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Variability in Juvenile Hormone production by locust corpora allata kept *in vitro* for long periods

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Abstract. Juvenile Hormone III (JH-III) production by corpora allata (CA) of sexually mature female locusts (*Locusta migratoria migratorioides* (R. & F.)) was maintained *in vitro* for up to 30 days in an agar-solidified medium. Hormone production was measured periodically with a short-term radiochemical assay. Low-activity CA increased their activity significantly after 24–48 h incubation in the long-term medium, but high-activity glands did not. Variations in activity were considerable among glands tested on the same day and among measurements from the same gland on different days. Farnesoic acid-stimulated rates of JH-III production were always higher than the basal rates, suggesting that the CA were not maximally activated. However, freshly excised low-activity CA, whose hormone production increased in the long-term conditions, showed similar farnesoic acid-stimulated rates of JH-III production to those of freshly excised high-activity glands, suggesting that at the time of excision of the corpora allata rate-limiting step(s) preceding farnesoic acid biosynthesis were inhibited or refractory to stimulation *in vivo*.

Key words. *Locusta migratoria*, corpora allata, long-term incubation, Juvenile Hormone biosynthesis.

Introduction

The corpora allata (CA) of insects produce Juvenile Hormone (JH) which has various regulatory roles in development and reproductive biology (Engelmann, 1970; Tobe, 1980; Tobe & Stay, 1985). Several publications have shown or implied that the CA can be maintained successfully *in vitro* for rather long periods (Judy *et al.*, 1973a, b, 1975; Müller *et al.*, 1974; Jennings *et al.*, 1975). These authors incubated several glands in the same tube, and pooled the media from different tubes containing glands kept for various time periods, but did not assess the activity of individual glands. In fact, only Judy *et al.* (1973a) provided direct evidence that CA incubated *in vitro* at 20–25°C were producing JH for up to 15 days. More recently, Wilhelm *et al.* (1987) studied activity of individual CA by incubating the glands for up to 4 days, but these authors added haemolymph to the medium.

Studies of CA activity *in vitro* are generally conducted in short-term radiochemical assays, during which JH production is presumed to represent rates *in situ* (Tobe & Stay, 1985). These rates were found to be highly variable between CA taken from sexually mature female locusts, even when the females were at similar physiological stages (Pratt & Pener, 1983; Gadot & Applebaum, 1985). It was suggested that a short-term regulatory nervous mechanism, which is pulsatory in nature, might cause variations in the activity of locust CA (Feyereisen, 1985). Herein we report on long-term incubation of locust CA in conditions that allow maintenance of glands for up to a month *in vitro*, in the absence of any insect-derived factors other than those from the CA. We also report on the variable activity of these glands under the standard conditions of the long-term incubation, as assessed periodically by the short-term radiochemical assay. Some results have already been reported in a conference paper (Gadot *et al.*, 1992).

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Materials and Methods

Animals. Experiments were conducted with sexually mature females, aged 20–30 days after fledging, from a breeding colony of the African migratory locust, *Locusta migratoria migratorioides* (R. & F.). For details on the origin of stock animals see Lazarovici & Pener (1977). Females were kept with males under crowded conditions with continuous illumination at ambient temperatures and were fed Kikuyu grass (*Pennisetum clandestinum*) and flaked oats.

Radiochemical assay of JH production by incubated CA. Assays of JH-III production by the CA were carried out before, and periodically during, the long-term incubation. Corpora allata were dissected under sterile conditions and cleaned thoroughly from attached tissue and nerves. Single glands were placed into the medium with the aid of a steel loop. Usually, eight to eighteen glands excised on the same day, originating from locusts kept in the same cage, were used in each set of experiments. The activities of single CA were monitored under sterile conditions in minimal essential medium for 2–3 h by the radiochemical assay of Pratt & Tobe (1974), as modified by Gadot & Applebaum (1985). Radiolabelled JH-III was extracted from the assay medium with hexane, separated by thin-layer chromatography and quantified by liquid scintillation spectrometry. The proportion of JH-III from the total radioactivity was calculated as the ratio of the radioactivity in the JH-III zone in thin-layer chromatography and the radioactivity in an equivalent aliquot from the same hexane extract (after subtraction of blanks of ^{3}H-methionine). Other more polar radiolabelled compounds, presumably JH-III diol or compounds that co-migrated with a JH-III diol standard on thin-layer chromatography, were also detected in the hexane extracts by the thin-layer chromatography as were previously shown in incubation medium of fresh locust CA (Gadot & Applebaum, 1985; Gadot *et al.*, 1987). Farnesoic acid (70% pure, Sandoz Crop Protection, California, U.S.A.)-stimulated rates of JH-III production, which represent the maximum capacity of the CA to produce JH-III, were measured on several occasions before and during the long-term incubation. Farnesoic acid is a late precursor of JH-III and therefore earlier rate-limiting step(s) in JH-III production are bypassed (Tobe & Pratt, 1976). Corpora allata were incubated for 2–3 h in the short-term assay medium fortified with 100 μM farnesoic acid, after determination of basal rates of JH-III production (Gadot & Applebaum, 1986).

