On the Specificity of Juvenile Hormone Biosynthesis in the Male *Cecropia* Moth *

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The accessory sex glands (ASG) of adult male *Cecropia* contain an enzyme that methylates juvenile hormone acids (JH-acids) in the presence of S-adenosyl-L-methionine (SAM). The methyltransferase is highly specific. The reaction rates decrease in the order JH-I-acid, JH-II-acid and JH-III-acid; in each case the natural enantiomer is esterified predominantly. Methyltransferase activity with the same substrate specificity was also demonstrated in adult female corpora allata (CA). Male CA have only marginal methyltransferase activity.

The CA of male *H. cecropia* contain substantial amounts of JH-I-acid and JH-II-acid (minimum: 5 pmol/pair). When kept in organ culture, they release JH-acids into the medium. Radiolabeled propionate and mevalonate are incorporated efficiently into the carbon skeletons of the JH-acids. The enzyme system performing these transformations cannot be forced to produce JH-III-acid even in the presence of high mevalonate concentrations, though homomevalonate may enhance biosynthesis of JH-I-acid and JH-II-acid more than tenfold.

It becomes evident that the regulation of JH titer balances with regard to the homologous structures during insect development is not merely a question of the availability of low molecular weight precursors, but in addition that of highly specific enzymes acting as regulatory entities in the later steps of the biosynthetic sequence.

Introduction

Four juvenile hormones (JH-O, JH-I, JH-II, and JH-III, Fig. 1) have been detected in insect material of various origin (see [1–3], and references cited therein). The relative concentrations of the homologous hormones may differ not only between insect species but also between the individual stages of postembryonic development [4–6]. It has been suggested that not only the total JH-titer but the concentrations of the individual components may be a prerequisite for the regulation of differentiation at substeps in the postembryonic developmental program. This raises the question of the evolutionary significance of the structural diversity of the juvenile hormones, and furthermore of the biochemical mechanism underlying JH-titer regulation during the life history of an insect. Some of the regulatory factors are most likely involved in the control of specific enzyme systems associated with JH biosynthesis.

In early 4th instar larvae of the saturniid moth *Hyalophora cecropia* (L.) the principal juvenile hormone is JH-II. Its concentration in hemolymph is 17 pg/ml with no JH-I or JH-III detectable (< 3 pg/ml), whereas in total body extracts of the same stage 15 pg JH-I/eq.[+] and 32 pg JH-II/eq. were found (Peter, Dahm, and Röller, in preparation). In adult
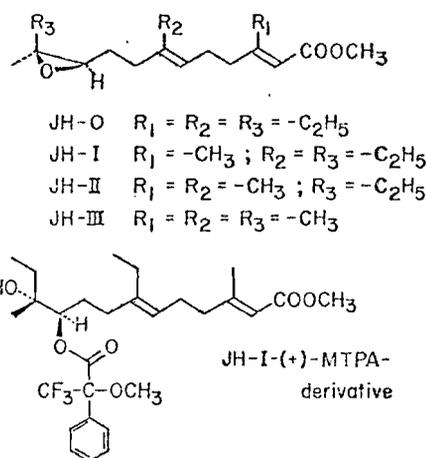
Fig. 1. Structures of the natural juvenile hormones and of a (+)-α-methoxy-α-trifluoromethylphenylacetic acid [(+)-MTPA] derivative. The (10 R, 11 R)-diastereomer is derived from natural (10 R, 11 S)-JH-I.

males the main component, however, is JH-I (1−3 µg/eq.) accompanied by 10−30% JH-II and about 1% JH-O [3]. The rather large amounts of hormones found in the adult male shortly after adult eclosion certainly reflect a high biosynthesis rate from propionate and acetate, presumably *via* homomevalonate [7, 8] and thus render this species a valuable object for biochemical investigations. The juvenile hormones are accumulated exclusively in the adult male accessory sex glands (ASG) [9]. The process of accumulation may involve the methylation of precursor acids (Fig. 1) by transfer of the methyl group from L-methionine [10]. Exogenously applied juvenile hormones are at least in part hydrolyzed and remethylated. ASG *in situ* accumulate preferentially JH-I, to a lesser extent JH-II, and discriminate strongly against JH-III [1]. A methyl group transferring enzyme was observed in homogenates of the ASG, the substrate requirements in this system are S-adenosyl-L-methionine (SAM) in addition to the precursor acid [11].

