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Transesterification of juvenile hormone occurs in vivo in locust when injected in alcoholic solvents

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Abstract. Catabolism of juvenile hormone was studied in vivo in the African locust by injection of the labelled natural enantiomer (10R) (12-^{3}H) JH-III. Due to the poor solubility of JH in aqueous solution, it was injected in a water–miscible solvent. Ethanol was chosen for its apparently low toxicity towards the locust. In these experimental conditions, reverse phase liquid chromatography procedure (RP-HPLC) coupled with on line radiodetection, revealed an apolar metabolite of JH-III. This compound was found both in adult females and in fifth stadium larvae. We demonstrate that this metabolite resulted from substitution of the carboxyl methyl group of JH-III by some hydrophobic moiety. This compound co-migrates in our RP-HPLC system with the JH analog epoxy-ethyl farnesoate (JH-III ethyl ester) obtained by KCN-catalysed transesterification of JH-III in ethanol. Both JH-III ethyl ester of chemical origin and biological compounds extracted from locusts give the same spectra when analyzed by gas chromatography-mass spectroscopy (GC-MS). Transesterification of JH-III was not observed with locust tissues incubated in vitro but occurred in vivo even if JH was injected in other alcoholic solvents such as propanol. Our data suggest that transesterification of JH-III occurred in vivo and underline the role of injecting solvent in in vivo studies.
Key words. Transesterification; insect; JH metabolism; ethyl-JH.

Juvenile hormone (JH) is an important isoprenoid hormone playing a crucial role in insect growth and development. The hemolymphatic JH titre determines the availability of the hormone at the level of target tissues and results from two dynamic processes, i.e. JH biosynthesis by the corpora allata and JH degradation[1,2]. JH biosynthesis has been extensively studied over the last decade. Its degradation has been investigated to a lesser degree and much of the work done to date is based on in vitro studies. However, recent data on JH catabolism in vivo using labelled JH have revealed new pathways for JH catabolism[3,4] and have increased interest in such approaches.

As JH is a lipophilic molecule with limited solubility in aqueous media, labelled JH is usually injected in a small volume of water-miscible organic solvents such as ethanol or acetone. The labelled tracer is expected to label the endogenous pool of JH and to provide information on JH metabolism in situ. In the course of our studies on JH catabolism in the African locust *Locusta migratoria migratorioides*, we found that acetone at doses higher than 5 µl per last instar larvae resulted in locust death. Because ethanol could be injected in volumes of up to 50 µl without apparent disturbance of the animal, we have carried out a first set of experiments using ethanol. However, in the present paper, we demonstrate that alcoholic solvent, even at very low concentration, induces in vivo transesterification of JH-III.

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Materials and methods

Animals. *Locusta migratoria migratorioides* were reared at 30 °C under crowded conditions. They were fed every morning on fresh wheat and bran.

Chemicals. JH-III was purchased from Sigma. Labelled (10R)-[12-^{3}H]JH-III (555 Bq/pmol) was a gift from Prof. Glenn Prestwich (Stony Brook, New York). Labelled (10RS)-[10-^{3}H]JH-III (444 Bq/pmol) was purchased from NEN. Farnesol (trans-trans) was a gift of Prof. René Feyereisen (Tuscon, Arizona). The ethyl ester JH-III was produced at 70 °C over night, in 95% ethanol solution using potassium cyanide (4 mg /ml) as a milder catalyst for ester exchange[5]. Ethyl ester JH was purified on RP-HPLC, Hepes buffer system and extracted with hexane.

In vitro biosynthesis of (10R)-[^{14}C methyl]JH-III. (10R)-[^{14}C methyl]JH-III was produced by incubating individual pairs of corpora allata from adult female locusts in TC-199 containing ^{14}C-methyl-methionine (Amersham, UK; final conditions 0.3 mM; 80 dpm/pmol) and farnesol (300 µM)[6]. JH-III extracted with hexane was subjected to normal phase liquid chromatography (NP-HPLC). Radioactivity was monitored

using solid scintillation on a Berthold LB 506 detector and peaks corresponding to (10R)-[^{14}C methyl]JH-III were collected.

