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# THE JUVENILE HORMONES OF CECROPIA

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The corpora allata (CA) of Hyalophora cecropia larvae produce two juvenile hormones (JH), JH-I and JH-II, and maintain a stage specific titre of these two hormones. During metamorphosis, the CA of males apparently lose the ability to synthesize JH. The adult CA produce instead the corresponding epoxyacids which are converted to JH in the accessory sex glands (ASG) by a specific methyltransferase. The methyltransferase is first detectable between days 15 and 16 of adult development. This coincides with the period when ASG have become competent to methylate in vivo injected JH-I-acid and JH-II-acid and to accumulate the corresponding JH. During mating, the accumulated JH is transferred along with other seminal material to the bursa copulatrix of the female.

Both male and female adult Cecropia CA produce JH-acids in vitro but the amounts secreted by the male are two orders of magnitude greater. The concentrations of JH-acids in CA extracts differ in a similar manner. However, homogenates of female CA contain substantial methyltransferase activity (as for example do CA of adult Manduca sexta) unlike male CA which show only marginal activity. Whether or not the female CA secrete JH and/or JH-acid in vivo is not clear. In the presence of competent ASG, co-cultured with CA in vitro, the secreted JH-acids are transformed into JH. The prefered substrate for the methylating enzyme in the ASG is (10R,11S)-JH-I-acid, and to a lesser extent (10R,11S)-JH-II-acid. The enzyme strongly discriminates against JH-III-acid. The substantially higher biosynthetic capacity of male CA coupled with the unique ability of the ASG to methylate and store JH accounts for the more than two orders of magnitude higher JH content in the male as opposed to the female. The sexually dimorphic secretory activity of the corpora allata was found to be independent of the internal milieu during the course of metamorphosis.

One of the early experimental methods for determination of JH activity has been termination of pupal diapause in Cecropia by implantation of active CA or injection of juvenile hormone preparations. JH-I-acid also causes initiation of development in debrained, allatectomized diapausing pupae that results in formation of pupal-adult intermediates. Our data suggest that JH-acid has both prothoracicotropic and morphogenetic properties in Cecropia pupae.

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The study of juvenile hormones (JH) has a history extending over more than twenty years beginning with the observation of Williams (1956) that the male Cecropia moth is a rich source of JH. In spite of noteworthy contributions from many investigators during the intervening years the theme is still not exhausted and occasionally yields unexpected and quite remarkable results. Williams (1963) and also Schneiderman and Gilbert (1964) had shown that without corpora allata (CA) no JH is formed in the male, and since the CA themselves, upon implantation in receptive hosts, exerted high juvenile hormone activity, there were no reasons to doubt that JH is synthesized and released by the male CA. However, our recent findings that JH in male Cecropia was labeled with [methyl-$^{14}$C]-methionine even in absence of the CA led to a search for the specific tissues which store JH. The discovery that the JH of adult male Cecropia moths is almost entirely accumulated in their accessory sex glands (ASG) (Shirk et al., 1976) made it necessary to re-evaluate the roles of both the CA and the ASG in the accumulation of JH. In this review we will discuss the biosynthetic capabilities of these two organs, the way in which they are distinguished from those of related species, the contributions of the CA and the ASG for the formation and accumulation of JH in the male Cecropia moth, and finally the possible functional significance of this unique saturniid JH system.

## Relative Concentration and Identity of Juvenile Hormones

Biological assays have been the basis for estimation of JH concentration, most noteworthy the Galleria wax test either in its original form (Schneiderman et al., 1965), or in the modified version by DeWilde et al. (1968). Table 1 shows relative JH activities in developmental stages of Hyalophora cecropia. The standard for activity here used is the Cecropia Unit (CU) which is the activity of 1 mg of a standard Cecropia extract; later it was estimated to correspond to about 3 ng JH-I (or JH-II) (Meyer et al., 1970). In the developing male JH became detectable two days before emergence and reached after 7 days a total amount of 0.6 µg/animal. This value is in good agreement with our data. We often have isolated from adult males between 0.5 and 2 µg JH-I (occasionally more than 10 µg). JH concentrations in mature and immature stages are best compared on the basis of fresh weight: male adults 1 µg JH per g; eggs and newly hatched larvae 20 ng/g; and 5th instar larvae and diapausing pupae about one tenth of that concentration. The observation that eggs from allatectomized females had no detectable JH (with the implication that JH in eggs is derived from female CA) should be reinvestigated with modern chemical methods of JH detection.

