The juvenile hormones / edited by Lawrence I. Gilbert. -

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ON THE IDENTITY OF THE JUVENILE HORMONE IN INSECTS

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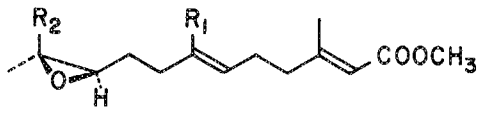
INTRODUCTION

The secretion of the corpora allata, the juvenile hormone (JH), has a morphogenetic function during the larval life of an insect and may act as a gonadotropin in the adult. On the basis of transplantation experiments, it had been concluded that the hormone of the corpora allata is stage and order unspecific (Wigglesworth, 1970; Novák, 1966). Consequently, the name "juvenile hormone" was retained irrespective of its endocrinological function during a specific postembryonic stage. Piepho (1950) even had suggested that the juvenile hormones of all insects be chemically related, if not identical.

During the past eight years three natural compounds, claimed to be juvenile hormones, have been isolated from insect sources. In order of their historical appearance, we will call them JH I (1, Fig. 1) (Röller et al., 1967; Dahm et al., 1967), JH II (2) (Meyer et al., 1968), and JH III (3) (Judy et al., 1973a). Numerous experiments have demonstrated that these compounds can indeed be produced by corpora allata, and there is extensive literature about their biological activity in various life stages and different orders of insects. In view of this, it is surprising that at the present time we cannot say with certainty which of the three is the morphogenetic hormone of any insect species. With a single exception (Lanzrein et al., 1975), all identifications are based on work with adult insects or their corpora allata in vitro. Consequently, they yield at best information about the gonadotropic hormone but not necessarily about the morphogenetic hormone. Our present dilemma in identifying the hormone of a specific species and postembryonic stage has its roots in the finding that the three



1	$R_1 = R_2 = -C_2H_5$	JH-I
2	$R_1 = -CH_3$, $R_2 = -C_2H_5$	JH-II
3	$R_1 = R_2 = -CH_3$	JH-III

Fig. 1. Structures of the known juvenile hormones. In all experiments with synthetic hormones, the racemates were used.

hormones, as predicted, are closely related chemically, but that contrary to earlier assumptions they can be readily distinguished by their physiological activities.

In an extension of an experiment reported earlier (Röller and Dahm, 1968), we injected various amounts of JH I, JH II and JH III, diluted in olive oil, into full-grown eye-class 1 larvae of Galleria mellonella (scoring according to Piepho, 1938). A dose of 1 µg JH I caused more than 50% of the injected larvae to molt into larval-like intermediates. Ten µg JH II were less effective; and after application of 100 µg JH III most animals finally molted into normal pupae. Obviously JH III, even in a most unphysiological dose of 100 µg, is not able to mimic the effect of three brain-corpora cardiaca - corpora allata complexes transplanted from third or fourth instar larvae into full-grown larvae (wandering last larval instar) as reported by Piepho (1942). For the evaluation of JH activity, the Tenebrio assay (Karlson and Nachtigall, see Röller and Dahm, 1968; Bjerke and Röller, 1974) and the Galleria wax test (de Wilde et al., 1968) have been used quite frequently. Both tests do not involve transportation of the applied material by the circulatory system; it is deposited close to the target tissue which it reaches by diffusion. Complications owing to JH-degrading enzymes in the hemolymph should, therefore, be absent from these test systems. Nevertheless, in the Galleria wax test the specific activity of JH III is lower than that of JH I or JH II by a factor of 100. In the Tenebrio assay the difference is even larger, the specific activities of JH I and JH III are separated by four orders of magnitude (Table 1). The latter result is particularly puzzling since JH III is the only hormone that could be isolated from extracts of reproductive adults of this species (Trautmann et al., 1974a) or from in vitro cultures of their corpora allata (Judy et al., 1975). A plausible, but not necessarily correct explanation of the apparent discrepancy between occurrence and morphogenetic activity of JH III would be the assumption that at least some insect species use different JH homologs as morphogenetic and gonadotropic hormones.

TABLE 1. SPECIFIC ACTIVITIES OF RACEMIC JUVENILE HORMONES IN THE GALLERIA WAX TEST AND IN THE TENEBRIO ASSAY.

	GU/µg	TU/µg
JH I	200×10^3	800
JH II	200×10^3	30
JH III	2×10^3	0.05

GU = Galleria Unit; amount required to elicit a positive response in 50% of the animals scored. TU = Tenebrio Unit; amount required to elicit a positive response in 40% of the treated pupae.

With one exception (Lanzrein et al., 1975), JH I and JH II have been identified only in lepidopterous insects (Röller et al., 1967; Meyer et al., 1968; Dahm and Röller, 1970; Röller and Dahm, 1974) or in cultures of their corpora allata (Roller and Dahm, 1970; Judy et al., 1973a; Jennings et al., 1975a). JH III alone was found in orthopteran, coleopteran and a hymenopteran species (Trautmann et al., 1974a,b) and also in cultures of orthopteran and coleopteran corpora allata (Judy et al., 1973b, 1975; Müller et al., 1974). Disregarding all other evidence, these findings might suggest that the original JH of insects is JH III, and that JH I and JH II are special evolutionary achievements in Lepidoptera and possibly some other insect orders.

It should be possible to test the two hypotheses outlined above by transplantation experiments. Corpora allata producing exclusively JH III should not be able to replace the original gland in an insect where only JH I or JH II show appreciable morphogenetic activity. The reverse experiment would yield no information since, as far as known, the biological activities of JH I or JH II are always equal or greater than that of JH III. Examination of the literature from this point of view reveals that within the Lepidoptera, the stage unspecificity of the corpora allata secretion is well documented (Piepho, 1942; Williams, 1959; Fukuda, 1962, 1963).

In a series of classical experiments demonstrating order unspecificity, Piepho (1950) implanted ten corpora allata with attached corpora cardiaca of Tenebrio larvae or four corpora allata of adult Carausius morosus Br. (Orthoptera, Phasmida) into full grown Galleria larvae and observed the same effects as after

transplantation of corpora allata from larvae of Achroia grisella (Fabr.), Anagasta kuehniella (Zeller), and Bombyx mori (L.). However, the juvenilizing action of these glands was weak and not comparable to that of implanted corpora allata from Galleria larvae (Piepho, 1942). Inhibition of metamorphosis was restricted to the regenerating epidermis at the implantation site which is particularly sensitive to JH. The observed effects might have been easily induced by JH III. A striking experiment, on the other hand, has been reported by Yashika (1960). He implanted ten brain - corpora allata complexes of adult Ctenolepisma villosa (Apterygota) into diapausing pupae of H. cecropia from which the brain with its corpora cardiaca and corpora allata had been extirpated within five hours after pupation. Seven out of ten pupae experienced a second pupal molt while the other three developed into, what Yashika called, "imperfect adults". All 14 controls, implanted with brain only, developed into normal adults. In view of the relative specific activities of the three JH's in other lepidopterous insects, it is very doubtful whether JH III producing corpora allata would cause the effects observed by Yashika.

