtained over the observation period from 2 to 6 weeks after injection are shown. If we consider the emergence rate over the 2–4 weeks post-injection period, the tendency and rate of emergence appear the same as in Table 1. In this case, most of the control pupae (only debrained pupae and sham injected pupae) transformed into adults between 5 and 6 weeks after November 5, that is 9–10 weeks after brain removal. Injection of Fe₂(SO₄)₃ led 71% of the pupae to adult form within 4 weeks. After injection of CuCl₂, Zn(CH₃CO₂)₂ and MgSO₄ adult formation appeared in 45%, 32%, and 37% of the pupae respectively. Glutamic acid led 40% of the injected animals to imaginal stage, a dose response being evident.

¹ Partially purified as follows: fresh adult silkworm head→acetone powder→extract with 2% NaCl→90°C for 3 min→supernatant→treatment with phenol→phenol layer→removal of phenol with ether→precipitation with rivanol (see, NISHITSUTSUJI-UWO, 1972).

Table 1. AN EXAMPLE OF BIOASSAY ON *Bombyx* ♂ (DATA TAKEN 2 TO 4 WEEKS AFTER INJECTION)

Injection solution (40 μ l injection)	Dilution fold	No. of pupae injected	No. of available cases	Not-developed within 2-4 weeks		(Adult form) Days required for emergence
				dead	alive	
Brainless control ^a	—	—	15	0	0	23
Brain hormone ^b 4mg prot./ml	× 1	10	9	0	0	17, 18, 18, 18, 19, 19, 19, 19, 19
	× 4	10	10	1	1	17, 18, 19, 19, 19, 19, 20, 20
	× 16	10	10	0	5	18, 18, 18, 19, 19
	× 64	10	8	0	7	17
0.05M Acetic acid	× 1	10	10	0	8	20, 22
	× 4	10	10	1	6	20, 20, 24
	× 16	10	8	0	6	21, 23
	× 64	10	10	0	7	18, 20, 20
0.05M KCl	× 1	10	10	0	4	18, 19, 19, 20, 20, 23
	× 4	10	10	0	7	19, 19, 21
	× 16	10	10	0	7	19, 19, 23
0.05M CaCl ₂	× 1	10	10	1	5	16, 19, 19, 19
	× 4	10	10	0	6	16, 19, 19, 23
	× 16	10	10	0	7	18, 20, 20
0.05M MgSO ₄	× 1	10	9	0	4	17, 18, 19, 20, 20
	× 4	10	10	0	1	16, 18, 18, 18, 18, 18, 18, 19, 19
	× 16	10	10	0	1	19, 19, 19, 19, 19, 19, 20, 20, 20
0.05M FeCl ₃	× 1	10	8	0	0	18, 18, 18, 18, 19, 19, 19, 19
	× 4	10	9	0	3	18, 18, 19, 20, 20, 23
	× 16	10	2	1	0	18
	× 64	10	0	—	—	all died within a week

Date of brain removal: Early June 1971. Date of injection: July 14, 1971.

^a July 14, 1971, when injection was performed in other series, was regarded as zero-day.

^b See text.

Results of bioassay on *Bombyx* and *Samia*

Summarized data on *Bombyx*- and *Samia*-tests are arranged in Table 3. Fig. 1 shows the individual results of injection of a non-hormonal agent over the concentration range indicated in Table 4. These data were taken from the results of bioassays in which non-hormonal substances were injected into test animals over the past 2 years. The data have been aggregated irrespective of the concentration of injection solution, because in general no regular dose response was observed in present experiments.

1) Intact or sham control. When the brain removal of *Bombyx* was carried out within 10 min after pupation, 5-13% of the male pupae became moths 16-30 days after the operation. There after, the remaining pupae developed one by one with a large individual variation (ISHIZAKI, 1972). The rate and the range of adult formation seemed to vary according to the rearing conditions and the season (KOBAYASHI *et al.*, 1958).