Bioassays in our laboratories were concerned with the hormone titers in hemolymph of 3rd, 4th, and 5th instar larvae (Table 2) (Meyer and Lüscher, 1973). If the response in the wax test is elicited primarily by JH-I or JH-II (JH-0 and JH-III have much lower specific activities) one may expect to find in hemolymph of earlier instars

TABLE 1. RELATIVE JUVENILE HORMONE CONCENTRATIONS IN VARIOUS LIFE STAGES OF HYALOPHORA CECROPIA

| | CU/animal[a] | CU/g fresh weight |
|---|---|---|
| Unfertilized eggs | 0.04 | 8 |
| Unfertilized eggs from allatectomized females | 0 | 0 |
| 1st instar larvae (newly hatched) | 0.03 | 7 |
| 5th instar larvae | 4 | 0.6 |
| Diapausing pupae | 6 | 1 |
| Chilled pupae to 17 day-old developing adults | 0 | 0 |
| 20 day-old developing males | 25 | 14 |
| 22 day-old newly emerged males | 120 | 200 |
| 7 day-old adult males | 180 | 420 |

From Gilbert and Schneiderman, 1961. a) One Cecropia Unit (CU) is estimated to be equivalent to 3 ng JH-I or JH-II.

TABLE 2. JH-ACTIVITY IN HEMOLYMPH EXTRACTS OF CECROPIA LARVAE

| | | | | | |
|---|---|---|---|---|---|
| 3rd instar, | 0-1 day-old | < 900 | 5th instar, | 0-4 day-old | 2.5 |
| | 2-3 day-old | 1800 | | 5-8 day-old | 4.4 |
| | 4-5 day-old | 960 | | 9-13 day old | 0.8 |
| 4th instar, | 0-1 day-old | 180 | | | |
| | 2-3 day-old | 380 | | | |
| | 5-6 day-old | 92 | | | |

From Meyer and Lüscher, 1973. Results in Galleria Units (GU) per g Hemolymph. 1 GU is equivalent to 0.7-2 pg (±)-JH-I (or JH-II).

ng amounts of JH, which is comparable to concentrations determined in hemolymph of 4th instar Manduca sexta larvae by analytical chemical methods (Peter et al., 1976, Schooley et al., 1976). The JH concentration throughout the 5th instar appeared to be lower (Table 2) than peak values in early 5th instar M. sexta (see also Fain and Riddiford, 1975). First information about the nature of the JH of H. cecropia larvae comes from organ cultures of CA from 4th instar larvae. Under the stimulatory influence of mevalonate and homomevalonate in the culture medium JH-II and JH-I were produced in the ratio of 4:1 (Dahm et al., 1976). Analyzing haemolymph from various stages of Samia cynthia, a saturniid moth which has a similar peculiar JH system as

H. cecropia, Schooley et al., (1976) found in 4th instar larvae 80, 820 and 160 pg/ml JH-I, JH-II and JH-III, respectively, and <10, 120 and 30 pg/ml in 5th instar larvae. With our own analytical method we determined in a sample of early 4th instar larvae hemolymph of Cecropia 17 pg/ml JH-II (in view of the bioassay data an unexpectedly low value, JH-I and JH-III were in this experiment below the detection limit of about 3 pg/ml). In total body extracts from animals of the same stage and age 15 pg/eq. JH-I and 32 pg/eq. JH-II were found. The identification is quite reliable since in the final chromatographic analysis background peaks with retention times similar to JH-derivatives were not in evidence (Fig. 1). The quantity of lipid extract from this experiment (1.12g from 56 larvae) corresponds well with data compiled by Gilbert and Schneiderman (1961). However, the value for concentrations of JH determined in the various samples by analytical methods and by bioassay are quite disparate which indicates clearly the need for an improved quantitative method of JH analysis.

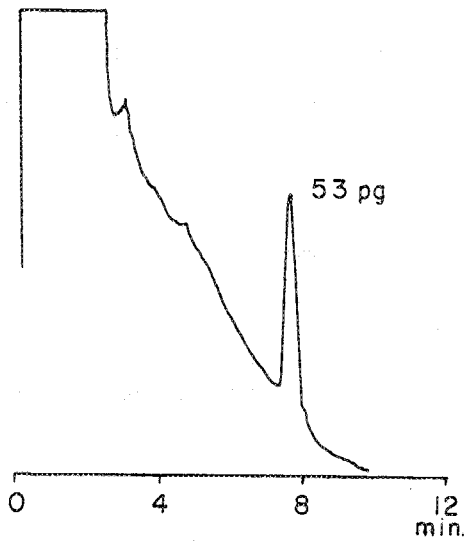



Fig. 1. GLC (ECD): JH-II derivative from total body extracts of 4th larval instar H. cecropia.