In vivo catabolism study. (10R)-[12-^{3}H]JH-III dissolved in ethanol (10 µl, approx 100,000 dpm, 1.5 pmol) was injected into the abdomen through the intersegmental membrane. After incubating at 27 °C for 1 hour, the insects were homogenized in 5 ml of acetonitrile containing triethylamine (2 drops per 100 ml acetonitrile) using an Ultra Turrax T25 homogenizer at 13,500 rpm, for 30 s at 4 °C. After centrifugation (5 min, 7,000*g* at 4 °C), 1 ml of the supernatant was evaporated under a stream of nitrogen and the redissolved sample was injected into the RP-HPLC system.

Radioactivity monitoring. Radioactivity was monitored by on line solid scintillation (50% efficiency for ^{14}C) or liquid scintillation (25% efficiency for ^{3}H and 80% for ^{14}C) after adding scintillation cocktail (Flow Zinker, 3 ml/min).

Reverse phase liquid chromatography (RP-HPLC). RP-HPLC procedure was performed using a polymer column (PLRP-S, 5 µm, 100 Å Polymer Laboratories) on Beckman System Gold HPLC system, essentially as described by Halarnkar and Schooley[7]. Samples were separated on an acetonitrile gradient in Hepes buffer at pH 7.4 at a flow rate of 1 ml/min using two elution methods:

– a linear gradient from 5% to 80% acetonitrile in 20 min.

– a 3-slope gradient running acetonitrile from 5% to 60% in 15 min followed by 60% to 70% in 5 min, then 70% to 80% in 2 min.

Normal phase liquid chromatography (NP-HPLC). We used an isocratic procedure (1 ml/min) on a 250 × 4.6 mm silica column (Chrompack) using hexane/isopropanol (93/7) saturated in water as the mobile phase.

Gas chromatography and mass spectroscopy (GC-MS) analysis. GC-MS procedure is essentially as previously described[8]. GC was performed on a CP-Sil 5CB capillary column (25 m, 0.25 mm) at 200 °C, 1 ml/min (Chrompack). Mass spectrometer (MS) (Nermag) was used as a detector in chemical ionisation mode (CI+), using ammonia as reagent gas.

Results and discussion

To investigate JH catabolism in the African locust *Locusta migratoria migratorioides* in vivo, we injected a minute amount of (10R) (12-^{3}H)JH-III (around 2 pmol), the natural form of JH, into this species. Because of the poor solubility of JH in aqueous solution, it was injected dissolved in a water-miscible solvent. Ethanol was chosen because of its apparent low effect on insect physiology, evaluated by insect survival. Adult locusts can survive up to 50 µl pure ethanol while 5 µl acetone killed them (not shown).

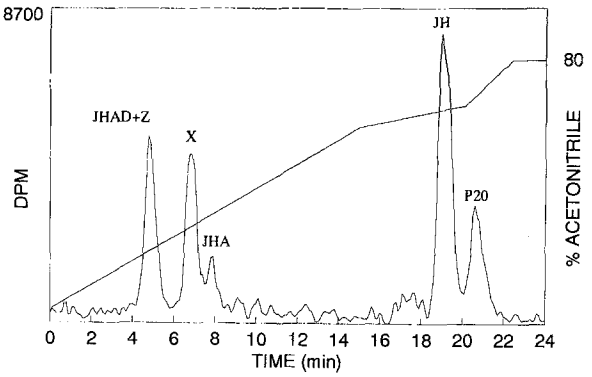

Figure 1. Radio-RPLC chromatograph of (10R)-[^{3}H]JH-III and its metabolites, 1 h after injection into 10-day-old adult female of *L. migratoria*. Sample was prepared as described in 'Materials and methods'. Peaks were identified according to the retention time of the corresponding standard. Acetonitrile gradient (three slope) was represented by the upper solid line.