After sham injection, in which the 'dauer pupae' were anaesthetized with cold and the needle was inserted without the injection of any solution, the ratio of adult

Table 2. ANOTHER EXAMPLE OF BIOASSAY ON *Bombyx* ♂ (DATA TAKEN 2 TO 6 WEEKS AFTER INJECTION)

Injection solution	Dilution fold	No. of pupae injected	No. of available cases	Not-developed within 2-6 weeks		(Adult form) Days required for emergence
				dead	alive	
Brainless control ^a	—	—	13	0	3	22^c , 22 , 35, 35, 37, 37, 37, 38, 38, 38
Sham injection	—	40	38	0	14	25 , 33, 33, 33, 35, 35, 35, 35, 35, 35, 35, 37, 37, 37, 37, 37, 37, 37, 42, 42
H ₂ O	—	15	15	0	6	26 , 33, 33, 33, 35, 35, 37, 37, 40
0.1M Fe ₂ (SO ₄) ₃	× 1	10	0	—	—	all died within a week
	× 4	10	9	2	0	21 , 23 , 24 , (21) ^b , 27 , 35, 37
	× 16	10	9	0	0	22 , 22 , 22 , 22 , 22 , 22 , 23 , 23 , 23
	× 64	10	10	1	0	23 , 23 , 23 , 23 , 23 , 24 , 24 , 33, 35
0.1M CuCl ₂	× 1	10	0	—	—	all died within a week
	× 4	10	0	—	—	all died within a week
	× 16	10	10	1	0	19 , 19 , 19 , 19 , 19 , 19 , 22 , 22 , 25
	× 64	10	10	0	1	33, 33, 33, 33, 35, 35, 35, 37, 40
0.1M Zn(CH ₃ CO ₂) ₂	× 1	10	0	—	—	all died within a week
	× 4	10	9	1	2	21 , 21 , 33, 35, 39, 51
	× 16	10	10	0	2	23 , 23 , 24 , 35, 37, 37, 39, 39
	× 64	10	9	0	3	23 , 23 , 23 , 26 , 35, 35
0.1M MgSO ₄	× 1	10	9	0	0	17 , 19 , 19 , 19 , 23 , 23 , 33, 33, 35
	× 4	10	9	0	1	21 , 23 , 26 , 26 , 33, 33, 35, 37
	× 16	10	10	0	0	21 , 21 , 22 , 33, 33, 35, 35, 35, 37, 39
	× 64	10	10	0	1	23 , 33, 33, 35, 35, 35, 37, 42, 42
Glutamic acid 1 mg/ml	× 1	10	9	0	0	19 , 21 , 21 , 21 , 21 , 22 , 33, 35, 35
	× 4	10	8	0	0	19 , 21 , 21 , 25 , 33, 33, 35, 37
	× 16	10	9	0	1	22 , 22 , 26 , 33, 33, 33, 35, 37
	× 64	10	9	0	2	22 , 33, 35, 35, 37, 37, 39

Date of brain removal: Early October 1971. Date of injection: November 5, 1971.

^a November 5, 1971, when injection was performed in other series, was regarded as zero-day.^b () died on the way to imaginal differentiation.^c Numbers in bold-face type refer to emergence within 2-4 weeks.

formation seemed similar to that of intact dauer pupae.

On the other hand, development of either intact or sham-injected debrained pupae of *Samia* hardly ever occurred.

2) Injection of solutions used in the BH extraction and purification processes. Deionized water, 2% NaCl, 0.1 M Tris-HCl pH 7.6, 0.05 M Pyridine-AcOH pH 6.0, and 0.05 M acetic acid were often used in the BH extraction and purification processes. After injection of these solution, some debrained pupae developed into imaginal form within 4 weeks. The rate of development was higher than that of intact debrained pupae. The emergence rate for *Bombyx* was 17%; and that for *Samia* was 1%.

3) Injection of solutions containing light-metal ions. None of the 0.1 M solutions caused the injected animal to die. By injection of these solutions at a series of

Table 3. TOTAL EMERGENCE RATE AFTER INJECTION OF NON-HORMONAL AGENTS

Non-hormonal substance injected	<i>Bombyx</i> -test			<i>Samia</i> -test		
	Total no.	No. of adult form	% of emergence	Total no.	No. of adult form	% of emergence
Intact and Sham injection	140	10	7	150	0	0
H ₂ O, AcOH, buffers	197	33 (2)	17 (1)	421	5	1
Na ⁺ , K ⁺ , Ca ⁺⁺ , Mg ⁺⁺	273	95 (7)	35 (3)	327	6 (1)	2
Fe ⁺⁺ , Fe ⁺⁺⁺	100	68 (6)	68 (6)	82	3 (2)	4 (2)
Cu ⁺⁺	74	23	31	98	2 (2)	2 (2)
Zn ⁺⁺	74	31	42	40	0 (1)	0 (3)
Fe, Cu, Zn ions (total)	248	122 (6)	49 (2)	220	5 (5)	2 (2)
Amino acids	72	30 (1)	42 (1)	40	0	0
Acetone	40	12	30	40	9	23
Others	72	13	17	20	1	5

() number or percentage of specimens which died on the way to adult formation.