We now have investigated the substrate specificity of the methyl group transferring enzyme system from homogenized ASG of adult male *Cecropia* that have been allatectomized as pupae, and therefore do not contain endogenous juvenile hormones. In addition, we report some experiments concerning the specificity of the corpora allata with regard to the utilization of mevalonate and homomevalonate for the biosynthesis of the carbon skeletons of juvenile hormones in *Cecropia*.

Results and Discussion

Selectivity of JH-acid methylation in ASG-homogenates

Racemic juvenile hormone acids were prepared by enzymatic hydrolysis of racemic JH-I, JH-II and JH-III with *Manduca sexta* 5th larval instar hemolymph [1, 2]. The esterase present in this hemolymph does not discriminate between the homologous structures nor between the enantiomers as was determined by GLC-calibration of JH-acid mixtures after their reconversion to JH with diazomethane. A sample of [7-ethyl-³H]JH-I-acid was esterified with diazomethane and the amount of label in the two diastereomeric(+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) derivatives (Fig. 1) determined by the method of Nakanishi [13]. Both

diastereomers contained equal amounts of label (50 ± 2%).

Mixtures of the racemic JH-I-, JH-II-, and JH-III-acids were incubated with aliquots of JH-free homogenized ASG and [methyl-³H]SAM (Table I). The isolation procedure for the juvenile hormones involved extraction of the incubation mixtures after addition of unlabeled carrier JH-I, JH-II, and JH-III, followed by TLC and HPLC-separations. The amount of radioactivity associated with the methyl ester group of the juvenile hormones through incorporation of label from [³H]SAM thus gives a rough approximation of the quantities of juvenile hormone formed. It is assumed that a dilution of the radiolabeled SAM with endogenous SAM from the ASG preparations can be neglected. The values in Table I are not corrected for recovery of carrier JH which, however, was generally in the range of 75 ± 10%, as estimated from the response of the UV-detector during HPLC. In relative terms (% of JH-acid methylated), the main product is JH-I. Under the experimental conditions it is produced in 1.5−2 times higher yield than JH-II. The enzyme system appears to discriminate strongly against JH-III-acid since the relative amount of label incorporated is only approximately 15% of that found in JH-I. Generally, the percentage of JH-I-acid methylated is in the range of 50%. The small amount of label in the control experiment (1.5 of Table I) most likely results from contamination, since all experiments listed in Table I were performed in parallel and no special

Table I. Substrate specificity of methyltransferase in homogenized, JH-free, adult male accessory sex glands (ASG) of *H. cecropia*. Racemic JH-acids were incubated for two hr in 125 µl of ASG homogenate, 10 µl 0.1 M phosphate buffer pH 7.5, and 8.92 µCi[³H]-SAM (10 Ci/mmol) in 10 µl 0.001 N H_2SO_4. The ASG (0.25 eq. per expt.) were from 20 day-old pharate adult males which had been allatectomized as pupae.

Experiment No.	1.1	1.2	1.3	1.4	1.5
JH-I-acid:					
pmol acid added	184	122	88	0.88	0
pmol ³H incorporated	75.7	45.8	35.7	0.60	(0.18)
% methylated	41.1	37.6	40.5	68.4	—
JH-II-acid:					
pmol acid added	250	418	304	3.04	0
pmol ³H incorporated	67.6	86.6	69.1	0.51	(0.11)
% methylated	27.1	20.7	22.7	16.8	—
JH-III-acid:					
pmol acid added	192	207	165	1.65	0
pmol ³H incorporated	13.5	12.2	9.3	0.17	(0.06)
% methylated	7.0	5.9	5.6	10.4	—

care was applied during the experimental procedures to exclude possible cross contamination even at the lowest detection level. Experiment 1.4 was performed with the smallest amounts of precursor acids (1% of that in experiment 1.3): the value of 68% for JH-I-acid methylated in this experiment would be reduced to 48% after subtraction of the background value from experiment 1.5.

