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IN VITRO BIOSYNTHESIS OF JH III BY THE CORPORA ALLATA OF ADULT FEMALES OF *BLATTELLA GERMANICA* (L)

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Abstract—A radiochemical assay which fulfills the required validation criteria has been used for quantification of the *in vitro* biosynthesis of JH III by the *corpora allata* of adult females of *Blattella germanica* throughout the 7 days of the first reproductive cycle. The presence of JH III has been independently confirmed by HPLC and mass spectrometry. Results indicate that rates of JH release increase repidly from day 3 to day 6, which is correlated with oocyte growth. The highest levels of JH release (2.58 ± 1.11 pmol/hr per pair) were obtained from day-6 females. The time course of JH production by CA from day-6 females showed that CA released JH at a linear rate for at least 9 hr. From these results, it can be concluded that titers at high production ages and linearity ranges are satisfactory enough to be used in studies on the regulation of JH production in this species.

Key Word Index: *Blattella germanica*, juvenile hormone III, *in vitro* biosynthesis, *corpora allata*

INTRODUCTION

Design of appropriate methods for determination of juvenile hormone (JH) levels and for monitoring the dynamics of its production by *corpora allata* (CA) has promoted progress on those problems related to the regulation of reproduction by these hormones.

The radiochemical assay described by Tobe and Pratt (1974) (see also Tobe and Stay, 1977; Tobe and Feyereisen, 1983; Feyereisen, 1985, for successive improvements and reviews) has provided a wide body of information on CA activity during the reproductive cycle. The order Dictyoptera has been one of the most thoroughly studied in this sense and following this approach, Weaver and Pratt (1977) studied the species *Periplaneta americana*, which shows the less modified type of oviparity. On the other hand, Tobe and Stay (e.g. 1977, see also Stay and Tobe, 1981; Tobe *et al.*, 1985) have investigated the viviparous cockroach *Diploptera punctata*, and Lazrein *et al.* (1978) the ovoviviparous *Nauphoeta cinerea*, which show a highly modified model of reproduction. In the present investigation we have studied several parameters of the *in vitro* biosynthesis of JH by the CA of *Blattella germanica*, which represents an evolutionary intermediate type of oviposition strategy, during the first reproductive cycle. The results obtained could serve as a basis for further investigations on extrinsic and intrinsic CA control factors, in the context of a more applied research on this important insect pest.

MATERIALS AND METHODS

Insects

Adults of *B. germanica* were reared at 26(±1)°C as described elsewhere (Bellés and Piulachs, 1983). Newly molted virgin females isolated from the colony were used at appropriate ages, which were rigorously determined by using the basal oocyte length as the main criterion (Bellés and Piulachs, 1983).

Dissections and measurements

Individual CA–CC complexes were dissected and transferred to the incubation medium. Special care was taken to remove traces of adjacent tissues. Dissections were performed in a sterile environment and the specimens were surface-sterilized with 90% ethanol before dissection. The ovaries of the same specimens were dissected under Ringer solution and the lengths of the basal oocytes were measured using an ocular micrometer. After the incubation, the two diameters (d_1 and d_2) of each CA were measured and the gland volume (V) was calculated according to the formula: $V = 4\pi/3[1(d_1 + d_2)/4]^3$

Compounds and analytical techniques

All glassware was coated with 1% solution of silicone emulsion 201. JH I, JH II and JH III were obtained from Sigma. Methyl farnesoate and [5-$^2H_2$]JH III were prepared in our laboratory. [10-^{3}H(N)]JH III (11 Ci/mmol) was obtained from New England Nuclear and [methyl-^{3}H]-methionine (80–85 Ci/mmol) from Amersham.

Thin layer chromatography (TLC) analyses were performed on Merck silicagel plates with concentrating zone. High performance liquid chromatography (HPLC) analyses were carried out with a Waters modular system. Radioactivity was measured with a LKB 1217 Rackbeta scintillation counter and with a Rita TLC rediosCanner (Isomess, Ruhrallee, W. Germany). Gas chromatography–mass spectrometry analyses using chemical ionization with methane as reagent gas and selected ion monitoring (GC–MS–CI–SIM) were carried out with a Hewlett-Packard 5988 apparatus. The system was fitted with 25 m × 0.2 mm (i.d.) glass capillary column packed with dimethylsilicone. Under these conditions, the detection limit for the peak of JH III at m/z 235 was 37 pg.

