THE EFFECTS OF JUVENILE HORMONE ANALOGUES ON THE EMBRYONIC DEVELOPMENT OF SILKWORMS*

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Recent studies suggest that juvenile hormone can block the embryonic development of insects.[1] In the experiments in question, freshly laid eggs of the "linden bug" (*Pyrrhocoris apterus*) were brought into contact with the so-called "paper factor"—a juvenile hormone analogue known to be extremely effective in preventing the postembryonic metamorphosis of the Pyrrhocoridae.[2-4] After exposure to the hormonally active material, the eggs initiated embryonic development in an apparently normal manner; then, at some stage after the formation of the germ band, development stopped with the still-living embryo short of completion. In additional experiments,[5] the same phenomenon was duplicated by treating *Pyrrhocoris* eggs with a potent synthetic juvenile hormone material described by Law *et al.*[6]

These findings provide the first indication that embryonic development is subject to inhibition by the very same hormonally active materials which are effective in blocking the metamorphosis of postembryonic insects.[2-8] In the present investigation this proposition has been re-examined in experiments performed on two species of silkworms.

Materials and Methods.—Experimental animals: Eggs were collected from *Hyalophora cecropia* and *Antheraea pernyi* by placing mated female moths in individual paper bags or in glass jars containing discs of Whatman's filter paper. Over a period of several days the moths deposited their eggs, the ventral surface of each egg being cemented to the paper by a brownish glue. The eggs, still attached to the paper, were collected at 0.5- or 12-hour intervals, placed in Petri dishes, and incubated at 25 ± 0.5°C and 60 ± 5 per cent humidity under 11 hours of daily illumination. Untreated "control" eggs behaved as follows: 90–95 per cent of *cecropia* eggs hatched in 9.5–10 days; 96–99 per cent of *pernyi* eggs, in 8.5–9.5 days.

Hormonal materials: Since silkworms are completely insensitive to the paper factor,[2, 3] we tested two materials known to be highly effective in blocking the metamorphosis of silkworms—*cecropia* oil extracted with ether from the abdomens of male *cecropia* moths[7] and the synthetic juvenile hormone described by Law *et al.*[6] The specific formulations were as follows: (1) Crude *cecropia* oil was used without dilution; 5 mg gave a 3 + reaction in the standardized *polyphemus* assay for juvenile hormone.[8] (2) Chromatographed *ceropia* oil was used without dilution; 0.1 mg gave a 3 + assay. (3) Crude synthetic hormone; 0.025 mg gave a 3 + assay. The material was dissolved in methanol. (4) Preparations of synthetic hormone dissolved in crude or purified *cecropia* oil.

Moths were injected just below a spiracle of the second abdominal segment by means of a 27-gauge hypodermic needle in conjunction with a 1-ml Agla micrometer syringe (Burroughs Wellcome Co.). For topical application onto the dorsal surface of eggs, droplets (0.16–0.32 μl) were dispensed from a 30-gauge

needle sealed to a 0.1-ml microsyringe (Hamilton Co.), the volumes being measured by the Agla micrometer.

Results.—Eggs from injected moths: The hormonally active materials were injected into 23 female *cecropia* moths, 5 prior to mating and the rest immediately after mating. After the beginning of oviposition, the eggs were collected at 12-hour intervals and incubated at 25°C.

Eggs laid by these treated moths often failed to hatch; this was particularly true for moths receiving the higher doses of the more active hormonal materials (Table 1). Eggs deposited within the first three hours after the injection were less affected because of the time required for the injected material to be dispersed and brought into contact with the eggs in the oviducts. So, also, a delay of one or

TABLE 1

DEVELOPMENT OF EGGS LAID BY FEMALE *cecropia* MOTHS INJECTED WITH JUVENILE HORMONE MATERIALS

Dosage (mg)		Eggs oviposited	Per cent hatched	Developmental Stage of Unhatched Eggs (%)		
				Blastoderm	Definitive germ band	Recognizable first-instar larva
		Injection before Mating				
Synthetic hormone	Crude *cecropia* oil					
—	73	114*	91	5.5	0	4.5
Preparation 1						
1.25	25	237	23	75	0	2
2.5	50	125*	97	0	1	2
4.5	90	183*	0.5	79†	14†	6†
5	100	80*	0	90	4	6
	Inactive *cecropia* oil					
—	200	271	79	1	1	19
		Injection after Mating				
	Crude *cecropia* oil					
—	50	229	88	1	0	11
—	100	88	71	8	5	16
—	150	47	79	11	4	6
—	200	37	5	92	0	3
1.25	25	85	36	56	4	4
2.5	50	165	0	75‡	25‡	0
2.5	50	212	0	98‡	2‡	0
Preparation 2						
6	30	198	84	2	0.5	13.5
10	50	38	16	0	3	81
10	—§	265	56	1.8	0.4	42
	Purified *cecropia* oil					
—	30	306	95	4	0	1
—	50	240	29	6‡	2‡	63‡
Preparation 3						
5	50	191	23	0	28	49
5	50	124	49	2	0	49
5	50	30	20	0	40	40
Preparation 4						
5	50	130	50	23	0	27
6	60	198	83	2	1	14
7.5	75	241	17	23	1	59
	Inactive *cecropia* oil					
—	50	207	98	0.5	0	1.5