Formation of the diastereomeric (+)-MTPA-derivatives of JH-I and JH-II from experiment 1.1 (Table I) and their analysis by HPLC revealed that the methylating enzyme not only exhibits substrate selectivity with regard to homologous structures, but that it is also enantioselective. The diastereomeric mixture of the JH-I-(+)-MTPA-derivatives (58 100 dpm) was subjected to HPLC separation: 47 700 dpm (82.1%) were associated with the (10 R, 11 R) and 6900 dpm (8.2%) of (10 S, 11 S)-diastereomer. Analogously, 83 000 dpm of the JH-II derivative yielded 72 550 dpm (86.6%) of the (10 R, 11 R) and 6900 dpm (8.2%) of (10 S, 11 S)-derivative. Two principally different mechanisms may explain the observations: either the JH-acid methyltransferase is a remarkably substrate- and enantioselective protein, or several proteins are interacting by selective protection of the naturally configurated higher homologes, leaving the others subject to degradative metabolism.

However, no JH-binding protein has been found in the ASG of *Cecropia* (Weirich, unpublished). Some preliminary experiments indicate that upon incubation of [7-ethyl-³H]JH-I-acid with crude ASG-homogenate the natural (10 R, 11 S)-enantiomer is

methylated rapidly and in high yield, whereas catabolic enzymes from the microsomal fraction, presumably epoxyhydratases, act only slowly on the unnatural (10 S, 11 R)-enantiomer. Since no binding protein, exerting a protective function, is present, we believe that the observed selectivity in the formation of juvenile hormones from their precursor acids is best explained by the substrate specificity of the methyl transferase. As will be discussed below, the same substrate selectivity was observed in the methylation of JH-acids by an enzyme system from homogenized CA where the presence of a JH-binding protein is unlikely. More detailed studies, however, will be necessary before a firm conclusion can be reached.

Biosynthesis of JH-acids in corpora allata of H. cecropia

Generally, the source of juvenile hormones in insects are the corpora allata (CA). To this time no report describes the isolation of JH or even of JH-activity from extracts of the endocrine organs themselves in spite of the fact that CA may be active when implanted in a suitable host or that they may secrete JH when kept in organ culture. Homogenates of CA from female *Manduca sexta* contain an enzyme which converts JH-acid and added [methyl-³H]-S-adenosyl-methionine ([³H]SAM) to labeled JH. *De novo* biosynthesis of JH-acids does not seem to occur in such homogenates under the experimental conditions described and, if no JH-acids are added,

Table II. Conversion of endogenous and synthetic JH-acids to JH in homogenates of corpora allata (CA) from male and female adult *Cecropia*. Volume of incubation mixtures: 20 µl per CA eq.[a].

Experiment No.	2.1	2.2[b]	2.3	2.4[b]	2.5	2.6
Corpora allata from	10 ♂	3 ♀	5 ♀	3 ♂ + 3 ♀	4 ♂	10 ♀
µCi [³H]SAM added	4.5	5.0	5.0	5.0	4.5	4.5
i. e. pmol SAM	506	562	562	562	506	506
incubation time [h]	4.5	3.0	5.5	3.0	2.5	2.5
pmol JH-I-acid added	0	0	0	0	88	88
pmol ³H incorporated	0.049	1.19	0.55	0.50	0.090	20.42
pmol JH-I/CA eq.	0.005	0.40	0.11	0.08	0.023	2.04
pmol JH-II-acid added	0	0	0	0	304	304
pmol ³H incorporated	0.052	0.79	0.66	0.14	0.089	23.68
pmol JH-II/CA eq.	0.005	0.26	0.13	0.02	0.022	2.37
pmol JH-III-acid added	0	0	0	0	165	165
pmol ³H incorporated	< 0.001	< 0.001	< 0.001	< 0.001	0.007	0.628
pmol JH-III/CA eq.	nil	nil	nil	nil	0.002	0.063

[a] Animal equivalent; refers to CA pair.
[b] The $12\,000 \times g$ supernatant was used in experiments 2.2 and 2.4.

the observed incorporation of label reflects the presence of endogenous JH-acids as well as the presence of the methyltransferase [1, 14, 15]. The conversion of a selected number of alkenoic acids by homogenates of corpora allata from *Manduca sexta* was studied by Reibstein *et al.* [14]. The enzyme does alkylate JH-I-acid and JH-III-acid, but not oleic-, 2-hexadecenoic-, 9,10-epoxystearic-, or 9-keto-2-decenoic acid. The substrate specificity of the enzyme towards the homologous JH-acids and their enantiomers has not been investigated.

