The Juvenile Hormones in Blood of Larvae and Adults of *Manduca sexta* (Joh.)

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Juvenile Hormone, *Manduca sexta*

The juvenile hormones (JH) in extracts of *Manduca sexta* blood were converted to 10-(2,4-dichlorobenzoyloxy)-11-methoxy derivatives and quantitatively determined by gas chromatography with electron capture detection. Early IVth instar larvae contained 0.62 ng JH-I, 1.1 ng JH-II and 0.07 ng JH-III per ml blood. Early Vth instar larvae, and adult of both sexes contained the hormones in concentrations from 0 to 0.24 ng/ml blood. No hormone was detected in late Vth instar (wandering) larvae. The detection limit in our experiments was $\leq$ 0.003 ng/ml blood.

Three materials with juvenile hormone (JH) activity have been isolated from extracts of insects or from cultures of their corpora allata: JH-I (**1a**)[1], JH-II (**1b**)[2], and JH-III (**1c**)[3]. At the present time none of these compounds has been identified unambiguously as the authentic morphogenetic hormone of an individual insect species. With one exception[4], the source of the isolated hormone was always an adult. We now have developed a procedure which allows qualitative and quantitative identification of subnanogram amounts of JH-I, JH-II, and JH-III in the blood of insects. The tobacco hornworm, *Manduca sexta* (Joh.), has become one of the best studied species in insect endocrinology. It was therefore a most suitable object for testing the new method.

JH-III was first identified as a natural product in organ cultures of corpora allata from adult female *Manduca sexta*[3]. In addition to JH-III, the culture media contained JH-II as a major component[3]. We have found that freshly excised corpora allata initially produce more JH-II but that the JH-II production declines much faster than that of JH-III. Concluding that corpora allata *in vitro* are probably lacking a precursor for the biosynthesis of JH-I and JH-II, we added mevalonolactone and homomevalonolactone to the medium. Under these conditions all three hormones are produced and JH-II remains the major product as long as the gland is active. Corpora allata of larvae were found to be much less active but to secrete the hormones in the same pattern. Clearly, *Manduca* is able to produce all three hormones *in vitro*. Now it was of interest to identify the hormones in the blood of various postembryonic stages of this species.

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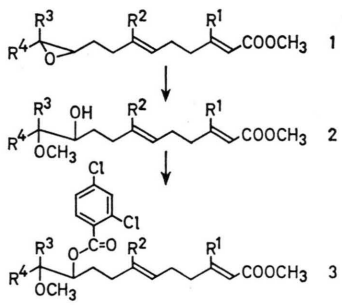

	R^1	R^2	R^3	R^4
a	$-CH_3$	$-C_2H_5$	$-C_2H_5$	$-CH_3$
b	$-CH_3$	$-CH_3$	$-C_2H_5$	$-CH_3$
c	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
d	$-C_2H_5$	$-C_2H_5$	$-CH_3$	$-C_2H_5$

1a=JH-I **1b**=JH-II **1c**=JH-III **1d**=*ttt*-JH-0

Materials and Methods

Manduca sexta were reared in our laboratory on an artificial diet[5]. Blood (usually 3–5 ml) was collected in water at 0 °C and immediately extracted with ethyl acetate. At this point 34.2 ng (91400 dpm) [methoxy-^{3}H]-*t,t,t*-JH-O (**1d**) were added to each sample as an internal standard. Throughout the procedure, solutions in organic solvents were concentrated or evaporated to dryness at room temperature by a stream of dry nitrogen. The extract of blood from female adults contained $\simeq$ 100 mg oily material which was repeatedly extracted with methanol (separation of the phases by centrifugation at -20 °C and $4000 \times g$). The combined

methanol extracts of this preparation and the evaporation residues (3 – 15 mg) of all other extracts were resolved by TLC on silica gel HF_{254} (E. Merck, Darmstadt) with benzene/5% ethyl acetate. The juvenile hormones were eluted with ethyl acetate and kept for 90 min at 22 °C in 0.1 ml methanol containing 0.014% perchloric acid. After addition of 1 ml 2% aqueous sodium chloride, the mixture was extracted with ethyl acetate and the extract resolved by TLC with benzene/15% ethyl acetate. The material in the zone corresponding to the 10-hydroxy-11-methoxy derivatives (**2**) was eluted with ethyl acetate and treated with 5 µl 2,4-dichlorobenzoyl chloride in 0.1 ml pyridine for 6 h at 22 °C in the dark. The reaction mixture was evaporated to dryness by a stream of dry nitrogen, the residue extracted with ether and resolved by two successive TLC separations with benzene/15% ethyl acetate and benzene/5% ethyl acetate. The derivatives (**3**) of the individual juvenile hormones were finally resolved by High Pressure Liquid Chromatography on a 60 × 0.4 cm µ-Porasil column (Waters Associates Inc., Milford, Massachusetts) with hexane/3.5% ethyl acetate/0.02% 2-propanol. The retention volumes of the hormone derivatives were calculated from that of the radiolabelled *t,t,t*-JH-O-derivative. Individual fractions from this separation were evaporated, the residues redissolved in 25 µl 2,2,4-trimethylpentane/20% ethyl acetate and analyzed by GLC on a Hewlett Packard Model 5710A gas chromatograph equipped with a 15 mCi ^{63}Ni electron capture detector. Argon with 5% methane was used as a carrier gas. The recovery of the internal standard derivative as measured by GLC and by scintillation counting indicated an overall yield of 50 ± 10%.