* Signifies moths which were hand-mated after failure to mate spontaneously.
† Based on microscopic examination of 50% of unhatched eggs.
‡ Based on microscopic examination of 25% of unhatched eggs.
§ In this one experiment 50 mg of peanut oil was used as the vehicle for the synthetic juvenile hormone.

more days in oviposition was generally associated with an increase in the percentage of eggs that hatched; this fact reaffirms the rapid breakdown of the hormonally active materials, especially of the *cecropia* hormone contained in the *cecropia* oil.[9]

Of the five moths injected prior to mating, only the individual which received the lowest dose spontaneously mated when placed overnight with males. Therefore, in order to obtain fertile eggs, it was necessary to "hand mate" the other females. This effect was not encountered when a control female was injected with twice the volume of inactive *cecropia* oil prior to mating.

After 12 days of incubation, the unhatched eggs were submerged in Ringer's solution and the chorion peeled away with jeweler's forceps. Eggs derived from moths that received less than 200 mg of crude *cecropia* oil or the less active synthetic preparations nearly always contained fully formed first-stage larvae, many of which had failed to initiate or to complete blastokinesis—a developmental anomaly that will be discussed below in further detail. In the case of unhatched eggs deposited by moths that received the higher doses of the more potent juvenile hormone materials, embryonic development commonly stopped in the blastoderm stage (Table 1), corresponding to the 18th hour of normal embryonic development. Despite the absence of an embryonic axis ("germ band"), these eggs often showed extraembryonic membranes and an organization of the yolk into discrete cells intermingled with clumps of whitish material. Certain other eggs proceeded to the formation of a recognizable germ band and then stopped, generally at the stage of blastokinesis.

Topical application to freshly laid eggs: Microdrops of synthetic hormone were applied to *cecropia* and *pernyi* eggs during the first four hours after oviposition. As summarized in Table 2, hatching was depressed by the lowest dose tested and completely suppressed by the application of 0.48 μg per egg. By contrast, at least 90 per cent of control eggs hatched after treatment with 0.64 μg of peanut oil.

Topical application after preliminary incubation: Eggs were collected at 30-minute intervals and incubated at 25°C for specific periods before they received a topical application of synthetic hormone (0.32 μg for *cecropia* and 0.64 μg for *pernyi*). Groups of 20–50 eggs were used until the 32nd hour, and groups of 15–25 eggs thereafter.

In the results summarized in Figures 1 and 2, it is clear that as far as the hatching of the eggs is concerned, an initial period of high and rather uniform sensitivity was

TABLE 2

EFFECTS OF SYNTHETIC JUVENILE HORMONE TOPICALLY APPLIED TO FRESHLY LAID EGGS OF TWO SPECIES OF SILKWORMS

Dose per egg (μg)	*Hyalophora cecropia*		*Antheraea pernyi*	
	No. of eggs	Per cent hatching	No. of eggs	Per cent hatching
0.08	20	70	—	—
0.16	30	23	—	—
0.32	167	10	100	11
0.48	10	0	60	0
0.64	—	—	100	0
Control Eggs Receiving Peanut Oil				
0.32	42	93	—	—
0.64	10	90	50	94

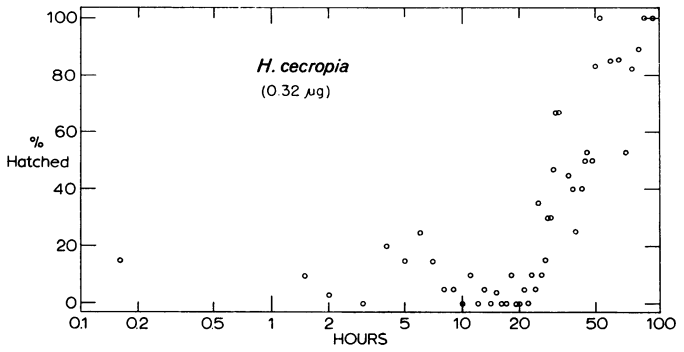

FIG. 1.—Per cent of *cecropia* eggs that hatched when 0.32 μg of crude synthetic juvenile hormone was topically applied to each egg at the indicated hour of incubation. Each datum up to 30 hr is based on 20 eggs and thereafter 15–20 eggs.