Incubation of CA homogenates from adult male and female *H. cecropia* with [³H]SAM (Table II) shows that production of JH from endogenous acids in homogenates of female organs is one to two orders of magnitude higher than in the corresponding male system. The difference between male and female CA becomes even more apparent when synthetic (racemic) acids are added to the homogenates (expt. 2.5 and 2.6). It may be calculated from the data in Exp. 2.6 that the preferred substrate is JH-I-acid (23.2% methylated) in favor of JH-II-acid (7.8%) and even more so of JH-III-acid (0.4% methylated). The enzyme system in female CA thus exhibits the same substrate specificity as does the methyltransferase in male ASG. The failure of adult male CA to methylate JH-acids efficiently is in marked contrast to the rather high amounts of juvenile hormones found in the animal. It appears as a possibility that JH-acids are biosynthesized but not methylated in the CA and that they are secreted in substantial quantities into the hemolymph or, *in vitro*, into the culture medium. In the overall biosynthesis scheme emerging, methylation would occur in the ASG only.

The methyltransferase in homogenates of JH-free ASG, either from 18−20 day old pharate adults or from 1−2 day old males that had been allatectomized as pupae, was used for the detection of JH-acids by the incorporation of label from [³H]SAM. Thirty-six pair of corpora allata-corpora cardiaca complexes from 1−2 day old adult male *H. cecropia* were homogenized in phosphate buffer and immediately extracted with ethyl acetate. After incubation of the evaporated extracts with ASG-homogenate and 10 µCi (1.33 nmol) [³H]SAM, analysis for radiolabeled JH was performed as usual. The results indicated the presence of 5.41 pmol JH-I-acid/eq., 2.11 pmol JH-II-acid/eq., and 0.015 pmol JH-III-acid/eq. Again, the calculations are based on the

assumption that the specific activities of the radiolabeled SAM and of the juvenile hormones formed are equal. In addition, no corrections for yield were made, and substrate specificity of the ASG-enzyme is not considered, thus the data represent minimum amounts.

Derivatization of the JH-I and JH-II from the experiment described above again demonstrated that the hormones had 10R, 11S-configuration: 91.7 ± 1.2% of the total radioactivity eluted with the faster moving (10R, 11R)-(+)-MTPA derivatives of the JH-I and JH-II. Earlier we have described the isolation of JH-I from CA cultures of male *H. cecropia* [16]. These results could not be reproduced, at least not under the culture conditions presently used: the CA show only marginal biosynthetic activity for JH-I and JH-II. They secrete, however, JH-acids into the culture medium (Table III). The occurrence of *de novo* biosynthesis of JH-acid was demonstrated by the incorporation of [1-¹⁴C]propionate and [2-¹⁴C]mevalonate. The medium was extracted with ethyl acetate after five days, unlabeled carrier JH and JH-acids were added to the extracts and separated by TLC. In all experiments, the JH-fraction was devoid of label (< 20 dpm). Conversion of the JH-acids into JH with diazomethane was followed by TLC and HPLC resolutions. With propionate as the precursor, the main product is JH-I-acid, whereas in the presence of mevalonate JH-II-acid predominates. JH-III-acid, if formed at all, constitutes less than 1.5% of the acid mixture. Again, the data in Table III represent minimum amounts of JH-acids biosynthesized, since the specific activity of the products and the yields of the chemical conversions were not determined. Proof of the absolute configuration by the usual procedure via (+)-MTPA-derivatives established the natural (10R, 11S)-configurations for JH-I-acid and JH-II-acid in Expt. 3.1