In recent experiments with CA cultures of Cecropia male and female 5th instar larvae we found no difference between the sexes and - without mevalonate/homomevalonate stimulation - again JH-II was the major product besides some JH-I. In preparations from Cecropia larvae we have not detected JH-III which makes the rather high concentration of JH-III in S. cynthia larvae mentioned above (Schooley et al., 1976) rather puzzling.

The identification of JH in larval stages of H. cecropia has yielded no unusual results. Next let us examine the situation in the adult moths. Males contain µg amounts of JH; JH-I and JH-II approximately in the ratio of 4:1 and, as most recently shown (Bergot et al., 1980), accompanied by about 1% JH-0 (Fig. 2). Sometimes traces of JH-III are found, particularly in in vitro experiments, but this hormone has not been identified unambigously as a component of the natural JH of Cecropia. Following the discovery of JH-II, we isolated the JH active principle from lipid extracts of male S. cynthia, a close relative of Cecropia, carefully monitoring each purification step with both the Tenebrio assay and the Galleria wax test, and found no indication for an active substance other than JH-I and JH-II (Röller and Dahm, 1974). The dimorphism in some

saturniid moths with regard to JH content has been known since Williams' work (1961). However, quantification and identification of JH from whole body extracts of saturniid moths has encountered some technical problems which are worth noting. Gilbert and Schneiderman (1961) had reported difficulties with extracts of female Cecropia. Waxy particles in the oily extract caused toxic effects in the standard injection test with chilled pupae of Antheraea or Callosamia. The particles could be removed by sedimentation. We find test results from crude extracts of low specific activity quite unreliable. It should be noted that female moths contain much less lipid than males. The differences in JH activity between males and females are obvious when expressed as either units/g extract or units/animal equivalent (Table 3, columns 2 and 3). However, only after simple purifications which removes the bulk of lipids the real quantitative difference in adult Cecropia becomes apparent (Table 3, column 1). Analysis of adult

TABLE 3. RELATIVE JUVENILE HORMONE CONCENTRATIONS IN EXTRACTS OF ADULT SATURNIDAE

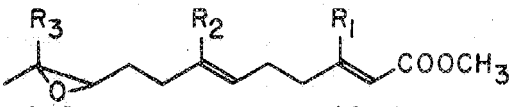
| | | X1000 GU/animal[a] | CU/animal[b] | CU/g extract[b] |
|---|---|---|---|---|
| Hyalophora cecropia, | males | 200 | 180 | 1000 |
| | females | 0.5 | 17 | 125 |
| Samia cynthia, | males | 180 | 45 | 670 |
| | females | 5 | 6 | 250 |
| Rothschildia orizaba, | males | 1 | 30 | 335 |
| Antheraea polyphemus, | males | 5 | 6 | 65 |
| | females | - | 5 | 50 |
| Attacus atlas, | males | - | 60 | 335 |
| | females | - | 15 | 145 |

a) D. Meyer et al., unpublished. One Galleria unit is equivalent to approximately 2 pg JH-I or JH-II. b) from Gilbert and Schneiderman, 1961. One Cecropia unit (CU) is estimated to be equivalent to 3 ng JH-I or JH-II. The activities in column 1 were determined from purified preparations, those in columns 2 and 3 from crude extracts.

Cecropia extracts by our ECD method (Peter et al., 1976) confirmed these bioassay results: 730 ng JH-I, 70 ng JH-II, possibly 7 ng JH-III for a male and 3.4 ng JH-I, 0.7 ng JH-II, and possibly 0.05 ng JH-III for a female. We had not been able to isolate JH from female Cecropia, female S. cynthia, and male Rothschildia orizaba with techniques which had been successful for male Cecropia. This result is in accordance with our own bioassays which indicated low biological activity in the partially purified extracts.

Paguia et al., (1976) have attempted to isolate JH from another saturniid moth, Attacus atlas (sex not specified). The specific activity of the crude extract (Galleria wax test) was one half that of an comparable extract of H. cecropia.

The main activity co-chromatographed with JH-III (not JH-I) during purification, but it was not found to be JH-III. The active principle has yet to be identified which is surprizing in view of the very high biological activity determined for the extracts.



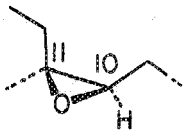

$R_1, R_2, R_3$: $-C_2H_5$ (±)-JH-0

$R_1$: $-CH_3$; $R_2, R_3$: $-C_2H_5$ (±)-JH-I

$R_1, R_2$: $-CH_3$; $R_3$: $-C_2H_5$ (±)-JH-II

$R_1, R_2, R_3$: $-CH_3$ (±)-JH-III

10R,11S




(+)-JH-I
(natural JH-I)

Fig. 2. Structures of Juvenile Hormones. The configuration of natural JH-II and JH-0 may be presumed to be identical with that of JH-I; JH-III has also 10R configuration.