Since interpretation of published results does not lead to an unambiguous identification of the morphogenetic corpus allatum hormone, it is necessary to reconsider the nature of the evidence essential for identification of a chemical entity with the physiologically defined hormone. It appears that three criteria must be met to identify the JH chemically without any ambiguity. First, the compound in question must be produced by the corpora allata. Second, its titer in the circulatory system must rise and fall in synchrony with the processes controlled by the hormone. Third, when under appropriate experimental conditions the natural hormone has been deleted from the animal, the artificially supplied chemical must be able to substitute fully for the authentic hormone.

When it could be assumed that the juvenile hormone in all insects is identical, results of experiments with various species could be generalized for interpretation. Since the concept of the single hormone is challenged, it is necessary that all the criteria are met experimentally in one defined stage of a specific insect species. Considerations and experiments concerning the origin, titer, and function of JH are presented in the following.

RESULTS AND DISCUSSION

Juvenile Hormones Produced by Corpora Allata In Vitro

The identification of methyl 10,11-epoxy-farnesoate as a JH (JH III) is based mainly on the finding that it is produced by adult corpora allata of several species under in vitro conditions.

The isolation of small amounts of this compound from total extracts of plant feeding insects would not have, without prejudice, led to the conclusion that it functions as a hormone in the respective species. An important piece of evidence missing in these investigations is the demonstration that JH III, while present in some developmental stages, is absent in others where its absence can be postulated on the basis of general physiological considerations. With regard to the in vitro systems, it is quietly assumed that corpora allata under such artificial conditions produce the same endocrine products as in vivo. We cannot yet explain why corpora allata of Manduca sexta in vitro incorporate mevalonate into JH II and JH III (Schooley et al., 1973) while H. cecropia seem to be unable to use this compound as a precursor for JH I and JH II (Metzler et al., 1971). During our work on the biosynthesis of JH in Cecropia, we made another observation which might be relevant to the question of possible differences in the biosynthetic activity of corpora allata in vivo or in vitro. Male moths usually are vigorous to an age of about five days; thereafter they deteriorate rapidly. They normally contain besides JH I 10 to 30% JH II. However, very old males which had been injected with [S-methyl-^{3}H]-methionine and incubated for a few days contained more labeled JH II than JH I (Fig. 2). It is reasonable to assume that the aging moths fail to synthesize enough of the special precursor for the ethyl side chains of the hormones, in consequence of which the corpora allata produce relatively more JH II. With this consideration in mind we re-examined corpora allata of female M. sexta under in vitro conditions (Judy et al., 1973a).* JH III was the major product, but the time course of JH-production proved to be interesting (Table 2). During the first days after explantation, the glands release more JH II than JH III into the medium, but JH II synthesis ceases soon while the rate of JH III synthesis initially increases. The result seemed to indicate that a specific precursor for JH II and possibly JH I is in short supply in the in vitro system. Since the studies of the Zoecon group (Schooley et al., 1973; Jennings et al., 1975b) as well as our own investigations (Gowal et al., 1975; Peter and Dahm, 1975) had indicated that in vitro as well as in vivo the

* In this and all other in vitro experiments we used as the basic culture medium Grace's insect tissue culture medium without methionine (GIBCO, Grand Island Biol. Co., NY), supplemented with 1% bovine albumin (Fraction V from bovine plasma, Metrix, Div. of Armour Pharmaceutical Co., Chicago, IL) (Judy et al., 1973a). In most experiments (S-methyl-^{14}C)-methionine (spec. activities in the range between 47 and 58 Ci/mol) was added in order to label the hormones. After incubation, the media were extracted several times with ethyl acetate, at which point depending on the experiment unlabeled JH's were added as carriers. The hormones were purified by TLC on methanol-washed Silica Gel HF-254 (E. Merck, Darmstadt, Germany) with benzene/5% ethyl acetate and resolved by HPLC-II (see Table 10).

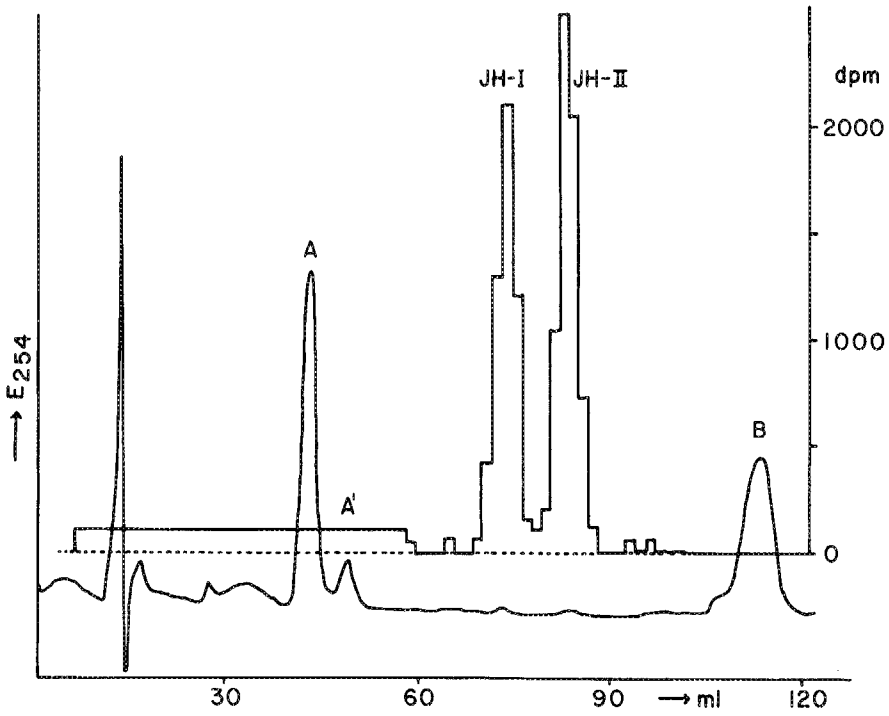


Fig. 2. Juvenile hormones from a 9 day-old male Cecropia moth. The moth had been injected on day 6 with 50 µCi [S-methyl-^{3}H]-methionine. After the usual purification procedure (see Table 10), the hormones were separated on an 0.23 x 300 cm Porasil T (Waters Associates, Inc., Milford, MA) column with hexane/2.8% ethyl acetate/ 0.04% 2-propanol. The peaks A, A', and B on the UV-trace are those of reference compounds: A and B are 0.4 µg each of nonanal- and acetone 2,4-dinitrophenylhydrazone, A' is an impurity in A. The preparation contained 1.0 µg (5200 dpm) JH I, 1.2 µg (6400 dpm) JH II, and no JH III (GLC-analysis and liquid scintillation counting).