In vitro *incubation methods*

Incubations were carried out in Millipore-filtered TC-199 medium containing L-methionine (0.1 mM), glutamine, Hanks' salts, HEPES buffer (25 mM) plus Ficoll (20 mg/ml), and to which radioactive methionine (diluted to a final L-methionine sp. act. of 100 mCi/mmol) was added.

Individual CA–CC complexes were incubated in 200 μl of medium, at 30(±0.5)°C in the dark and with gentle shaking. When JH release was measured over different time periods,

glands from 6-day-old females were used. At prescribed intervals, the glands were transferred to fresh radiolabeled medium and the medium from each interval was analyzed. In the experiments carried out to study the relationship between animal age and JH release, glands were incubated for 8 hr.

At the conclusion of the incubation, 25% of the glands selected at random from each experiment, were routinely checked by electron microscopy to confirm that they had not suffered damage.

Radiochemical assay for JH III determination

The procedure used for extraction and purification of the released hormone was slightly modified from those previously reported (Pratt and Tobe, 1974; Tobe and Stay, 1977). All assays were performed in triplicate. Accordingly, JH III (4 μg, carrier and U.V. spot tracer) and *n*-decane (7 μl, added to prevent losses of hormone throughout evaporation of solvents) were added to an aliquot of medium (50 μl) which contained ethanol (100 μl). Chloroform was then added (500 μl) and the mixture was vortexed (2 min) and centrifuged (2 min at 14,000 *g*) after which the aqueous layer was removed and re-extracted with chloroform (500 μl). The combined organic fractions were washed with water (200 μl) to eliminate most of the residual methionine and concentrated under N_2 to approx. 30 μl. The residue was spotted onto a TLC plate and chromatographed using 85:15 hexane–ethyl acetate. The spot corresponding to JH III was scraped off and radioactivity was measured. The efficiency of the overall process, using tritiated JH III, was found to be 84.1 $\pm$ 1.2% ($n = 8$).

In the experiments carried out to assess the release of methyl farnesoate, 5 μg of the cold standard were added to the medium before extraction and radioactivity of the corresponding TLC spot was also measured.

Identification of JH III produced in vitro

First, CA–CC complexes ($n = 12$) from 6-day-old females were incubated for 8 hr. Aliquots were independently subjected to the extraction procedure without previous addition of cold JH III carrier. Individual extracts were joined into three different pools corresponding to 4 CA–CC pairs each and to oocyte lengths (mm) of 1.87 $\pm$ 0.17 (A), 1.97 $\pm$ 0.07 (B) and 1.78 $\pm$ 0.16 (C), respectively. Then, concentrated samples were spotted onto TLC plates, chromatographed with 70:30 hexane–ethyl acetate and radioactivity was measured with a radioscanner (30 min per spot, Ar-5% CH_4 at 1 ml/min). Zones of samples A, B and C corresponding to JH III were scraped off, extracted with 4:1 pentane–ethyl acetate and radioactivity of an aliquot was determined. Then extracts were centrifuged, concentrated under N_2 and stored at -20°C. For quantification purposes, samples A and B were subjected to further HPLC purification under the conditions below. GC–MS–CI–SIM analyses were performed by redissolving the samples in 2-methylheptane followed by addition of the corresponding amount of bisdeuteriated JH III used as internal standard.

In a complementary experiment CA–CC complexes ($n = 5$; oocyte length = 1.73 $\pm$ 0.11 mm) from 6-day-old females were incubated for 3 hr. Aliquots (100 μl) were independently subjected to the extraction procedure without previous addition of JH III as carrier. Extracts were concentrated under N_2, resuspended in methanol (25 μl) and stored at -20°C. HPLC analyses were carried out with a 30 × 0.39 cm (i.d.) column packed with Spherisorb ODS-2 (10 μm, Tracer Analítica, Barcelona). Elution conditions, using a methanol–water gradient from 75:25 to 95:5, were set to allow optional analysis of methyl farnesoate at the same flow rate as that used for JH III. Eluates corresponding to 1 min fractions were collected and radioactivity was measured. Recovery for JH III in the HPLC purification step was 94.5 $\pm$ 3.08% ($n = 8$).

RESULTS

Analytical requirements for validation of the radiochemical assay

Previous results obtained in our laboratory from GC–MS–CI–SIM analysis of purified hemolymph extracts of *B. germanica* adult females have shown that JH III is the only JH present within the detection limits of the system (Camps *et al.*, unpublished information). Thus, the *in vitro* study has been based upon the assumption that JH III is the major, if not the only, hormone released by CA of adult females of this species. However, this assumption demanded additional confirmation which was carried out by two distinct procedures.