The juvenile hormone derivatives were identified by isothermal GLC on three of the following five columns (180 cm, glass): 3% SE-30 (275 °C), 3% OV-1 (265 °C), 3% OV-17 (290 °C), 3% XE-60 (255 °C) and 10% UC-W98 (300 °C). Authentic samples, prepared by the same method and identified by mass spectrometry, served as reference compounds. They were calibrated by UV-colorimetry; the absorption coefficients [ε_{220}: 24,000 ± 1500; ε_{230}: 20,800 ± 1300 (methanol)] had been determined through the derivative of [7-ethyl-^{3}H]-JH-I with known specific ^{3}H-activity. Under the conditions used for the analysis a peak of 5 mm height was produced by 5 pg of the dichlorobenzoate **3c**. The recovery of small quantities throughout the procedures had been checked in pilot experiments where 0.25 – 1.0 nanogram of the hormones had been added to the naturally hormone free blood extracts of late Vth instar larvae. In order to detect possible contamination of samples with synthetic hormones, each set of experiments included controls with and without juvenile hormone.

Results and Discussion

The highest concentration of juvenile hormones was found in blood of early IVth instar larvae; it was considerably lower in early Vth instar larvae (Table I). No JH could be detected in wandering Vth instar *Manduca*; the detection limit in this experiment was ≦ 0.003 ng/ml blood. This result corresponds well with titer determinations by the *Galleria* wax test (K. Judy, private communication) and a *Manduca* assay (L. Riddiford, private communication). Also, blood of late Vth instar larvae has a high JH-esterase activity which makes the presence of any JH at this stage and age unlikely [6]. In practically all experiments, the three juvenile hormones were found side by side in varying concentrations. At present no interpretation is possible with regard to the juvenile hormone composition in the different stages. It may be of interest that we have found in early IVth instar larvae of *Hyalophora cecropia* 0.15 ng JH-II per ml blood and were unable to detect JH-I or JH-III. Before any conclusions could be reached, it would be necessary to show that the ratios JH-I : JH-II : JH-III are closely controlled or, alternatively, that in allatectomized larvae physiological effects of JH-mixtures depend

Table I. Juvenile hormones in blood of *Manduca sexta*; *nil:* detection limit ≦ 0.003 ng/ml blood.

No. animals	Stage	Blood collected [ml]	JH	Concentration [ng/ml]
82	early IVth larval instar (1 – 2 day-old)	3.5	I	0.62
			II	1.1
			III	0.07
28	early Vth larval instar (1 – 2 day-old)	5.1	I	0.24
			II	0.11
			III	0.12
17	Vth larval instar, fully grown (4 – 5 day-old)	10.8	I	*nil*
			II	*nil*
			III	*nil*
116	female adults (1 – 2 day-old)	4.9	I	0.06
			II	0.14
			III	0.11
60	male adults (1 – 2 day-old)	1.5	I	0.10
			II	0.04
			III	*nil*, <0.02

on the ratios of their components. It has been shown, however, that the morphogenetic activity of JH-III in *Manduca* pupae is 300 times lower than that of JH-I and JH-II [7]. In females of *Manduca* the JH controls egg maturation [8] and in males, presumably, the function of accessory sex glands, but the relative gonadotropic activities of JH-I, JH-II and JH-III have not been determined.

In hemolymph of the roach *Nauphoeta cinerea*, depending on stage and age, also varying concentrations of JH-I, JH-II and JH-III were detected [4]. From the relative concentrations in nymphs and in adults it is suggested that JH-I and JH-II may be responsible for the morphogenetic and JH-III for the gonadotropic action. Hopefully, these identifications can be confirmed, since in total extracts of several adult roaches including *Nauphoeta* only JH-III [9] was found. JH-III was also the only hormone detected in larval and adult corpora allata cultures of a number of roach species [10 and own work on *Periplaneta americana* (L.), *P. fuliginosa* (Serville) and *Blaberus discoidalis* (Serville), unpublished].

The method described in this publication is technically too difficult to be considered as a routine procedure for the determination of JH-titers. In selected cases, however, where the effort appears to be justified, it allows qualitative identification and quantitative determination of the juvenile hormones I, II and III.

We thank the Zoecon Corporation, Palo Alto, Calif., for gifts of JH-0, JH-II and JH-III. Mrs. Jane Moore collected the hemolymph of *Manduca*. Throughout the investigation, we exchanged information with Prof. C. A. Salemink, University of Utrecht. Using a different derivative, his research group also developed a method for detection of juvenile hormones by gas chromatography with an electron capture detector [11].

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