ethyl side chains of JH I and JH II are introduced through incorporation of 3'-homomevalonate or its biochemical equivalent, it seemed logical to supply the corpora allata cultures with this intermediate. Addition of 1 mg homomevalonolactone (synthesized in our laboratories) per ml medium stimulated JH II biosynthesis considerably, and JH I was produced in amounts comparable to those of JH III. Even when equal amounts of homomevalonolactone and of mevalonolactone were added, JH II synthesis was stimulated much more than that of JH III; also, JH II was the major product as long as the glands remained active (Table 3). A similar effect was observed when corpora allata of female adult Heliothis virescens (Fabr.) were kept under the same conditions. These glands have

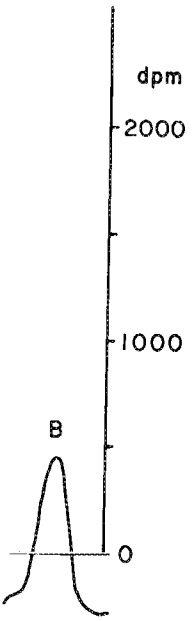


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TABLE 2. PRODUCTION OF JUVENILE HORMONES BY CORPORA ALLATA OF FEMALE ADULT MANDUCA SEXTA IN VITRO.

Day in culture	Experiment I [dpm/day x gland pair] JH II	JH III	Experiment II [dpm/day x gland pair] JH II	JH III
1st - 2nd	770	630	1,300	970
3rd - 4th	300	590	480	1,280
5th - 6th	70	1,470		
7th - 12th	∿2	∿2		
13th - 16th	nil	nil		

The culture medium contained 2 µCi [S-methyl-^{14}C]-methionine per ml.

TABLE 3. PRODUCTION OF JUVENILE HORMONES BY CORPORA ALLATA OF FEMALE ADULT MANDUCA SEXTA IN VITRO.

Day in culture	[dpm/day x gland pair] JH I	JH II	JH III
1st - 4th	220	7,300	2,550
5th - 8th	nil	690	34
9th - 12th	nil	20	6

The culture medium contained 2 µCi [S-methyl-^{14}C]-methionine, 1 mg mevalonate, and 1 mg homomevalonate per ml.

been reported to produce in vitro JH I and JH II (Jennings et al., 1975a); in our system they also release JH III (Table 4). Addition of the mevalono- plus homomevalonolactone to the medium had little effect on the production of JH III, but increased the synthesis rate of JH II four-fold and of JH I seven-fold.

TABLE 4. PRODUCTION OF JUVENILE HORMONES BY CORPORA ALLATA OF FEMALE ADULT HELIOTHIS VIRESCENS IN VITRO.

Experiment I				Experiment II: mv + hmv			
Day in culture	[dpm/day x gland pair] JH I	JH II	JH III	Day in culture	[dpm/day x gland pair] JH I	JH II	JH III
1st - 4th	76	530	300	1st - 3rd	550	2,180	450
5th - 8th	66	480	190	4th - 6th	510	2,080	300

All culture media contained 2 µCi [S-methyl-^{14}C]-methionine per ml. mv + hmv: the medium contained 1 mg mevalonate and 1 mg homomevalonate per ml.

Koyama et al. (1973) have shown that pig liver farnesyl pyrophosphate synthetase can utilize the normal isoprenyl pyrophosphates and their C_6-homologs rather indiscriminately to produce farnesyl pyrophosphate homologs. In contrast, the corpora allata system seems to be very specific. Under the experimental conditions, JH II would probably not have been distinguished from its 10'-desmethyl-7'-methyl isomer, but all JH variants with an ethyl instead of a methyl group at C-3 would have been easily detected. Cum grano salis we come to the conclusion that even in the presence of homomevalonolactone the corpora allata cannot be forced to the biosynthesis of a host of sesquiterpene-like compounds, but that they produce only those structures for which they are naturally programmed.

This appears to be confirmed by results of in vitro experiments in which the corpora allata produce exclusively JH III. Corpora allata of adult Periplaneta americana released 10 ng JH III per day and gland pair during the first few days after explantation. The specific incorporation ratio of label from [S-methyl-^{14}C]-methionine and 2-[^{14}C]-mevalonolactone is between 10 and 100%, of 1,2-[^{14}C] acetate about 10%, and of 1-[^{14}C]-propionate about 1% (Table 5).

TABLE 5. PRODUCTION OF JH III BY CORPORA ALLATA OF MALE PERIPLANETA AMERICANA IN VITRO.

Precursor [2 µCi/ml medium]	[µCi/µmol]	JH III [dpm/day x gland pair]
[S-methyl-^{14}C]-methionine	58	5,000
2-[^{14}C]-mevalonolactone	11	1,900
1,2-[^{14}C]-acetate	54	3,300
1-[^{14}C]-propionate	53	260

The culture medium was supplemented with 50 µg (unlabeled) methionine per ml.

Propionate should not be incorporated directly (Judy et al., 1973a; Peter and Dahm, 1975) but only after degradation to smaller fragments. Corpora allata of males and females release similar amounts of JH III except when females bear ootheca ready for deposition (Table 6). Addition of mevalonolactone increases the JH III yield about ten-fold while homomevalonolactone has no effect. With the exception of JH III, no trace of a compound with JH-structure was ever detected; the detection limit in some experiments was less than 0.1% JH III.

The same selectivity for incorporation of mevalonate and rejection of homomevalonate was found in adult corpora allata cultures of Blaberus discoidalis (Serville) (Table 7), P. fuliginosa (Serville), T. molitor L., and diapausing and non-diapausing beetles of Leptinotarsa decemlineata (Say). After incubation, the cultures contained only JH III. When the time course of JH production was determined, the corpora allata of all investigated species decreased JH synthesis drastically within the first two weeks in spite of the fact that they can be kept in culture for months without visible deterioration. So far we have found no way to reactivate inactive glands in vitro.