In a first series of experiments, we injected labelled (10R)-[^{3}H]JH-III dissolved in 10 µl pure ethanol into 10-day-old adult females. One hour later, insects were homogenized and an aliquot was injected into a RP-HPLC system using linear acetonitrile gradient on Hepes buffer at pH 7.4[7]. Within one hour, radioactivity was obtained in four peaks which were identified as JH-III, JH-III acid, JH acid diol and an unknown peak (named X). The peak eluting at Rt similar to JH-III acid diol also contained an unknown compound named Z that could not be separated from JH-III acid diol at pH 7.4[4]. For the purpose of this study, it is interesting to note the presence of a shoulder for the peak corresponding to the labelled JH-III. In order to elucidate the possibility of an additional peak close to JH-III, we switched from a linear gradient to a three slope gradient allowing better separation around JH-III. As shown in figure 1 this procedure clearly separates JH-III (Rt = 19:06) from an additional peak named P20 with Rt = 20:54. The three slope gradient RP-HPLC procedure was

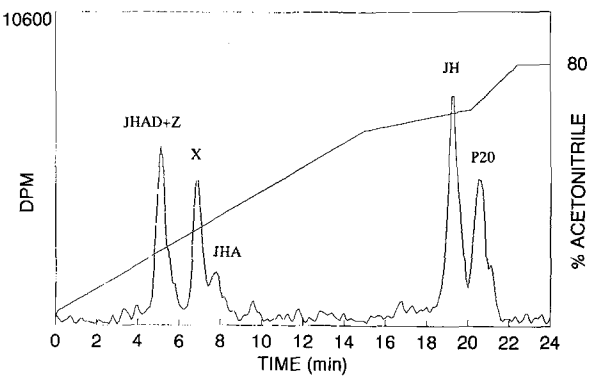

Figure 2. Radio-RPLC chromatograph of (10R)-[^{3}H]JH-III and its metabolites, 1 h after injection into 7-day-old last instar larvae of *L. migratoria*. Sample was prepared as described in 'Materials and methods'. Peaks were identified according to the retention time of the corresponding standard.

used in the following experiments in order to identify the non-polar compound P20.

P20 was observed in adult females from different ages. It was also found when JH-III was injected into last instar larvae (dissolved in 5 µl pure ethanol) (fig. 2). P20 could be detected up to two hours after JH injection but radioactivity associated with P20 decreased with time, suggesting some kind of degradation of this product. By selective grinding of tissues at various time up to two hours after JH injection in vivo, we found that only a small amount of P20 was observed in the gut but a substantial quantity of P20 was detected in the hemolymph and other tissues of the insect, which suggests that P20 is not some form of JH end product excreted by the gut. All together, these data prompted us to elucidate the chemical structure of P20.

In this attempt, we prepared a large sample by injecting 12 larvae with [^{3}H]JH-III. Thirty minutes after injection, the larvae were ground into hexane/water 1/1. Unchanged JH-III together with P20 were recovered in the hexane phase as revealed by injection of an aliquot on the RP-HPLC system. This sample containing both JH-III and P20 was used to investigate some chemical properties of the unknown molecule.

Rt for JH-III and P20 were not affected by changes in the pH of the mobile phase when using a citrate buffer at both pH 5.4 and 3, suggesting that P20 is not a carboxylic acid[4]. When the mixture containing both JH-III and P20 was submitted to hydrolysis, peaks corresponding to JH-III and P20 were replaced by two peaks with Rt = 12:24 and 13:00. The peak with Rt = 12:24 was JH-III diol (Rt = 12:24). The peak with Rt = 13:00 was hypothesized to result from P20 that has been hydrolysed in the same way as JH-III.

JH-III is a good substrate for porcine liver esterases of commercial origin (Sigma) resulting in JH-III acid formation (Debernard, unpublished). When the JH-III/P20 labelled mixture was incubated for 1 hour with porcine liver esterases, it resulted in a single labelled peak with Rt similar to JH-III acid. Probable degradation of both JH-III and P20 into JH-III acid suggests that both are esters of JH-III acid with different moieties.

To complete our investigations, we injected into the locusts [12-^{3}H]JH-III together with [^{14}C methyl]JH-III. [^{14}C methyl]JH-III was prepared by incubating adult locust corpora allata in TC-199 medium complemented with [^{14}C methyl] methionine and farnesol (200 µM)[6]. [^{14}C methyl]JH-III was extracted from incubation medium and purified on NP-HPLC. The labelled peak with Rt similar to JH-III was detected using solid scintillation monitoring and collected. Because JH-III and P20 are not discriminated on NP-HPLC, an aliquot of the collected fraction was run on RP-HPLC to check the purity of the JH-III and the absence of P20. Thirty min after injection of [12-^{3}H]JH-III and [^{14}C methyl]JH-III, the locust was ground in hexane/water, hexane phase submitted to RP-HPLC and radioactivity was monitored for both ^{3}H and ^{14}C. As shown in figure 3 both JH-III and P20 were found in the ^{3}H channel, but only JH-III was detected in the ^{14}C channel.