## The Male Accessory Sex Glands

The genital tract of the male moth consists of testes, vas deferens, accessory sex glands (ASG), common duct, and ejaculatory duct. We now know that the JH in males of H. cecropia is concentrated almost exclusively in the ASG (Shirk et al., 1976) and also that this organ is able to introduce the label from methionine into the JH molecule.

When [2-$^{14}$C]-JH-I had been injected into intact moths, about 10% of it could be recovered from the total extract after 25 hr (Metzler et al., 1972). When [$^{14}$C-methoxy]-JH-I and [$^{14}$C-methoxy]-JH-III had been injected into intact or allatectomized (decapitated) males, 25% and about 1% of the label was recovered with JH-I and JH-III, respectively. The hormones, isolated from the total extract, also carried the label from simultaneously injected [$^{3}$H-methyl]-methionine. When µg-amounts of JH-I and JH-III were injected they were labeled in the ratio 10:1 in favor of JH-I (Dahm, et al., 1976). The exchange of the ester methyl group of JH against the labeled methyl group of methionine occurs only in presence of the ASG and is independent of the CA. Consequently it could be presumed that the conversion of JH-acid to JH, observed much earlier (Metzler et al., 1972) is also an accomplishment of the ASG and not of the CA. It is likely that the natural substrate of the ASG is the JH-acid and not JH since Weirich and Wren (1976) found that adult Cecropia have high esterase activity, but lack a carrier protein to protect JH (Weirich, un-

published). It is also possible that the pattern of hormones in the ASG does not resemble the pattern secreted by the CA because the ASG discriminated between JH-I-acid and JH-III-acid.

One of the last steps in the biosynthesis of JH is the formation of the methyl ester. Reibstein and Law (1973) have shown in adult female Manduca CA that S-adenosyl-methionine (SAM) is a substrate in the methyl transfer to JH-acid which is catalyzed by an enzyme in the glands. Weirich and Culver (1979) have characterized a similar enzyme (SAM-JH-Methyltransferase) which is found in the ASG but not in other tissues of the genital tract of Cecropia. The ASG exhibit high enzyme activity already 8 days before eclosion. This is the same time when ASG *in vivo* become competent to methylate injected JH-I-acid and to accumulate JH (Shirk, *et al.*, in preparation). Therefore, the onset of JH accumulation depends on activation of CA and availability of JH-acid. The methylating enzyme is not found in ASG of *Manduca sexta* and *Antheraea Pernyi*, moths which do not accumulate JH (Weirich and Culver, 1979).

The selectivity of the methyl transfer reaction with regard to JH-I-acid, JH-II-acid and JH-III-acid has been studied in *in vivo*, *in vitro*, and in homogenates of ASG (Table 4). Methylation with [$^3$H]-SAM should give JH of the same specific activity as the precursor and the yield may be calculated directly from the radioactivity of the products. The high percentage of conversion tends to mask differences

TABLE 4. RELATIVE SPECIFICITY OF JH-ACID METHYLATION BY MALE ACCESSORY SEX GLANDS (ASG) OF CECROPIA MOTHS

| | JH-I | JH-II | JH-III |
|---|---|---|---|
| ASG *in vivo* [a)] | 16,7000 dpm | 23,800 dpm | 1,600 dpm |
| ASG *in vitro* [b)] | 76,400 dpm | 6,400 dpm | nil |
| ASG homogenate [c)] | 41% | 27% | 7% |

ASG from moths, allatectomized as pupae, to ensure absence of endogenous JH. a) moth injected with 10 µg of each JH-acid and [methyl-$^{14}$C]-methionine. b) ASG cultured with 10 µg of each JH-acid and [methyl-$^{14}$C]-methionine. c) ASG-homogenate incubated with 184 pmol JH-I-acid, 250 pmol JH-II-acid, 192 pmol JH-III acid, and [methyl-$^3$H]-SAM (results represent % conversion). In all experiments the racemic JH-acids were used.

in reaction rates, but the preference of the enzyme for JH-I and JH-II over JH-III is clearly evident. The difference is most noticable in reaction with ASG *in vitro*. Isotopic dilution of the radiotracer is probably low and the data would indicate about 2% conversion of JH-I-acid and 0.2% conversion of JH-II-acid. It is remarkable that in this experiment no trace of labeled JH-III was recovered. In *in vivo*

experiments with intact animals we have previously observed isotopic dilution of the methionine precursor in the range of 1000 (Metzler et al., 1971) and conversion of JH-I-acid in the range of 10% (Metzler et al., 1972). The high yield of JH-II in comparison to JH-I is of interest. In this context it may be worth mentioning that in some experiments with ASG homogenates the E,E,trans-isomer of JH-0-acid had been included and that the yield of methylation was similar to that of JH-II-acid.