Successful culture of corpora allata from immature insects has not yet been reported by other laboratories. The glands are less active than those of adults but they also can be stimulated by addition of mevalonolactone/homomevalonolactone to the medium (Fig. 3). So far, JH I and JH II have been produced only by

TABLE 6. PRODUCTION OF JH III BY CORPORA ALLATA OF ADULT PERIPLANETA AMERICANA IN VITRO.

Day in culture	JH III [dpm/day x gland pair]				
	♂	♂, mv	♀	♀, mv	♀, ♂
1st - 4th	2,600	23,100	5,600	40,600	24,000
5th - 8th	1,900	2,600	2,000	5,800	15,200
9th - 12th	1,020	170	830	360	9,700
13th - 16th	620				
17th - 20th	160				

All culture media contained 2 µCi [S-methyl-^{14}C]-methionine per ml. mv: 1 mg mevalonate/ml medium. o: female, bearing ootheca ready for deposition.

TABLE 7. PRODUCTION OF JH III BY CORPORA ALLATA OF ADULT BLABERUS DISCOIDALIS IN VITRO.

Corpora allata of	JH III [dpm/day x gland pair]	
		mv + hmv
Female adult	2,500	4,700
Male adult	3,900	5,600

All culture media contained 2 µCi [S-methyl-^{14}C]-methionine per ml. mv + hmv: The medium contained 1 mg each of mevalonate and homomevalonate per ml.

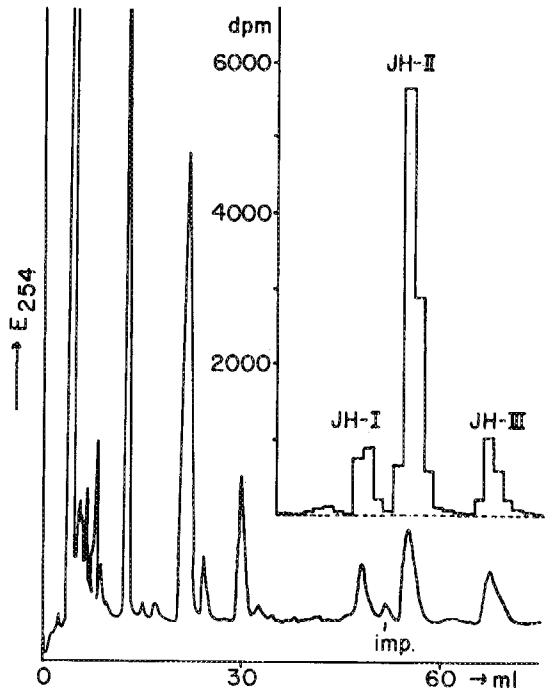

Fig. 3. High pressure liquid chromatography of [^{14}C]-labeled JH from corpora allata cultures of M. sexta larvae. Eight brain - corpora cardiaca - corpora allata complexes of freshly molted 5th instar larvae had been cultured for three days in the presence of 2 µCi [S-methyl-^{14}C]-methionine, 1 mg mevalonate, and 1 mg homomevalonate per ml incubation medium. Unlabeled juvenile hormones (containing an impurity) had been added to the preparation as carriers: they appear as peaks in the UV-absorption curve.

corpora allata of lepidopterous larvae (Table 8). While our results corroborate the view that JH I and JH II are restricted to a few species or orders of insects, they still cannot be taken as firm evidence. Free mevalonate and homomevalonate may after all not be intermediates in the biosynthesis of the natural hormones. Also, if homomevalonate is an unnatural product, e.g. in P. americana, why should the JH-synthesizing enzymes discriminate against it so strongly? In order to determine the specificity of the concerned enzymes more rigorously, we have begun to investigate JH biosynthesis in corpora allata homogenates. Under conditions described by Reibstein and Law (1973), homogenates and supernatants of M. sexta corpora allata produced JH II and JH III (Fig. 4), while in similar preparations from P. americana only JH III was detected.

TABLE 8. PRODUCTION OF JUVENILE HORMONES BY CORPORA ALLATA OF INSECT LARVAE IN VITRO.

Species	Instar	[dpm/day x gland pair] JH I	JH II	JH III
Galleria mellonella	7th	nil,<5	250	nil, <5
Hyalophora cecropia	4th	280	980	nil,<30
Manduca sexta	5th	68	400	72
Manduca sexta[a]	5th	nil,<1	7	4
Periplaneta americana, male	b	nil,<5	nil,<5	630
Periplaneta americana, male[a]	b	nil	nil	210
Periplaneta americana, female	b	nil,<5	nil,<5	830
Periplaneta americana, female[a]	b	nil	nil	310
Blaberus discoidalis[c]	b	nil,<5	nil,<5	700[d]
Blaberus discoidalis[a,c]	b	nil	nil	1000

The incubation medium contained per ml 2 µCi [S-methyl-^{14}C]-methionine, 1 mg mevalonate, and 1 mg homomevalonate.

[a] Experiment without mevalonate and homomevalonate.

[b] Advanced stage but younger than last larval instar

[c] Sex was not determined.

[d] Yield lower than anticipated; the culture was contaminated with bacteria.

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× gland pair]

JH II	JH III
250	nil, <5
980	nil, <30
400	72
7	4
il, <5	630
il	210
il, <5	830
il	310
il, <5	700[d]
il	1000

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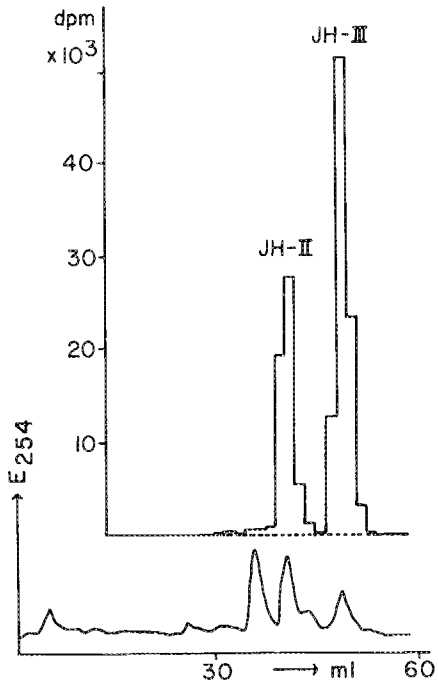


Fig. 4. Juvenile hormones produced by a homogenate of 10 corpora cardiaca - corpora allata pairs from adult female M. sexta. The incubation medium (Reibstein and Law, 1973) contained 0.5 µCi S-adenosyl-[S-methyl-^{3}H]-methionine (spec. act. 7.5 Ci/mmol).