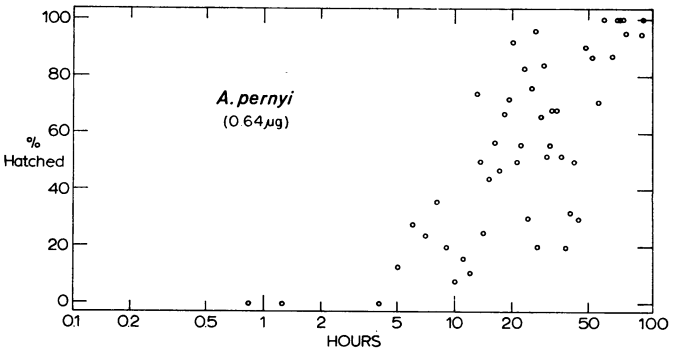

FIG. 2.—Per cent of *pernyi* eggs that hatched when 0.64 μg of crude synthetic juvenile hormone was topically applied to each egg at the indicated hour of incubation. Each datum up to 40 hr is based on at least 25 eggs and thereafter 15–20 eggs.

followed by one or two days during which sensitivity fluctuated to a surprising degree. Moreover, when treatment was postponed to or after the 96th hour of incubation, nearly all the eggs hatched at the normal time; this same insensitivity was observed even when the dose of hormone was increased fivefold.

Repeated topical applications: Fifty *cecropia* eggs were treated with 0.32 μg of synthetic hormone within 15 minutes after they were laid; this treatment was repeated at 24-hour intervals during the following nine days. Only 1 of the 50 eggs hatched. In control experiments in which eggs received daily doses of 0.32 μg of peanut oil, all 20 eggs hatched.

The developmental status of unhatched eggs: Random samples of each group of unhatched eggs were dissected in Ringer's solution. The vast majority contained dead or dying larvae, many of which were fully pigmented and seemingly ready to hatch. Their developmental condition, in short, was precisely the same as that described above for unhatched eggs deposited by moths that had been injected with the lower doses of the less active material. And, here again, most larvae showed abnormal orientations within the egg, signaling a partial or complete failure of blastokinesis.

Detailed studies were carried out on 284 unhatched *cecropia* eggs that had been treated with 0.32 μg of synthetic hormone during the first 12 hours of incubation. Of this total, 93 per cent contained first-instar larvae in a terminal phase of embryonic development. Of the remainder, 5 per cent showed no detectable development. Only 3 of the 284 eggs were scored as arrested in the germ-band stage. This result is therefore strikingly different from the arrest at the blastoderm stage, commonly observed when the eggs were exposed to potent juvenile hormone materials prior to oviposition.

Postembryonic development of hatched larvae: Many of the larvae which hatched from the treated eggs showed greatly reduced viability when reared on their normal food plants (*pernyi* on oak; *cecropia* on wild cherry). When exposed to synthetic hormone prior to the 48th hour of incubation, 50–70 per cent of the hatched larvae died within the first two days or during the first larval molt. These larvae often showed one or more anatomical defects.

The most common anomaly was a circular scar at the posterior edge of the mesothoracic tergum, marking the site of attachment of the yolk sac to the embryo. Hatched *pernyi* larvae frequently possessed tan instead of black pigmentation between the two dorsal tubercles of the first thoracic segment. Abnormalities were sometimes evident in the dorsal closure of the metathorax and the first one or two segments of the abdomen. *Cecropia* larvae were often characterized by misshapen or missing tubercles on one side of the fourth abdominal segment—an abnormality that sometimes included the fifth and, rarely, the sixth segment on the same side. Those larvae which survived to the third instar were usually undersized and abnormal in several additional respects. Of particular interest was the retention of certain patterns of pigmentation characteristic of earlier larval stages—a phenomenon which will be considered elsewhere in greater detail.

About 70 per cent of the larvae which hatched from eggs treated somewhat later in incubation (between the 48th and 96th hour) underwent normal development until at least the third larval instar. A pronounced decline in growth rate and viability was then evident and 55–80 per cent (depending on dosage) died prior to pupation.

As noted in Figures 1 and 2, nearly all eggs hatched when the hormonal treatment was postponed to or after the 96th hour of incubation. The same was true when the dose was increased fivefold. These latter individuals generally showed normal development throughout the first four larval stages. About 10 per cent died during the fourth molt, and the growth of the surviving fifth-stage larvae was greatly retarded. About 50 per cent spun cocoons and formed pupae which, especially in the case of *cecropia*, often retained vestiges of larval characters such as abdominal prolegs and dorsal tubercles.