Table 4. CONCENTRATION RANGE OF INJECTED SOLUTIONS

Injected solution	Concentration range/pupa		Total no. of injected pupae	
	μ moles	dosage μ g	<i>Bombyx</i>	<i>Samia</i>
Brainless pupa	—	—	69	150
Sham injection	—	—	80	15
H ₂ O	0	0	55	230
Acetic acid	0.032~2	1.9~120.1	40	127
Tris-HCl pH 7.6	0.032~4	3.9~484.4	40	77
Pyridine-AcOH pH 6.0	0.032~4	2.5~316.4	80	35
NaCl	0.2~13.6	11.6~795	120	95
Na-Acetate pH 6.0	0.096~6	7.1~444.1	—	73
KCl	0.064~4	4.8~298	30	28
CaCl ₂	0.032~2	4.7~294	30	28
MgSO ₄	0.064~4	15.7~986	70	48
MgCl ₂	0.064~4	13.0~813	40	20
Mg(CH ₃ CO ₂) ₂	0.064~4	13.7~858	40	20
MgCl ₂ +EDTA	0.032~2 +	6.5~407 +	40	20
	0.016~1	5.9~372		
FeSO ₄	0.064~4	17.8~1172	40	—
Fe ₂ (SO ₄) ₃	0.064~4	19.8~1240	40	20
FeCl ₂	0.064~4	15~939	40	20
FeCl ₃	0.064~4	17.3~1081	80	48
CuSO ₄	0.032~2	7.9~499	40	20
CuCl ₂	0.064~4	10.9~682	40	20
Cu(CH ₃ CO ₂) ₂	0.032~2	6.4~399	80	58
ZnSO ₄	0.064~4	18.4~1150	40	20
Zn(CH ₃ CO ₂) ₂	0.064~4	14.0~878	80	20
Aspartic acid	0.0038~0.24	0.66~41.8	40	20
Glutamic acid	0.0032~0.20	0.59~37.4	40	20
Acetone	4.2~268	244~15565	40	40
EDTA	0.0032~0.2	1.2~74.5	40	—
CH ₃ CO ₂ NH ₄	0.32~20	24.4~1500	40	20
Mercaptoethanol	0.64~4	4.9~312	40	10

dilutions, 18–54% (average 35%) of the test-*Bombyx* emerged and 0–2.5% (average 2%) of the test-*Samia* emerged. These emergence rates were twice as high as those after H₂O and buffer injections.

Fig. 1. Injection of non-hormonal agent.

4) Injection of solutions containing heavy-metal ions. A problem was to determine what concentration range of these solution to use; the animals often died if the concentration of the solution was too high, and with ferrous or ferric ions low concentrations of ion often led to death of the animal. The reason for this is at present uncertain. Consideration was also given to whether the use of strong acid salts or weak acid salts affected the results though no difference was found between the two.

After injection of cupric and zinc ions dauer pupae of *Bombyx*, 15–45% (average 31%) and 39–71% (average 42%) developed into adults, respectively. In the *Samia* test, however, only 0–3% developed after cupric injection and 0% after zinc injection. These ratios were similar to those seen after light-metal injections. On the other hand, injection of solutions containing ferrous or ferric ions led as many as 62–90% (average 68%) of *Bombyx* pupae to adult form, while even with *Samia* pupae, the emergence rate was 4% (2.5–6%).

5) Other substances. Aspartic acid and glutamic acid induced debrained pupae of *Bombyx* to adult development with the high rate of 40–43%. However, these amino acids had no effect on the injected *Samia* pupae.