Identification and Quantitative Determination of Juvenile Hormones by a Chemical Method

Since our in vitro experiments had revealed that JH III is not necessarily the major hormone of M. sexta, it was of interest to determine the JH composition in hemolymph of various postembryonic stages of this species. We decided to develop a procedure by which all three hormones are converted to halogen-containing derivatives which would allow final identification by gas chromatography with electron capture detection. Utilizing 10,11-bis-trifluoroacetoxy derivatives, Judy et al. (1973a) had already identified JH III in the hemolymph of fourth instar larvae, but attempts to verify the presence of JH I and JH II gave inconclusive results. In the meantime the same research group has improved the method by exploiting a different derivative and is now also in a position to analyze blood samples for all three hormones (Schooley et al., this volume). Selection of the larval stages used in our investigations was based on a JH titer curve which Judy (personal communication) obtained by means of the Galleria wax test. According to this

study the JH activity is high in early fourth instar larvae, moderately high in early fifth instar larvae, and very low in late fifth instar larvae. In addition to the penultimate and the last larval stages we included male and female adults in our experiments (Peter et al., 1976).

Hemolymph was collected in water of 0° and immediately extracted with ethyl acetate. At this point [methoxy-^{3}H]-ttt-JH-0 (Fig. 5, 4: R_1=R_2=R_4=-C_2H_5, R_3=-CH_3, [^{3}H]-labeled in the methyl ester group) was added to each sample as an internal standard. The extracts of hemolymph from adult females contained large amounts of oily material; most of it could be removed by precipitation in methanol at -20°C. The hormones (4) in the supernatant of this preparation, and in the other extracts directly, were purified by thin layer chromatography (TLC) and converted with perchloric acid in methanol to the 10-hydroxy-11-methoxy derivatives 5. After another TLC-purification, the appropriate fractions were treated with 2,4-dichlorobenzoyl chloride in pyridine. The derivatives 6 were purified by two subsequent TLC-separations and resolved by high pressure liquid chromatography (HPLC) (Fig. 6). The retention volumes of the hormone derivatives were calculated from that of the radiolabeled ttt-JH-0-derivative. The eluates of the HPLC separations were collected in such a way that each hormone derivative was spread over several fractions. The individual fractions were evaporated to dryness, the residues redissolved in 25 µl 2,2,4-trimethylpentane/20% ethyl acetate and analyzed by gas liquid chromatography (GLC) with an electron capture detector (Fig. 7).

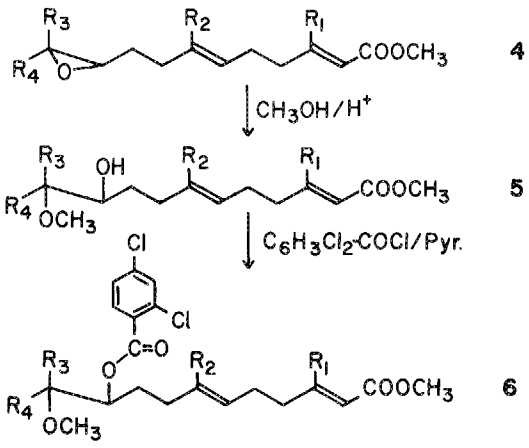
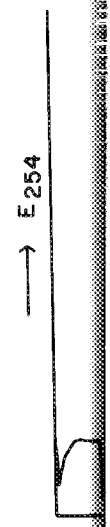


Fig. 5. Conversion of juvenile hormones to derivatives suitable for gas liquid chromatography with an electron capture detector.



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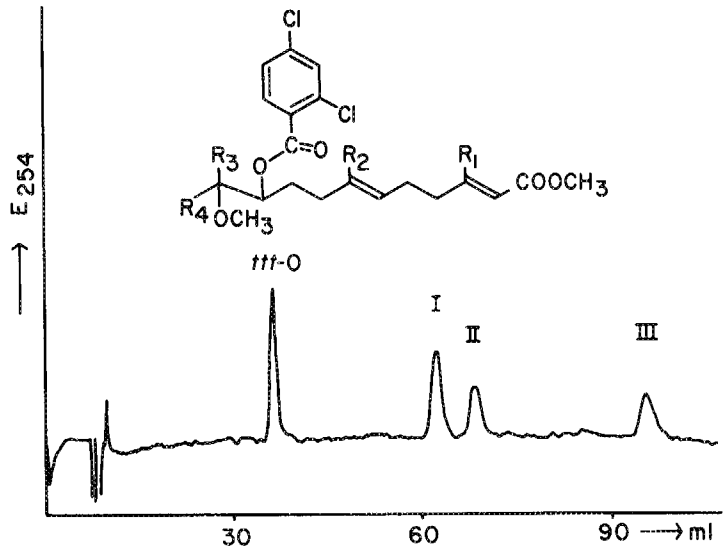

Fig. 6. Separation of the juvenile hormone derivatives 6 (0.5-1 µg each) by high pressure liquid chromatography. [0.4 x 60 cm µPorasil (Waters Associates Inc., Milford, MA), hexane/3.5% ethyl acetate/0.02% 2-propanol.] ttt-0, II and III are the derivatives of the respective JH. During analysis of natural hormones, the elution volumes were calculated from that of the [^{3}H]-labeled reference compound (ttt-0).

This procedure assured that material which on GLC had the retention time of one of the hormone derivatives 6 had also on the HPLC column the appropriate retention volume. Each chromatographic identification incorporates consequently the resolving power of both systems independently. To further assure accurate identifications, each fraction was analyzed on three of the following GLC columns: 3% SE-30, 3% OV-1, 3% OV-17, 3% XE-60 or 10% UC-W98. Authentic samples, prepared by the same method and identified by mass spectrometry, served as reference compounds. Under the conditions used for the analysis, a peak of 5 mm height was produced by 5 pg of the JH III derivative. The recovery of small quantities throughout the procedures was checked in pilot experiments where 0.25 - 1.0 ng of the hormones had been added to blood extracts of late 5th instar larvae which are devoid of natural hormone. In order to detect possible contamination of samples with synthetic hormones, each set of experiments included controls with and without JH.