Discussion.—The present study has documented the ability of juvenile hormone and hormonal analogues to block the embryonic development of silkworms. The effects, as we have seen, are maximal when the hormone is used in high concentration and is brought into contact with the eggs very early—in point of fact, while the ova are still in the oviducts of the female moth. In that event, embryonic development commonly proceeds to the blastoderm stage and then stops.

If the exposure is postponed until after the eggs have been oviposited, it is usually too late for juvenile hormone to interfere with the formation of a recognizable

first-instar larva. Despite this fact, many of these larvae failed to hatch; as illustrated in Figures 1 and 2, this was especially true of eggs exposed to juvenile hormone during the first day of incubation.

The vast majority of these eggs contained larvae whose orientations were abnormal due to their failure to initiate or to complete blastokinesis, a revolution of the embryo which normally occurs during the third day of incubation in *pernyi* and the fourth day in *cecropia*. Evidently, some aspect of this complicated locomotor activity is extremely sensitive to inhibition by juvenile hormone. And since the completion of blastokinesis is a necessary prelude to hatching,[10, 11] we attribute the failure of these larvae to hatch, as well as many of their anatomical defects, to an interference with blastokinesis.

When apparently normal larvae hatched from the treated eggs, the effects of the prior exposure were often evident at a certain stage in postembryonic development. The earlier the treatment, the sooner were abnormalities detected during larval life. Preliminary experiments[12] point to a similar phenomenon in the bugs *Pyrrhocoris apterus*, *Oncopeltus fasciatus*, and *Lygaeus kalmii*. These curious results suggest that the hormonal materials are able to interfere with the programing or latent storage of information for postembryonic development.

Of the findings reported here, the most clear-cut is the ability of the juvenile hormone materials to block embryonic development at the blastoderm stage when brought into contact with the eggs prior to fertilization. In additional studies to be described elsewhere,[12] this result has been duplicated in experiments performed on the bugs *Pyrrhocoris apterus* and *Lygaeus kalmii*.

Even the highest doses of the hormonal materials were unable to block development at the still earlier "cleavage island" stage—an observation which suggests that the information for blastoderm formation is already available to the unfertilized ovum. This conclusion is in agreement with recent findings on the eggs of some Coleoptera;[13] it is also supported by unpublished studies[12] demonstrating blastoderm formation in unfertilized *cecropia* eggs subjected to artificial parthenogenesis.

The ability to block the transformation of the blastoderm into a larval silkworm is strikingly reminiscent of the effectiveness of the same hormonally active materials in opposing postembryonic metamorphosis, for example, the transformation of a pupa into an adult moth.[8] In both cases, the set of genes which is operational at the time of treatment remains fully operational. But in the presence of high titers of juvenile hormone the organism is apparently unable to activate the next gene-set in its programed life history.[14]

Summary.—Embryonic development of silkworm eggs can be blocked as early as the blastoderm stage by exposing unfertilized eggs to potent preparations of juvenile hormone or juvenile hormone analogues. If the treatment is postponed until the eggs have been fertilized and oviposited, it is usually too late to block embryonic development before the completion of recognizable first-instar larvae which commonly fail to hatch. Larvae which hatch from treated eggs often show anatomical defects as well as curtailed viability and various abnormalities in postembryonic development. The implications of these findings are discussed in relation to the role of juvenile hormone in controlling the implementation of genetic information during embryonic and postembryonic development.

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[1] Sláma, K., and C. M. Williams, *Nature*, **210**, 329 (1966).
[2] Sláma, K., and C. M. Williams, these PROCEEDINGS, **54**, 411 (1965).
[3] Williams, C. M., and K. Sláma, *Biol. Bull.*, **130**, 247 (1966).
[4] Saxena, K. N., and C. M. Williams, *Nature*, **210**, 441 (1966).
[5] Sláma, K., and C. M. Williams, unpublished data.
[6] Law, J. H., C. Yuan, and C. M. Williams, these PROCEEDINGS, **55**, 576 (1966).
[7] Williams, C. M., and J. H. Law, *J. Insect Physiol.*, **11**, 569 (1965).
[8] Williams, C. M., *Biol. Bull.*, **121**, 572 (1961).
[9] Gilbert, L. I., and H. A. Schneiderman, *Trans. Am. Microscop. Soc.*, **79**, 38 (1960).
[10] Slifer, E. H., *Biol. Zentr.*, **52**, 223 (1932).
[11] Tirelli, M., *Zool. Jahrb. Abt. Anat. Ontog. Tiere*, **49**, 59 (1931).
[12] Riddiford, L. M., unpublished data.
[13] Lockshin, R., *Science*, **154**, 775 (1966).
[14] Williams, C. M., in *The Nature of Biological Diversity*, ed. J. M. Allen (New York: McGraw-Hill, 1961), p. 243.