Addition of 1 mg/ml each of unlabeled mevalonate and homomevalonate to the culture medium increased the rates of biosynthesis of JH-I-acid and JH-II-acid by one order of magnitude. In this case, the acids were detected enzymatically by the procedure involving ASG-homogenate and [³H]SAM (specific activity 8.9 Ci/mmol) as described above. In duplicate experiments, the amounts of JH-I-acid found were 55.6 and 41.8 pmol/eq., and of JH-II acid 55.5 and 36.3 pmol/eq. From the observed difference between the amounts of JH-I- and JH-II-acid methylated (1.43 ± 0.14 times more JH-I-acid than

Table III. Incorporation of [1-¹⁴C]-propionate and [2-¹⁴C]mevalonate (2 µCi/ml medium each) into JH-acids produced *in vitro* by corpora allata from adult male *Cecropia*. The acids were analyzed as JH after reaction with diazomethane.

Experiment No.	3.1	3.2	3.3	3.4
Number of CC-CA [a]	7	6	4	4
Precursor	propionate [b]	propionate [b]	mevalonate [c]	mevalonate [c]
JH-I:				
pmol incorporated	79.7	47.8	11.2	11.2
pmol/pair	5.61	3.99	2.79	2.79
JH-II:				
pmol incorporated	32.2	18.1	31.3	30.3
pmol/pair	4.60	3.02	3.91	3.79
JH-III:				
pmol incorporated	< 0.2	0.51	< 0.96	1.13
pmol/pair	nil	0.09	< 0.08	0.09

[a] Pair of corpora cardiaca – corpora allata complexes
[b] Specific activity: 53 mCi/mmol.
[c] Specific activity: 10.9 mCi/mmol.

JH-II-acid) it can be calculated that the acids actually are biosynthesized in nearly equal amounts. As was described above (Table I), the methyltransferase preparation from ASG methylated selectively JH-I-acid in favor of JH-II-acid by a factor of 1.5 – 2. No JH-III-acid was formed (≤ 0.001 pmol/eq.). Considering the substrate specificity of the ASG-enzyme system, JH-III-acid, if present in amount of 1% of the JH-I-acid, would have been detected easily.

These results confirm the view that the biosynthesis of the homologous juvenile hormones is controlled by highly specific enzymes. Because of their ability to discriminate strongly between mevalonate and homomevalonate or their metabolites, which function as intermediates in JH-biosynthesis, it is possible to stimulate selectively JH-I and JH-II formation in cultured CA from *e. g. Manduca sexta* and *Heliothis virescens*. In analogous systems from *Periplaneta americana* and other orthopteran species, JH-III production is increased but JH-III remains the only hormone formed. The JH-acid biosynthesis in *Cecropia* CA *in vitro* shows the same type of specificity.

It is interesting to note that an enzyme system utilizing mevalonate for the biosynthesis of farnesol does occur in unidentified tissues outside the corpora allata in *Cecropia* [17, 18]. Under conditions where mevalonate was incorporated efficiently into farnesol, we have not detected radiolabeled homo- or bis-homofarnesol after administration of radiolabeled mevalonate or propionate. The design of the experiment allowed the detection of homologous structures, if present, in a quantity of about 1% that of the farnesol (Peter and Peter-Katalinić, unpublished).

Homoterpenoid compounds, biogenetically derived from propionate, have never been isolated from natural sources other than insects. It appears that the CA are able to synthesize homoterpenoids with enzyme systems showing a high degree of specificity. Earlier work had shown that the juvenile hormones of male *cecropia* are accumulated in the ASG and that the ASG possess an enzyme system for the conversion of JH-acids to JH. The present contribution elucidates the specificity of this enzyme with regard to homologous structures and chirality. It shows that male CA *in vitro* biosynthesize JH-acids and that the immediate cause for release of these acids is a lack of methyltransferase activity. The source of JH in ASG might have been JH-acid, generated by hydrolysis of natural JH in hemolymph or peripheral tissue. While lacking direct evidence that the *in vitro* properties of male CA are not experimental artifacts, we conclude that this is not the case and that the male CA release also *in vivo* JH-acid and not JH. How much the specificity of the methyltransferase in ASG contributes to the composition of JH stored versus that of the acids released is not clear. We are proceeding with our investigation of factors leading to the sex dimorphism of CA activity in *cecropia*.