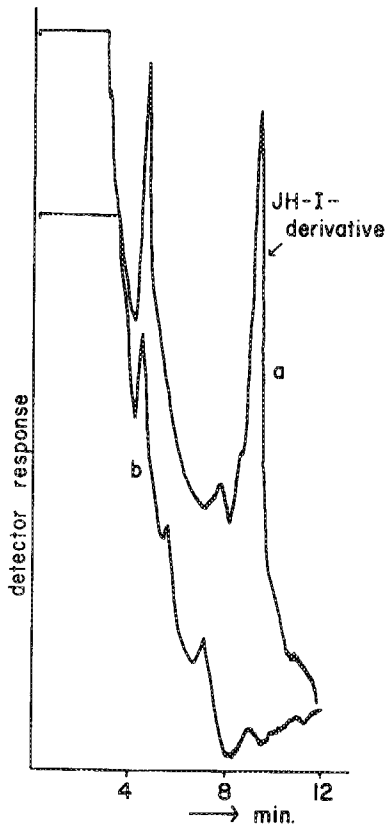

Fig. 7. JH I in hemolymph of M. sexta larvae. The hormones have been extracted from hemolymph, converted to the 10-(2,4-dichloro-benzoyloxy)-11-methoxy-derivatives, and separated by high pressure liquid chromatography. The fractions corresponding to the peak center of the JH I derivative (calculated from the radiolabeled reference compound, see Fig. 6) were injected in a gas chromatograph equipped with an electron capture detector [0.3 x 180 cm glass column, 3% OV-1 on 60/80 mesh Gas Chrom Q (Applied Science Lab., Inc., State College, PA), column temp. 275°C]. a) is a preparation from early fourth instar larvae; b) the corresponding preparation from late fifth instar larvae.

The highest concentration of JH was found in hemolymph of early fourth instar larvae; it was considerably lower in early fifth instar larvae (Table 9). No JH could be detected in wandering fifth instar larvae; the detection limit in this experiment was $\leq$ 0.003 ng/ml hemolymph. This result corresponds well with the earlier mentioned titer determinations by the Galleria wax test (K. Judy, personal communication) and a Manduca assay (L. Riddiford, personal

TABLE 9. JUVENILE HORMONES IN HEMOLYMPH OF MANDUCA SEXTA

No. animals	Stage	Hemolymph collected [ml]	JH	Concentration [ng/ml]
82	early 4th larval instar (1-2 day-old)	3.5	I	0.62
			II	1.1
			III	0.07
28	early 5th larval instar (1-2 day-old)	5.1	I	0.24
			II	0.11
			III	0.12
17	5th larval instar full-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	<0.02

nil: not detected, ≤0.003 ng/ml.

communication). It should be taken into account that hemolymph of late fifth instar larvae has a high JH esterase activity which makes the presence of any JH at this stage and age unlikely (Weirich et al., 1973). In all experiments, the three JH's were found together in varying concentrations. At present no interpretation is possible with regard to the JH composition in the different stages. Before any conclusions can be reached, one has to know whether the ratios JH I:JH II:JH III are closely controlled or whether in allatectomized larvae physiological effects of JH mixtures depend on the ratios of their components. It has been determined, however, that the morphogenetic activity of JH III

in Manduca pupae is 300 times lower than that of JH I and JH II (Riddiford and Ajami, 1973). In females of Manduca, JH controls egg maturation (Sroka et al., 1975) 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, varying concentrations of JH I, JH II and JH III were also detected (Lanzrein et al., 1975). 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 was found (Trautmann et al., 1974a). JH III was also the only hormone detected in larval and adult corpora allata cultures of a number of roach species. We will now apply the new method for detection of juvenile hormone in selected species. Of particular interest to us are L. decemlineata as a representative of Coleoptera and some insects of the orders Apterygota, Isoptera and Diptera. In early fourth instar larvae of H. cecropia we have found 0.15 ng JH II per ml hemolymph but were unable to detect JH I or JH III.

Accumulation and Storage of Juvenile Hormone in the Male Accessory Sex Glands of Hyalophora cecropia

The adult males of H. cecropia and of some related saturniid moths are distinguished by their ability to produce and store microgram quantities of JH (Röller and Dahm, 1968; Meyer et al., 1968; Röller et al., 1969; Dahm and Röller, 1970; Röller and Dahm, 1974). Consequently, Cecropia was the insect of choice for studying the biosynthesis of the hormones in vivo (Metzler et al., 1971, 1972; Gowal et al., 1975; Peter and Dahm, 1975). It has been shown that JH I is indeed synthesized by the corpora allata (Röller and Dahm, 1970) and that no hormone is sequestered in the abdomens of adults when the animals are allatectomized as pupae (Williams, 1963). To date, it has not been possible to detect a biological function of the large quantities of JH in adult saturniid moths. Allatectomized male and female Cecropia, crossmated, produced viable offspring (Williams, 1959).

The isolation of JH from insects other than H. cecropia and some related species is at the very least a tedious process. Since corpora allata in vitro do not necessarily produce the same compounds as in vivo, we had planned to use allatectomized male Cecropia moths as hosts for corpora allata of different insects and to rely on their JH collecting capability for accumulating the hormone. The procedure for isolating the JH from saturniid moths has been perfected during the past ten years and it is now possible

to recover amounts as low as 0.1 µg from single animals (Table 10). When the hormones have been labeled in vivo by injection of radiolabeled methionine (Metzler et al., 1971), the fractions of the second HPLC separation contain little radioactive material besides the hormones. The hormones recovered from this separation are gas chromatographically pure (Fig. 8).

TABLE 10. ISOLATION OF JUVENILE HORMONES FROM ABDOMENS OF MALE HYALOPHORA CECROPIA.

Abdomen	
↓	
0.2-0.8 g crude oil	Ether extraction
↓	
2-10 mg "Fr-III"[b]	Column chromatography (2.5 x 100 cm Sephadex LH-20[a], acetone:benzene = 1:1)
↓	
Juvenile hormones[d]	HPLC-I (0.9 x 60 cm EM-Gel Si-150[c], dichloromethane/12.5% chloroform/0.05% water)
↓	
JH I, JH II (JH 0, JH III)	HPLC-II (0.4 x 30 cm µPorasil[e], hexane/2% ethyl acetate/0.02% 2-propanol)

[a] Pharmacia Fine Chemicals AB, Uppsala, Sweden

[b] Nonanal- and acetone 2,4-dinitrophenyl-hydrazone serve as internal standards. The JH's emerge with the nonanal dinitrophenyl-hydrazone (total volume about 24 ml). "Fr-III" contains as a precaution against unexpected variations also the preceding ∿3 and following ∿6 ml.

[c] E. Merck, Darmstadt, Germany.

[d] The JH's are found in the fraction with 45-80% the retention volume of the internal reference compound methyl p-hydroxybenzoate.

[e] Waters Associates, Inc., Milford, MA.