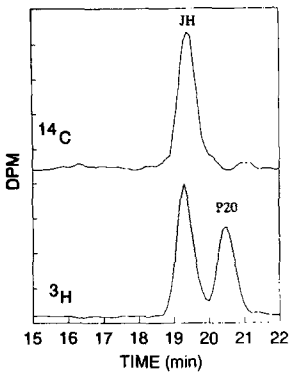

Figure 3. Radio-RPLC chromatograph of hexane extract of last instar larvae 30 min after injection of (10R)-[^{3}H]JH III and (10R)-[^{14}C-methyl]JH-III in 5 µl pure ethanol per larva.

All together, these data suggest that P20 is derived from JH-III by substitution of the carboxyl methyl group by an unknown radical that makes it less polar. At that point, we speculated that the unknown radical could be an ethyl group.

Ethyl ester JH-III (10-11 epoxy, ethyl ester farnesoate) was prepared by chemical transesterification of [12-^{3}H]JH-III and unlabelled JH-III in ethanol using KCN as a mild catalyst[5]. The labelled compound resulting from this transformation has the same chromatographic mobility in both RP-HPLC and NP-HPLC as P20. Biochemical transformation such as hydrolysis and esterase degradation also corroborate data obtained with P20 from biological origin.

Ethyl ester JH was further characterized by GC-MS (fig. 4A). Mass spectra in CI^+ mode with ammonia as reagent gas provide main fragments with m/z = 235, 236, 281, 298 interpreted as followed. 298: $MH^+ + NH_3$; 281: MH^+; 263: $MH^+ + H_2O$; 252: $MH^+ + NH_3$-C_2H_5OH; 235: MH^+-C_2H_5OH; 189: MH^+-(H_2O-CO C_2H_5OH), consistent with the expectedstructure.

Identification of ethyl ester JH-III of biological origin was carried out using a large pool resulting from injection of 10 µl each of ethanol solution containing (10RS)-(10-^{3}H)JH-III (25,000 dpm) mixed with 10 µg unlabelled JH-III into 30 fifth stadium larvae. After 1 hr, the insects were homogenized in hexane/water. The hexane phase was concentrated up to about 4 ml and then passed through a silica Sep-Pak (Waters). JH-III and P20 were eluted with hexane/ether (4/1) and dried. The sample was passed through a Vydac C18 (Amersham) reversed-phase cartridge and eluted with acetonitrile/water (70/30). JH-III and P20 were extracted from the aqueous phase with hexane, dried and injected into

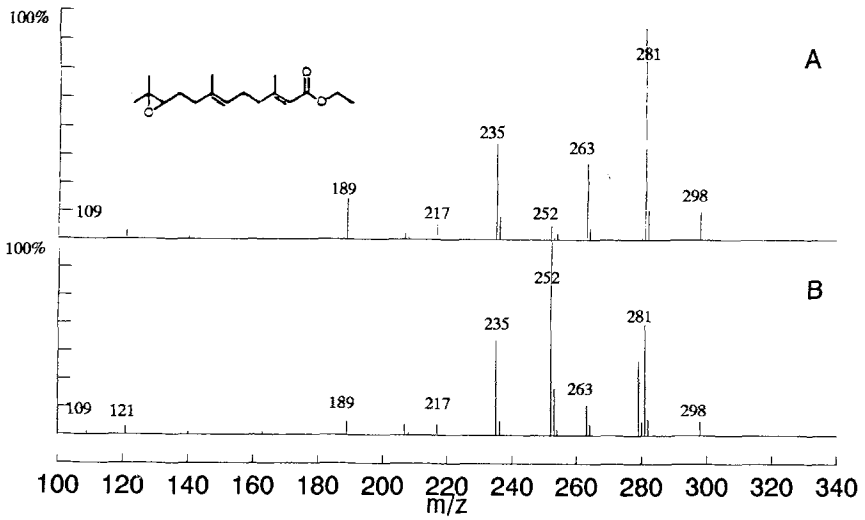

Figure 4. Identification of ethyl JH-III by GC-MS analyses using CI+ mode with ammonia as reagent gas. *A* Mass spectrum of JH-III ethyl obtained by KCN- catalysed-transesterification of JH-III in ethanol. *B* Mass spectrum of P20 extracted from insect 1 h after injection of (10R)-[^{3}H]JH-III in 5 µl pure ethanol per larva.