The stereochemistry of JH-I and JH-II produced from the racemic acids in ASG homogenates was determined through degradation to the diols, formation of the (+)-MPTA-derivatives, and HPLC, according to Nakanishi (1971). The hormones were found to have the natural 10R,11S-configuration (Peter et al., in preparation).

## The Corpus Allatum of the Adult Moth

Earlier we had isolated JH-I from cultures of CA of adult male H. cecropia in a medium containing pupal Cecropia blood (Röller and Dahm, 1970). Under conditions now generally used (see Dahm et al., 1976, and references therein) such CA did not produce JH and several attempts to stimulate the system by addition of pupal blood have also failed. After addition of 10 µg each of JH-I-acid, JH-II-acid, and JH-III-acid to the culture medium no significant amounts of the hormones could be recovered. In contrast CA of Periplaneta americana which normally produce only JH-III (see Dahm et al., 1976) are able to methylate all three acids without clear preference (Table 5). As discussed above, ASG under such conditions methylate

TABLE 5. INCORPORATION OF JH-ACIDS INTO JUVENILE HORMONES BY ADULT CORPORA ALLATA IN VITRO.

| Organ in vitro | dpm/day x gland pair | | |
|---|---|---|---|
| | JH-IA | JH-II | JH-III |
| CC-CA[a] of female Periplaneta americana | 8400 | 11400 | 14600 |
| CC-CA of male Hyalophora cecropia | 16 | 16 | 18 |
| ASG[b] of adult H. cecropia (allatectomized as pupae) | 76400 | 6400 | nil(<50) |

The culture medium contained radiolabeled methionine and 10 µg each of racemic JH-I-acid, JH-II-acid, and JH-III acid. a) CC-CA = corpora cardiaca-allata complex b) ASG = accessory sex gland

preferentially and efficiently JH-I-acid. These results support the hypothesis that the incorporation of JH-I-acid into JH-I in the intact male (Metzler, et al., 1972) is accomplished by the ASG and not by the CA. Further evidence to support this conclusion comes from experiments with CA-homogenates and labeled SAM (Table 6). Homogenates of CA from M. sexta produced all three hormones, confirming the results of Reibstein and Law (1973). CA of adult female P. americana and larval H. cecropia yielded the hormones identified for the respective species and developmental stage. CA of male Cecropia moths lack either JH-acids, the enzyme, or both.

TABLE 6. INCORPORATION OF LABEL FROM [methyl-$^3$H]-S-ADENOSYL-METHIONINE INTO JUVENILE HORMONES BY CORPUS ALLATUM HOMOGENATES

| CA of | | JH-I | JH-II | JH-III |
|---|---|---|---|---|
| 10 adult female M. sexta | dpm total | 750 | 52800 | 89500 |
| | pmol/eq | 0.005 | 0.317 | 0.538 |
| 5 adult female Periplaneta americana | dpm total | <5 | <5 | 1600 |
| | pmol/eq | - | - | 0.019 |
| 5 adult male H. cecropia | dpm total | <10 | <10 | <90 |
| | pmol/eq | - | - | - |
| 5 4th instar larvae of H. cecropia | dpm total | 170 | 90 | <5 |
| | pmol/eq | 0.002 | 0.001 | - |

Each incubation contained: 5 µl 0.1 M phospate buffer pH 7.6; 5 µCi[$^3$H]-SAM (7.5 Ci/mol) in 5 µl 0.001 N $H_2SO_4$, and the CA homogenate in 10 µl Grace's medium. Incubation time 5-6 hr.