When 1-2 day-old male Cecropia moths are injected with 50 µCi [S-methyl-^{3}H]-methionine and sacrificed 48 hr later, usually JH with [^{3}H]-activity between 10,000 and 100,000 dpm can be isolated. After depriving males of their corpora allata by decapitation before they were three hr old, we implanted corpora allata of other males or females and injected the radiolabeled methionine as a

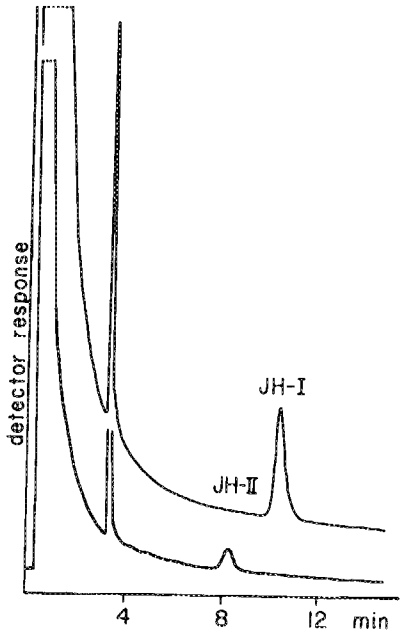

Fig. 8. Gas chromatograms of JH I and JH II isolated from a single male Cecropia moth by the procedure described in Table 10. The peak at 3 min represents 0.12 µg methyl palmitate added as an internal standard. [0.4 x 180 cm glass column, 3% QF-1 on 100/120 mesh Gas Chrom Q (Applied Science Lab., Inc., State College, PA), column temp. 160°C, flame ionization detector].

tracer. The implanted corpora allata appeared to function properly; large amounts of labeled JH could be isolated (Table 11). We have never been able to isolate either labeled or unlabeled JH from females; bioassay data indicate that they may contain only nanogram amounts of the hormone. The surprising result in this experiment was that allatectomized moths synthesized labeled JH. Since a wealth of endocrinological research had indicated that JH is produced in the corpora allata and not in other tissues, it seemed most likely that the labeled JH is not produced de novo but that the methyl ester group of the hormone, already present in the animal, is replaced by the methyl group of the labeled methionine. Degradation of JH in a number of insects involves loss of the methyl ester group as one of the first steps (Slade and Zibitt, 1972), while male Cecropia methylate the acid to the hormone in high yield (Metzler et al., 1972). Therefore it was likely that the methyl exchange was in some way connected with the ability of the moths to accumulate the hormone.

TABLE 11. [^{3}H]-LABELED JH I FROM DECAPITATED MALE CECROPIA MOTHS.

Preparation	Implant	JH I [dpm]
Intact male	none	55,000
Intact female	none	nil, 20
Decapitated male	none	7,000
Decapitated male	1 pair male cc-ca	71,000
Decapitated male	4 pair male cc-ca	128,000
Decapitated male	1 pair female cc-ca	21,000

Corpora cardiaca - corpora allata complexes of adult Cecropia moths were implanted in 5-16 hr old males which had been decapitated within 3 hr after adult eclosion. Two days later the moths were injected with 50 µCi [S-methyl-^{3}H]-methionine. They were sacrificed after a 48 hr incubation period.

When males were allatectomized as pupae during the early stages of adult development and were processed as adults in the usual fashion after being injected with the labeled methionine, no trace of labeled or unlabeled hormone could be detected. In some preparations of this nature, adult male or female corpora allata were implanted shortly after adult eclosion. These animals contained afterwards JH I as well as JH II (Table 12). It cannot yet be concluded whether female corpora allata in their natural environment also produce more JH II than JH I or whether the preponderance of JH II is a result of the experimental conditions. Some other surgical procedures in male or female pupae were without effect on the JH production.

In order to study the methyl ester exchange we injected unlabeled or [^{14}C]-labeled JH I and JH III together with [^{3}H]-labeled methionine in decapitated males (Table 13). When 5 µg each of unlabeled JH I and JH III were injected, the yield of [^{3}H]-JH I was as high as in intact males while the yield of [^{3}H]-JH II was similar to that in controls. The yield of [^{3}H]-JH III was relatively low. The same discrimination in favor of JH I was apparent when [methoxy-^{14}C] labeled JH I and JH III were injected in intact or decapitated males. About 20% of the [^{14}C]-JH I, but only 1% of [^{14}C]-JH III were recovered unchanged. The [^{3}H]-incorporation ratio in these experiments was in the usual range.

TABLE 12. JH I AND JH II FROM ADULT CECROPIA AFTER ALLATECTOMY OR OTHER SURGICAL PROCEDURES DURING THE PUPAL STAGE.

Preparation	JH I [dpm]	JH II [dpm]
male, allatectomized	nil	nil
male, N.C.C.[a] I and II cut	106,000	26,000
male, 1 pair ovaries implanted	71,000	10,000
female, N.C.C.[a] I and II cut	nil	nil
female, 4 pair male gonads implanted	nil	nil
male, allatectomized, [2 pair male adult cc-ca implanted][b]	10,800	9,900
male, allatectomized, [2 pair female adult cc-ca implanted][b]	320	1,090

The surgical procedures except implantation of cc-ca complexes were performed on pupae prior to initiation of adult development. The implanted ovaries and male gonads were taken from pupae of the same developmental stage. Fifty µCi [S-methyl-^{3}H]-methionine were injected 48 hr after adult eclosion. The moths were sacrificed after an incubation period of 48 hr. nil: not detectable, less than 20 dpm.

[a] nervi corporis cardiaci.

[b] The corpora cardiaca - corpora allata complexes were implanted during the first day after adult eclosion.

Obviously, some tissue or tissues in the moth are able to degrade and to resynthesize the juvenile hormone after it is secreted by the corpora allata. It had generally been assumed that JH is sequestered in the fat body (Williams, 1963), but when rechecking the literature on this point we found that convincing evidence had never been presented.

When we dissected male moths and tried to isolate JH from the various tissues, we quickly discovered that not the fat body but tissues of the reproductive tract are responsible for the accumulation of the hormone (Shirk et al., 1976). In order to facilitate

ER ALLATECTOMY OR

]m]	JH II [dpm]
	nil
0	26,000
0	10,000
	nil
	nil
)	9,900
	1,090

cc-ca complexes
development.
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ethionine were
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TABLE 13. REPLACEMENT OF THE METHYL ESTER GROUP OF JH BY THE S-METHYL GROUP OF METHIONINE IN INTACT AND DECAPITATED MALES OF HYALOPHORA CECROPIA.