Experimental

Radiolabeled substrates: *cf.* [7]. Sodium [1-^{14}C]-propionate (53 mCi/mmol), [2-^{14}C]mevalonolactone (10.9 mCi/mmol), and [methyl-^{3}H]-S-adenosyl-L-methionine ([^{3}H]SAM) (7.5–10 Ci/mmol) were purchased from Amersham-Searle; [7-ethyl-^{3}H]JH-I (11.8 Ci/mmol) was a product of NEN.

Preparation of JH-acids: Racemic JH-acids used as substrates in enzymatic reactions were prepared by the procedure of Weirich *et al.* [12]. Hemolymph was collected from 4 day-old *Manduca sexta* 5th instar larvae, centrifuged at $12000 \times g$ for 15 min at $0°$ and diluted with an equal volume of 0.16 M Tris-HCl buffer pH 7.7. Mixtures of synthetic juvenile hormones (20–40 µg, calibrated by GLC on 3% XE-60 and 3% EGSS-X) were incubated with 2 ml diluted hemolymph for 5 h at 27 °C in a shaking water bath. Extraction of the reaction mixture with ethyl acetate was followed by extraction of the combined ethyl acetate phases with 5% potassium carbonate solution. Adjustment to pH 4–5 with solid potassium dihydrogenphosphate preceded repeated extraction with ethyl acetate and final resolution by TLC (silicagel; hexane/25% ethyl acetate/5% acetic acid). The isolation procedure was monitored by internal standardization with 1.3 µg (119300 dpm) [7-ethyl-^{3}H]JH-I and finally by GLC-calibration of an aliquot in which the acids had been reconverted to juvenile hormones by an etheral solution of diazomethane. Overall yield: $62 \pm 4\%$. No discrimination between homologous structures was observed. The esterase does not discriminate between enantiomers, as was determined by the (+)-MTPA-procedure (see below). Racemic JH-acids, used as carrier compounds in incorporation experiments, were prepared by alkaline hydrolysis of juvenile hormone mixtures.

Isolation of radiolabeled juvenile hormone from incubation with ASG-enzyme system: The glands were dissected and after removal of fat body homogenized in an all glass Potter Elvejhem homogenizer in Grace medium. Aliquots of the homogenate were pipetted into conical centrifuge tubes containing the JH-acid sample to be analyzed. After mixing with 5–20 µl Tris-HCl buffer, [^{3}H]SAM was added (usu-

ally 5 µl in 0.001 N H_2SO_4) and the incubation left at ambient temperature for the time indicated. 5–20 µg unlabeled racemic JH-I, JH-II, and JH-III were added, the mixture extracted with ether and subjected to TLC (silicagel; benzene/5–15% ethyl acetate). Final analysis involved HPLC on a 0.4×30 or 0.4×60 cm µ-porasil column (Waters Assoc.) with hexane/2% ethyl acetate/0.02% 2-propanol. Individual fractions were assayed for radioactivity. If the amount of label present in the JH-zone from TLC exceeded 100000 dpm, an aliquot of this sample was diluted further with carrier JH before HPLC separation.

Isolation of JH and detection of JH-acids produced in organ culture: Unlabeled carrier JH and JH-acids were added to the extract of culture medium and resolved by TLC (silicagel; hexane/25% ethyl acetate/1% acetic acid). The juvenile hormone fraction was further resolved by TLC, followed by HPLC as described above. The JH acid fraction was processed by one of two different methods: either by transformation into JH with homogenates of ASG (see above) or, alternatively, by reaction with an excess of diazomethane in ether and subsequent TLC and HPLC analysis.

Preparation of (+)-MTPA derivatives [13]: JH-diols were prepared as described previously [7] and acylated with 10 µl (+)-MTPA chloride in 100 µl pyridine during 1–2 h at 23 °C. Evaporation of the reaction mixture was followed by extraction of the residue with ether and TLC (silicagel; benzene/15% ethyl acetate). Resolution of the diastereomers was performed by HPLC on a 0.4×60 cm µ-porasil column with hexane/8% ethyl acetate/0.05% 2-propanol.

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