The lack of SAM-JH-acid methyltransferase is evident from an experiment where JH-acids were supplied (Table 7). As in experiments with whole glands in vitro, the adult male CA are unable to incorporate the JH-acids. Homogenates of the female gland, however, accomplish the conversion to JH efficiently and show on the basis of percent conversion a preference for JH-I. Since ASG are a rich source of the methylating enzyme, we utilize glands from adult males, allatectomized as pupae, for quantification of JH-acids. The shortcoming of the method is the low sensitivity for JH-III-acid. It is still preferable to the alternative method, namely esterification with diazomethane which gave, because of the small amounts involved, rather uncertain yields. From adult male CA-homogenates, incubated with ASG-homogenates and [$^3$H]-SAM, we isolated 5 pg JH-I and 2 pg JH-II per CA-pair. Female CA contained about 2 orders of magnitude smaller amounts of JH-acid, predominantly JH-II-acid. Upon culturing

TABLE 7. INCORPORATION OF JH-ACIDS INTO JH IN CORPUS ALLATUM HOMOGENATES FROM ADULT CECROPIA

| CA-homogenates of: | | JH-I | JH-II | JH-III |
|---|---|---|---|---|
| 4 male | dpm total | 1780 | 1750 | 130 |
| | pmol/eq | 0.020 | 0.020 | 0.002 |
| 10 female | dpm total | 400000 | 464000 | 12300 |
| | pmol/eq | 1.80 | 2.10 | 0.06 |

The incubation mixture contained 88 pmole JH-I-acid, 304 pmole JH-II-acid, 165 pmole JH-III-acid, and [methyl-$^3$H]-S-adenosyl-methionine.

the CA of adult male Cecropia release the acids into the medium and de novo biosynthesis, under in vitro conditions, could be demonstrated by incorporation of labeled mevalonate and propionate. As is the JH biosynthesis in CA of other species, the production of JH-acids is greatly stimulated by addition of mevalonate and homomevalonate to the medium (Table 8). The biosynthetic capacities of adult male

TABLE 8. STIMULATION OF JH-ACID BIOSYNTHESIS IN CORPORA ALLATA IN VITRO FROM ADULT MALE CECROPIA MOTHS

| JH-acids/[$^3$H]-SAM | Expt. #1 | Expt. #2 |
|---|---|---|
| JH-I (dpm) | 2 196 000 | 1 649 000 |
| pmol/eq. | 55.6 | 41.8 |
| JH-II (dpm) | 2 192 000 | 1 432 000 |
| pmol/eq. | 55.5 | 36.3 |
| JH-III (dpm) | 20 | 20 |
| pmol/eq. | nil | nil |

The medium contained 1 mg/ml each of mevalonate and homomevalonate. Analysis of acids by conversion to JH with homogenate of male accessory sex glands of moth allatectomized as pupae and [$^3$H]-SAM.

Cecropia CA and ASC when cultured either separately or together are shown in Table 9. The system very likely reflects the normal in vivo events. In this experiment only

JH-I and JH-I-acid were determined. The compounds (after conversion of the acid to JH) were identified by HPLC through co-chromatography of the labeled products with unlabeled carrier. ASG cultured alone did not produce JH or JH-acid. CA produce and release JH-acid which in co-culture of CA and ASG is methylated and stored. It must be mentioned here that female CA, in spite of their content of methylating enzyme, in organ culture did not produce JH but JH-acid, albeit in much smaller amounts than the male gland. Ajami (1974) cultured brain-CC-CA complexes of female

TABLE 9. PRODUCTION OF JH-I-ACID BY CORPORA ALLATA (CA) AND ACCESSORY SEX GLAND (ASG) OF ADULT MALE HYALOPHORA CECROPIA IN VITRO.

| Organ(s) in vitro | Extract of | JH-I (pmol) | JH-I-acid (pmol) |
|---|---|---|---|
| 3 pair CA | medium | -- | 1.75 |
| | CA | -- | 0.04 |
| 3 pair CA + 1 ASG | medium | 0.35 | 0.06 |
| | CA | -- | -- |
| 1 ASG | medium | -- | -- |
| | ASG | -- | -- |

JH was detected through labeling with [methyl-$^{3}$H]-methionine in the culture medium; JH-acid was separated and identified after conversion to JH with [$^{3}$H]-SAM and ASG-homogenate. All ASG in this experiment were from moths which had been allatectomized as pupae.

Cecropia and reported high yields of label from [methyl-$^{14}$C]-methionine in JH-I. He notes, however, difficulties with the reproducibility of his results which, he suspects, are due to imperfect surgical techniques. It is possible that the release of JH-acid by female CA in vitro is an experimental artifact rather than the indication of the in vivo process.