RP-HPLC system (Hepes buffer 7.4). P20 was collected according to its retention time, extracted again and injected into the NP-HPLC system. The final collected fraction was checked for P20 content by injecting an aliquot into RP-HPLC and liquid scintillation counting. The sample was analyzed by GC-MS. As shown in figure 4B, the mass spectrum of P20 in general corresponds to the ethyl ester JH-III spectrum except for the peculiar intensity of a fragment with m/z = 252 that could result from some contaminant in the biological sample. GC-MS analyses provide strong arguments that the P20 detected in the biological sample is ethyl ester JH-III. This structure is perfectly consistent with all the data in the present paper using labelled P20 molecules.

According to these data, transesterification of JH-III occurs in vivo in the African locust when JH-III is injected dissolved in ethanol. We never detected transesterification of JH in vitro, when JH introduced in ethanol was incubated with hemolymph, individual tissues or group of tissues (5 µl ethanolic solution into 500 µl incubation medium). Ethyl JH was formed when JH was injected in vivo in ethanol volumes as small as 2 µl. Ethyl JH was also formed when JH-III was injected in an ethanol/water mixture until 10 µl of a 10:90 ethanol:water mix. To investigate the role of the injecting solvent in this mechanism, we have injected JH-III with propanol and acetone. We clearly found that ethyl JH-III was not formed when using acetone, but as mentioned above, the volume has to be very small (< 5 µl) to permit insect survival. When JH-III is injected with a small volume of propanol-2 (< 5 µl), ethyl JH-III is not detected but we observed a more hydrophobic product eluting with Rt 22 min. We suggest that this compound is JH-III propyl ester.

Transesterification of methyl ester in alcoholic solvent is widely used in chemical engineering. It has been reported to occur with JH during the course of extraction procedures. This chemical mechanism could explain the detection of ethyl homologs of JH when larvae of *Manduca sexta* were bled directly into cold ethanol (Bergot and Schooley, personal communication[9]). Reports of in vivo transesterification are sparse but it has been reported for cocaine (2-carboxyl methyl ester of cocaine) co-administered with ethanol in mice or humans leading to formation of the 2-carboxyl ethyl ester of cocaine that possesses a pharmacological activity comparable to that of cocaine[10]. It is interesting to note the involvement of microsomal carboxyesterases in this process[11]. The present work is the first evidence that a similar mechanism occurs in vivo in insects but the role of carboxyesterases has not yet been yet studied.

The physiological relevance of transesterification in insects is doubtful. Ethyl JH-III has been described as a JH analog exhibiting JH-like effects in several insects[12]. However, transesterification requires the presence of ethanol which is quite unusual in insects. Ethanol is produced by *Chironomus* larvae reared in anaerobic conditions[13] but nothing is known about the possible links with JH metabolism.

If the physiological relevance is questionable, the consequences of these data in experimental approaches are very important. Transesterification of JH could occur when JH is injected in an alcoholic solvent. Because ethyl JH is not separated by the usual methods used for studies in JH degradation, i.e. partition assay[14,15], NP-HPLC, radioactive collection using wide (> 30 min) fractions, ethyl JH could be simply ignored. The ethyl JH may greatly interfere due both to its effect as a JH

analog and to its own catabolism that could be different in rate from the catabolism of JH-III. When injected in locusts, ethyl JH is degraded by locust carboxyl-esterase resulting in formation of both JH-III acid and JH-III acid diol (not shown) but the relative rates of degradation of JH-III and ethyl JH-III has not been investigated. The present paper stresses the need for caution in the choice of solvent for in vivo studies of hormonal degradation and the unexpected mechanisms that they can generate.

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