Preparation	Material injected besides	JH I [dpm]		JH II [dpm]	JH III [dpm]	
(adult males)	50 µCi [^{3}H]-methionine	[^{3}H]	[^{14}C]	[^{3}H]	[^{3}H]	[^{14}C]
Intact	none	82,000	--	11,000	nil	--
Decapitated	none	2,800	--	1,900	--	--
Decapitated	5 µg JH I + 5 µg JH III	80,000	--	1,400	6,300	--
Decapitated	10^4 dpm [$^{14}CH_3O$]-JH I and [$^{14}CH_3O$]-JH III	11,500	2,500	8,900	1,300	131
Intact	same as above	52,000	1,400	8,600	167	14

The moths were decapitated within 3 hr after adult eclosion, injected at an age of 2 days, and sacrificed at an age of 4 days. nil: not detectable, less than 40 dpm.

the isolation of JH, it was labeled routinely by injection of [S-methyl-^{3}H]-methionine into the animals 48 hr prior to dissection. In a number of experiments we analyzed for JH not only radiochemically and gas chromatographically but also with the Galleria wax test (Table 14). JH could be identified by radiolabel and by gas chromatography only in tissues of the reproductive tract. According to the bioassay its concentration in the remainder of the body is at least three orders of magnitude lower. In another experiment (Table 15), the male accessory sex glands were dissected out and extracted separately from the remainder of the abdomens. JH was found exclusively in the extracts of the male accessory glands. The hormones appear to be associated with the luminal contents rather than the gland tissue (Table 16).

With regard to the biochemistry of the JH accumulation, we have already obtained a number of miscellaneous results which eventually will give a complete picture of the process. Some saturniids other than H. cecropia also accumulate JH in the male accessory sex glands. No trace of JH was detected by bioassay in the accessory sex glands of male M. sexta. The methyl exchange is not required for the accumulation; in vivo and in vitro the accessory glands are able to assimilate JH without chemical change. At the same time they have in vivo as well as in vitro the ability to assimilate the corresponding acid, methylate and store it, and also to exchange the methoxy group in JH already stored.

CONCLUSION

Our discovery that the accessory sex glands of male Cecropia have the unique ability to store large amounts of JH merits further intensive study. The endocrine situation in male Cecropia is similar to that in females in that the reproductive functions are independent of JH-control. The corpora allata of males are appreciably larger than those of females and have a much higher biosynthetic activity, as shown by our transplantation experiments. However, the marked difference between the sexes in JH content must to a large part be attributed to the exceptional nature of the male accessory sex glands. Although JH in the Cecropia moth has apparently no function, its accumulation may be a physiological relic, albeit exaggerated, of a process which in some other insect species is important for the maturation or activity of the accessory sex glands.

We have shown that larval and adult corpora allata of Manduca sexta under certain in vitro conditions produce JH I, JH II and JH III. Hemolymph of larvae and adults contains the same hormones in quantities corresponding to the JH-activity profile established by bioassays. The occurrence of all three hormones in various relative concentrations might suggest specific roles for the

TABLE 14. JUVENILE HORMONE IN DIFFERENT TISSUES OF ADULT MALE HYALOPHORA CECROPIA.

Moth #	Preparation	Biol. activity [GU][a]	JH I [dpm]	JH I [µg]	JH II [dpm]	JH II [µg]
1	Reproductive tract	20 x 10^6	32,000	1.4	12,000	0.4
	Remainder of carcass	<1 x 10^3	nil	--	nil	--
2	Accessory sex glands plus seminal vesicles	5 x 10^5	6,200	2.1	590	0.3
	Remainder of carcass	1 x 10^3	nil	--	nil	--

Two 48 hr old male moths were each injected with 2 µCi [S-methyl- H]-methionine. They were dissected and extracted after a 48 hr incubation period. nil: not detectable, less than 35 dpm.

[a] Galleria Unit (see Table 1).

TABLE 15. JUVENILE HORMONE IN THE ACCESSORY SEX GLANDS OF MALE HYALOPHORA CECROPIA.

Moth #	Preparation	JH I [dpm]	JH I [µg]	JH II [dpm]
1	Accessory sex glands	3,100	95%[a]	860
	Remainder of abdomen	nil	58%[a]	nil
2	Accessory sex glands	4,500	2.8	1,800
	Remainder of abdomen	nil	nil	nil
3	Accessory sex glands	42,900	1.8	nil
	Remainder of abdomen	nil	nil	nil
4	Accessory sex glands	4,000	3.9	350
	Remainder of abdomen	nil	nil	nil
5	Accessory sex glands	44,000	2.2	5,300
	Remainder of abdomen	nil	nil	nil

Five 24 hr old moths were injected each with 25 µCi [S-methyl-^{3}H]-methionine. They were dissected and extracted after a 48 hr incubation period.

[a] % recovery: based on the recovery of 10 µg unlabeled JH I added to the crude extracts of moth 1. nil: not detectable, less than 200 dpm and/or 0.2 µg.

individual hormones in morphogenesis and reproduction. However, the low morphogenetic activity of JH III coupled with the fact that it is only as effective as JH I or JH II in reproductive assays warrants cautious interpretation of the data. Whether or not the three hormones have qualitatively different morphogenetic functions may best be investigated in insects with polymorphic larvae and adults.

.ANDS OF MALE

JH II [dpm]
860
nil
1,800
nil
nil
nil
350
nil
5,300
nil

· µCi [S-methyl-
fter a 48 hr

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larvae and

TABLE 16. BIOSYNTHETICALLY LABELED JH I AND JH II FROM LUMINAL CONTENT AND GLAND TISSUE OF ACCESSORY SEX GLANDS FROM MALE HYALOPHORA CECROPIA.

Moth #	Preparation	JH I [dpm]	JH II [dpm]
1	Luminal content	10,700	3,600
	Gland tissue	1,800	400
2	Luminal content	24,600	3,800
	Gland tissue	1,100	100

Male moths were prepared as described in Table 15. The accessory sex glands were placed in insect Ringer solution and cut in pieces. The luminal contents were separated from gland tissue as well as possible by pressing the pieces with glass rods.

Corpora allata *in vitro* are surprisingly specific with regard to JH-synthesis. Homomevalonate and/or mevalonate in the medium are used as precursors and even stimulate JH-biosynthesis. However, production of JH I and of JH II was observed only in cultures of lepidopterous corpora allata, while those of other species produced exclusively JH III. JH-homologs or isomers other than JH I, JH II and JH III were never detected. We may conclude that the *in vitro* production of specific juvenile hormones indicates intrinsic biosynthetic capabilities of corpora allata from different species and more likely than not reflects the situation *in vivo*. Whether or not this conclusion is valid will be shown by chemical identification of juvenile hormones in hemolymph of such species whose corpora allata *in vitro* produce only one hormone.

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