Improvements in analytical methods for JH acids allowed us to examine the products of CA cultures more precisely than was formerly possible. In the experiments described in Table 10 we extracted the culture media (except experiment VII where the ASG was included), added carrier and separated the hormone and acid fractions by TLC. The hormones were resolved by HPLC. Experiments I and II show that determination of JH synthesis in CA cultures by TLC alone may not always be reliable. The acids were converted by methylation with diazomethane to the hormones, purified by TLC and resolved by HPLC. For analysis of JH and JH-acid production a number of radiolabeled

TABLE 10. JH AND JH-ACIDS PRODUCED BY CORPORA ALLATA (CA) IN VITRO FROM ADULTS OF VARIOUS SPECIES.

| Experiment: | I | II | III | IV | V | VI | VII | |
|---|---|---|---|---|---|---|---|---|
| CA-Culture: | ♂H.c. | ♂H.c. | ♀M.s. | ♀A.p. | ♂P.a. | ♂H.c. ♀P.a. | ♂H.c. +ASG | |
| Precursor | A | B | B | C | B | D | E[$^{3}$H] | + C[$^{14}$C] |
| TLC JH (dpm) | 175 | 220 | 11,600 | 17,350 | 3,330 | 68,220 | 4,130 | 2,720 |
| HPLC JH (dpm) | | | | | | | | |
| JH-I | <10 | <10 | 83 | 3,060 | <10 | 6,550 | 2,330 | 1,210 |
| JH-II | <10 | <10 | 4,060 | 9,570 | <10 | 2,860 | 830 | 400 |
| JH-III | <10 | <10 | 99 | (50) | 1,200 | 29,560 | <10 | <10 |
| TLC JH-Acid + $CH_2N_2$ (dpm) | 6,610 | 6,610 | 2,680 | 1,480 | 113 | 4,670 | 130 | 2,080 |
| HPLC (dpm) | | | | | | | | |
| JH-I | 2,920 | 1,990 | <10 | 146 | <10 | (nil) | (140) | 399 |
| JH-II | 1,220 | 1,070 | 570 | 570 | (40) | (nil) | <10 | 739 |
| JH-III | (41) | (172) | 30 | <10 | (98) | <10 | <10 | <10 |

Two to four CA were cultured for 5 days in Grace's medium with radiolabeled precursors for JH-biosynthesis. A: [3-$^{14}$C]-hydroxymethylglutaric acid; B: [$^{3}$H]-methylmalonic acid; C: [1-$^{14}$C]-propionate; D: [methyl-$^{14}$C]-methionine + unlabeled mevalonate and homomevalonate; E: [methyl-$^{3}$H]-methionine. The different hormones are not equally labeled by each precursor. Values in parantheses are of doubtful significance. H.c., H. cecropia; M.S., M. sexta; A.p., A. pernyi; P.a., P. americana.

precursors were used, some of which have not been used in CA cultures before. These precursors do not confer the label equally well to the different hormones (e.g. [1-$^{14}$C]-propionate can label JH-III only by tortuous metabolic pathways) which has to be taken in consideration for the interpretation of the results. CA of adult male Cecropia produce from hydroxymethylglutaric acid or methylmalonic acid only JH-I-acid and JH-II-acid, the hormones themselves were not formed (Experiment I and II). CA of M. sexta, always a useful reference standard, incorporate methylmalonic acid in JH-II but release a fair amount of JH-II-acid into the medium (Experiment III). CA of female A. pernyi (the male of this saturniid species has been mentioned above as not accumulating JH; also, its ASG is devoid of methyltransferase activity) are vigorous producers of JH-II and JH-I (Experiment IV); the ratio of JH to JH-acid is similar to that in Experiment III. Methylmalonic

acid cannot be incorporated into JH-III as a precursor without prior degradation to acetate. Its application should emphasize the labeling of JH-I and JH-II, and our preparation of this compound indeed shows this effect (Experiment III) when the known pattern of JH-biosynthesis by CA of M. sexta is taken in consideration. Even under these conditions the only product of CA from P. americana is JH-III (Experiment V). When CA of P. americana are co-cultured with CA of male H. cecropia, the JH-acids secreted by the CA of the moth are methylated by the enzyme of P. americana (Experiment VI). In this experiment only [methyl-$^{14}$C]-methionine was used as radioactive precursor, which leaves the acid fraction unlabeled. Experiment VII complements the results described in Table 9. Here we used double label, [1-$^{14}$C]-propionate for both JH-acid and JH (excluding JH-III and JH-III-acid), and [methyl-$^{3}$H]-methionine for JH (including JH-III). The ASG are able to convert the major portion of JH-acids produced by CA to the hormones, noticable again is the preference for JH-I-acid.

The ability of the ASG to concentrate and store JH explains to a large extent the difference in JH content between male and female Cecropia moths. The other factor contributing to the difference is the biosynthetic capacity of the respective CA. This is seen in earlier experiments in which CA were implanted into allatectomized males. Under these conditions the male CA produced 10 times more JH than the female CA (Dahm et al., 1976). Another difference noted in these experiments was that the female CA produced JH-II as the major endocrine product.