When 0.8–50% acetone was injected into the debrained pupae, a 30% emergence rate was seen in *Bombyx*, with no dose response. This effect was similar to that after light-metal ion injection. Special attention must be given in the case of *Samia*. As adult development of debrained *Samia* pupae was blocked so strongly that only 5–6 % of the pupae developed even when injected with solutions containing ferrous or ferric

Table 5. AN EXAMPLE OF BIOASSAY ON *Samia* WITH SPECIAL REFERENCE TO ACETONE-INJECTION (DATA TAKEN 5 WEEKS AFTER INJECTION)

Exp. no.	Injected solution (40 μ l injection)	No. of pupae injected	Not-developed within 2–5 weeks		Developed () days required for emergence
			dead	alive	
I	2 % NaCl	5	0	5	0
	0.5 % "	5	0	5	0
	0.13% "	5	0	5	0
	0.03% "	5	0	5	0
	330 μ g/ml BH ^a (RP)	5	0	0	5 (17, 18, 18, 18, 19)
	37 μ g/ml "	5	0	2	3 (18, 18, 18)
	4 μ g/ml "	5	0	5	0
	50 % acetone	5	0	2	3 (23, 24, 24)
	12.5% "	5	0	1	4 (19, 21, 21, 23)
	3.1% "	5	0	5	0
II	0.8% "	5	0	5	0
	50% methanol	5	0	5	0
	10% "	5	0	5	0
	50% acetone	5	0	2	3 (30, 31, 33)
III	10% "	5	0	4	1 (26)
	50% methanol	5	0	5	0
	10% "	5	1	4	0
	50% acetone	5	0	2	3 (29, 31, 32)
	10% "	5	0	3	2 (26, 30)

^a Partially purified brain hormone (extracted from adult *Bombyx*-head), see text.

ions, it is questionable why as many as 23% of the pupae injected with acetone developed. Table 5 shows an example of a bioassay on *Samia* with special reference to acetone injection. When 40 μ l of 10–50% acetone solution was injected 30% of the pupae underwent adult development, although the time required for emergence was longer than that after BH injection. Since adult development in *Samia*, unlike that in *Bombyx*, does not usually occur later than 3–4 weeks after injection, specimens developing 5 weeks after the injection may be regarded as positive cases, and the emergence rate thus increases to 55%. When the concentration of the acetone solution was less than 3.1% no development was induced.

Injections of mercaptoethanol and EDTA had effects similar to those injections of H₂O and buffer, both in *Bombyx* and *Samia*.

DISCUSSION

According to WILLIAMS (1967), when *cecropia* pupae previously chilled at 5°C for longer than 35 weeks receive non-specific agents by injection, a substantial number of brainless pupae initiate adult development. He proposed that this development results if the titer of endogenous hormone is barely subthreshold, from injury induced by the injected material.

In *Bombyx*, the time of the critical period of BH secretion responsible for adult development varies among races ranging from the feeding period of the 5th instar larva (FUKUDA, 1959) to shortly after pupation (KOBAYASHI, 1955). Therefore, even debrained 'dauer pupa' of *Bombyx*, a hybrid between Chinese No. 115 and Japanese No. 122, should have a barely subthreshold titer of endogenous brain hormone. The fact that debrained *Samia* pupae essentially did not develop following injections suggests that in the brainless *Bombyx* pupae endogenous brain hormone is so high that either a factor produced following injection, or the injected material itself, must function synergically with endogenous brain hormone in turning on the prothoracic glands.

It is noticed that the increase in emergence rate after injection over that of intact dauer pupa and sham injected pupae followed the series: H₂O and buffers < light-metal ions and amino acids < heavy-metal ions. The injection of solutions containing heavy-metal ions generally induced a great number of debrained pupae of *Bombyx* to undergo adult formation. Ferrous, ferric, and cupric ions especially seemed to have an effect on adult development, even in *Samia* pupae.

The fact that induction of adult formation occurred in an average of 17% of pupae even after the injection of H₂O or buffers also suggests that the *Bombyx* dauer pupa is not a suitable test animal for BH assay.

The dormant state of brainless *Samia* pupae seems to be stable; only 1 or 2% of pupae developed into imagos after injection of solutions used in BH extraction and purification, or solutions of light-metal ions. This behaviour satisfied the requirement necessary for use as a biological assay test-animal. Emergence after injection of ferrous or ferric ion solutions was seen even in *Samia*-pupae, and it would seem that these ions are involved in the adult development processes.

It is not clear why the injection of high concentrations of acetone induced stable *Samia* pupae to adult formation; but it can be emphasized that debrained *Samia* pupa is a most suitable test animal for BH assay.

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