First, HPLC analysis of a chloroform extract of culture medium after 3 hr incubation of CA from 6-day-old females showed a peak of radioactivity which coincided with that of JH III, whereas no radioactivity was found at zones corresponding to JH I and JH II.

The alternative identification of JH III was performed by mass spectrometry. In this case, after 8 hr incubation of CA from 6-day-old females, analysis of an extract of the radioactive TLC spot corresponding to the JH III zone (radiochromatography monitoring) by the combined GC–MS–CI–SIM technique, focusing ions at m/z 235 (M − 31) and 295 (M + 29) confirmed the presence of the released hormone. However, for the accurate estimation of JH titers, samples required further HPLC purification. Then, quantification of JH III of two samples (namely A and B, see Materials and Methods) by comparison of peaks at m/z 235 and 295 with those at 237 and 297 corresponding to the bisdeuteriated JH III internal standard gave a content of 1.54 and 6.29 ng, respectively, which permitted the evaluation of specific redioactivity for biosynthesized JH III (110 $\pm$ 6 mCi/nmol, $n = 2$). This value was in agreement with the specific radioactivity of tritiated methionine in the medium assuming a stoichiometry of 1 (molar incorporation ratio 1:1).

Release of JH III by the CA in vitro

Before studying the relationship between CA activity and insect age, it was necessary to define the period of linear release of JH III and to ascertain whether the quantity of accumulated JH would be sufficient for routine quantification. With this aim JH III release by CA from 6-day-old females (basal oocyte length = 1.78 $\pm$ 0.15 mm; $n = 17$) was determined at intervals of 3 hr throughout the first 12 hr of incubation, by transferring the glands to fresh radiolabeled medium and analyzing the medium for each interval. A supplementary determination at 24 hr was also carried out (Fig. 1). The comparison of the release rates in the first three periods (0–3/3–6/6–9) showed no statistical significant differences, whereas rates corresponding to the 9–12 hr period (and also to the 12–24 hr) were significantly lower (t-test, $P < 0.001$) when compared to the three preceding intervals. These results indicate that the rate of JH release remained linear during the first 9 hr of incubation.

Furthermore, the release of JH III by CA of *B.*

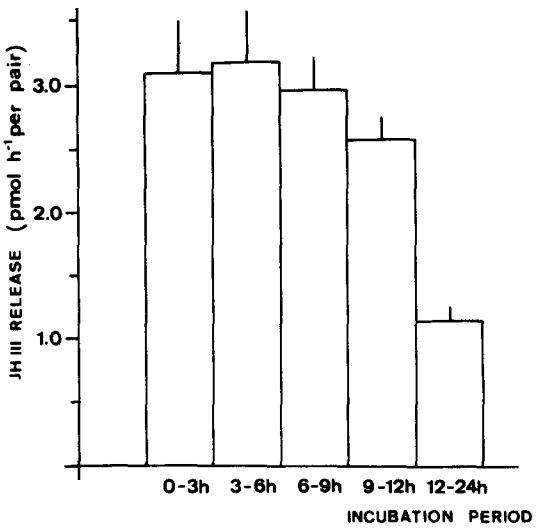

Fig. 1. Rates of JH III release in CA from 6-day-old adult females of *B. germanica* at different periods of incubation. Each column represents the mean of individual determinations of 17 separate pairs of CA. Vertical bars show the standard errors of the mean.

germanica was determined in females of different ages covering the 7 days of the first reproductive cycle (Fig. 2). Rates of JH release increase rapidly from day 3 to day 6, which is correlated with oocyte growth (Fig. 3) (yolk deposition begins between days 2 and 3 and oviposition occurs between days 7 and 8). The highest rates of JH release (2.58 ± 1.11 pmol/hr per pair; $n = 10$) were obtained from 6-day-old females; on day 7 the rate decreased to 1.31 ± 1.27 pmol/hr per pair ($n = 14$); and just before oviposition, rates of JH release were low, as in the previtellogenic stages. We were unable to detect any released methyl farnesoate, the biosynthetic JH precursor. CA volume was also measured at the conclusion of the incubation (Fig. 3) and showed certain parallelism with JH release (Fig. 2). Nevertheless, the correlation between these parameters did not fit a simple linear regression.

DISCUSSION

The results reported show that it is possible to follow the dynamics of JH III biosynthesis by CA from adult female *B. germanica* using the radiochemical *in vitro* techniques first described by Tobe and Pratt (1974). As suggested by Feyereisen (1985), identification of JH III as the hormone produced *in vitro* and demonstration of fixed 1:1 molar incorporation ratio of label from methionine constitute strong criteria for validation of the assay and have been satisfactorily accomplished in this assay.