A more detailed study of the development of the sexual dimorphism in CA activity was carried out by cross-transplantation experiments involving CA and Herold's organ (Anlage) for ASG. The results indicate that the development of the sex-specific biosynthetic pattern is independent of the internal milieu during metamorphosis (Shirk, Bhaskaran and Röller, in preparation).

## Function of Juvenile Hormone in Adults

The two preceeding sections have revealed an elaborate system of JH biosynthesis in the male Cecropia moth: production and release of the JH-acids in the CA, sequestration by the ASG, methylation by a rather specific enzyme, and storage in the lumen of the gland. During mating the hormones are transfered to the bursa copulatrix from which it can now be isolated (Shirk et al., 1980). When labeled methionine is injected in vivo, large amounts of radioactive JH accumulate in the ASG (10000 to 100000 dpm). Most of this JH is transfered during copulation and nothing or little remains in the ASG (Table 11). In the bursa copulatrix JH persisted even two days after copulation, but disappeared completely after four days. The eggs contained a high quantity of radiolabel which, however, was not associated with juvenile hormones. Whether or not this radiolabel is derived from juvenile

hormones remains to be investigated since a major portion, but certainly not all, of the radiolabel transfered during mating is incorporated in JH.

The transfer of JH to the female would lead to the suspicion that it has a regulatory function either in the female or in the progeny. Secretions of the ASG do not seem to be involved in the change from virgin to mated behavior of female Cecropia (Riddiford and Ashenhurst, 1973). Williams (1959) had already reported

TABLE 11. DISTRIBUTION OF JUVENILE HORMONE AFTER MATING OF CECROPIA FEMALES WITH MALES CONTAINING RADIOLABELED JUVENILE HORMONE.

| Preparation[a)] | Crude oil (dpm) | JH-I (dpm) | JH-II (dpm) | Time after copulation |
|---|---|---|---|---|
| Male ASG[b)] | | nil | nil | 0 |
| Female BC[b)] | | 9,600 | 1,600 | 0 |
| Male ASG[b)] | | 1,000 | nil | 0 |
| Female BC[b)] | | 10,100 | 2,500 | 2 days |
| Eggs | 83,700 | nil | nil | |
| Male ASG | | 3,250 | 450 | 0 |
| Female BC | | nil | nil | 4 days |
| Eggs | 19,500 | nil | nil | |

The JH in the male had been labeled by injection of labeled methionine. BC: bursa copulatrix; ASG: accessory sex gland; a) each set represents one mating pair; b) data from Shirk et al., 1980.

that allatectomy in the pupal stage had no apparent effect on the activities of the adult or the development of the next generation. We repeated the experiments and observed the next generation through pupation but did not discover any abnormalities. Therefore, the raison d'être of the accumulated JH remains unknown.

## Experiments with JH-I-Acid

Since our observations suggest that adult male Cecropia CA secrete JH acids, the profound endocrine effects of such CA after implantation in brainless diapausing pupae (Williams, 1959) are surprizing. Consequently we compared the effects of JH-I and JH-I-acid on Dauerpupae of Cecropia (Bhaskaran et al., 1980). The biological

activities of the two compounds were practically identical (Table 12). The doses applied were rather high but not unreasonably so; Williams obtained a 25% response with three implanted CA and we know that an adult male CA may produce several µg JH-acid within days. Since there is no difference between the activities of JH and JH-acid, it seems very unlikely that the JH-acid is methylated somewhere in

TABLE 12. EFFECT OF JH-I AND JH-I-ACID ON BRAINLESS DIAPAUSING CECROPIA PUPAE

| JH-I (µg) | 1 | 10 | 25 | 50 | 100 |
|---|---|---|---|---|---|
| animals developed | 20% | 25% | 40% | 50% | 90% |
| average score[a)] | 0.0 | 3.0 | 3.1 | 4.3 | 4.1 |
| JH-I-acid (µg) | 1 | 10 | 25 | 50 | 100 |
| animals developed | 15% | 20% | 15% | 55% | 80% |
| average score[a)] | 1.5 | 1.7 | 3.0 | 3.4 | 4.6 |

a) Data from Bhaskaran et al., 1980. Scoring of juvenilizing effects according to Williams, 1961.

the peripheral tissue and redistributed as JH to the target organs. We cannot exclude, however, that conversion occurs in the target tissue itself, since this would probably involve only minute fractions of the total amount injected. This possibility is under investigation. We are also looking for other phenomena in insect development which may be based on action of JH-acids rather than JH.

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