The highest release rates were obtained with CA from 6-day-old females (2.58 ± 1.11 pmol/hr per pair), but these values are relatively low in comparison with those reported for other species of cockroaches. For example, in *Periplaneta americana*, Weaver and Pratt (1977) found maximum rates of 20–25 pmol/hr per pair in 9-day-old females, and in *Nauphoeta cinerea*, maximum JH release observed was 70–80 pmol/24 hr, using females of the same age (Lanzrein *et al.*, 1978). The case of *Diploptera punctata* is exceptional, since maximum release rates of JH (up to 100 pmol/hr per pair in 4- to 5-day-old females) are the highest observed, not only in the order Dyctioptera but in all species studied to date (cf. Tobe and Stay, 1977).

As stated above, we intend to carry out intensive research on the activity of factors that could modulate JH production as well as the persistence of these effects. The designed experimental model comprises a 2–3 hr of incubation in the presence of a test compound which is preceded and followed by equal periods of incubation in control medium. Since the linearity of JH release along these three periods has been demonstrated, the model can be considered as appropriate and work on this line is now in progress.

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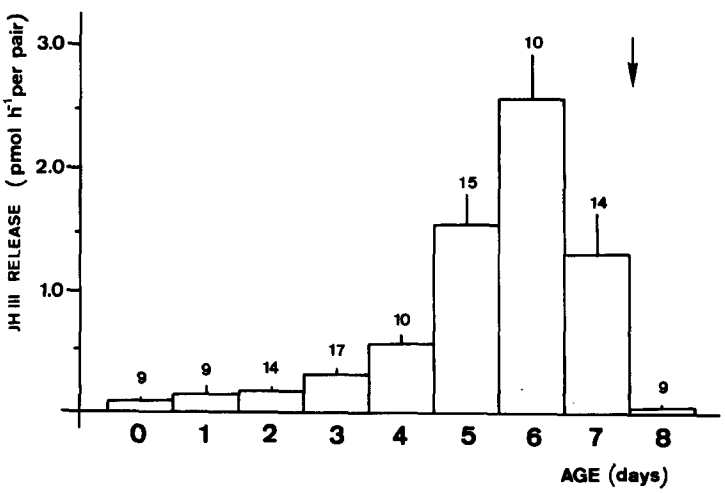

Fig. 2. Rates of release of JH III by CA during the first reproductive cycle of *B. germanica*. The number of individual determinations is indicated at the top of each column. Vertical bars show standard error of the mean. Oviposition time is shown by arrow.

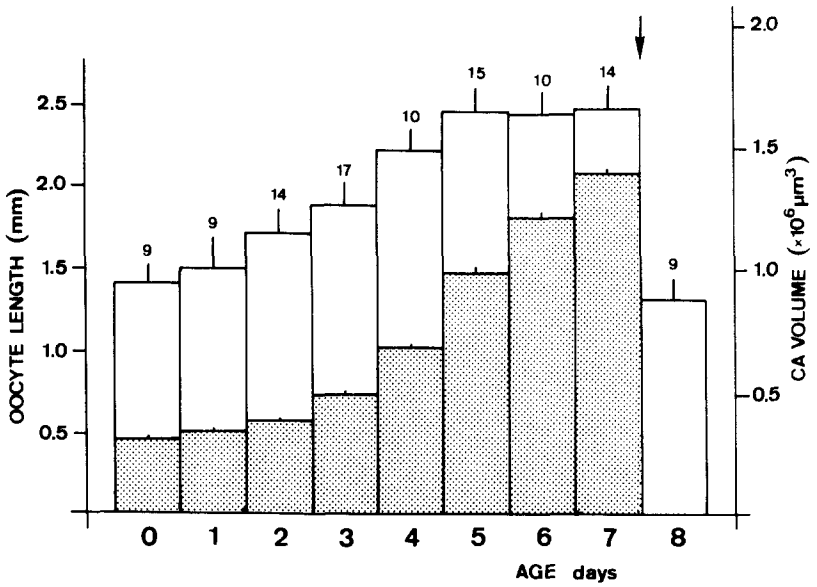

Fig. 3. Oocyte lengths (dotted columns) and CA volume (white columns) corresponding to the specimens of *B. germanica* used in the experiments for determination of the JH release rates during the first reproductive cycle. The number of individual determinations, the standard error of the mean and the oviposition time are indicated as in Fig. 2.

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