Long-term incubation. A jelly-like enriched medium was prepared fresh by mixing 100 μl of L-15B medium (Munderloh & Kurtti, 1989; with minor modifications as shown in Appendix A), fortified with glucose, tryptose phosphate broth (Difco), fetal bovine serum and gentamicine, with 200 μl of modified minimal essential medium, containing 1% agar in a 2 cm diameter watch glass. The final concentration of gentamicine in the incubation medium was 50 μg/ml, that of tryptose phosphate broth and fetal bovine serum was 6.7% each, and the agar concentration was 0.67%. This medium is denoted below as long-term medium. Stock solutions were prepared in advance and kept as indicated in Appendix A. Watch glasses with prepared media were either used immediately or kept in sterile conditions at 4°C and high humidity for short periods (2–3 days) before use. With the aid of a steel loop, a single CA was placed into the cooled solidified medium and the watch glass immediately covered with a tightly fitting glass cover which was sealed completely with hot paraffin to prevent evaporation of the medium. The CA were then incubated at 33°C. Periodically, the CA were removed from the long-term medium, assayed for JH-III production in minimal essential medium by the radiochemical assay (see above) and then returned to fresh long-term medium.

Statistical analysis. Non-parametric tests were employed in all cases where normality and/or homogeneity of variance could not be established (Siegel, 1956).

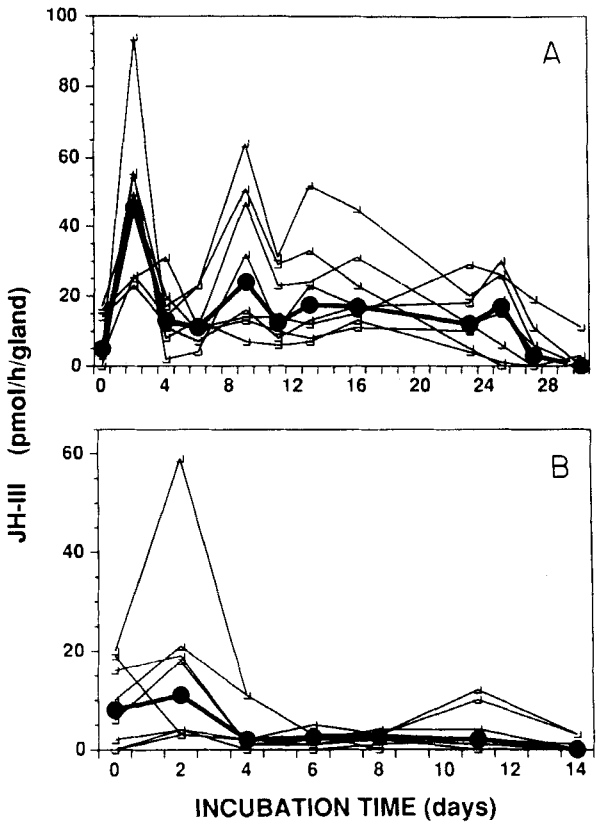

Fig. 1. Intra- and inter-gland variability in rates of JH-III production during long-term incubation of the CA. Each point (small squares) represents the rate of a single gland from the same set of experiments as measured by the radiochemical assay. Lines connect all the data-points for each gland. Medians (large dots connected by bold lines) are indicated for each age. (A) A set of experiments with glands showing relatively high rates of JH-III production. (B) Another set of experiments terminated after 14 days, with glands showing relatively low rates of JH-III production.

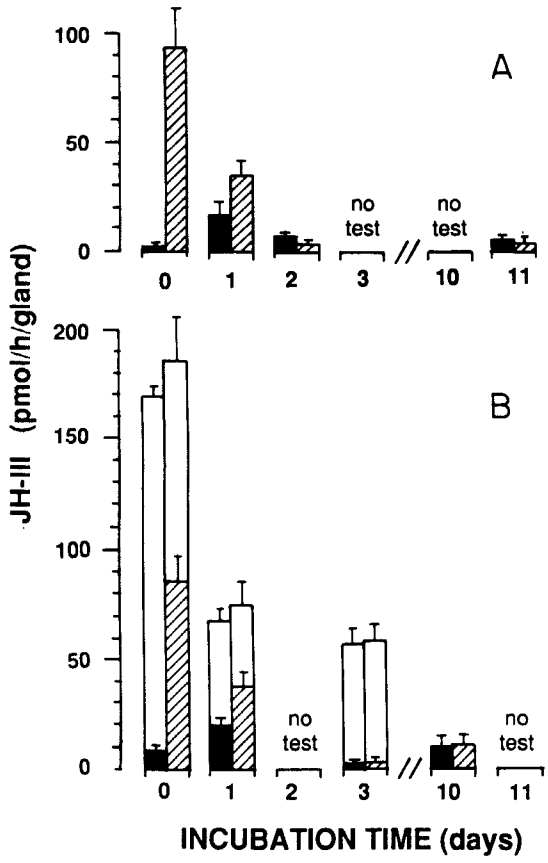

Fig. 2. Rates of JH-III production by CA in long-term incubation; averages (columns) and SE (bars on top of columns) are shown. Results are grouped according to the rate obtained in freshly excised (day 0) CA. Glands producing less than 20 pmol/h/gland on day 0 were taken as a group of 'initially low-activity' glands (black columns) and those producing more than this amount were taken as a group of 'initially high-activity' ones (striped columns). In each group and each set, the same individual glands were tested by the radiochemical assay on different days. (A) A set of experiments; $n = 5$ and $n = 3$, for initially low- and high-activity glands, respectively. (B) Another set of experiments; $n = 8$ and $n = 10$ for initally low- and high-activity glands, respectively. In this set, farnesoic acid-stimulated increase in the rates of JH-III production for days 0, 1 and 3 are also shown by white columns. Both A and B present selected sets of experiments with the highest proportion of initially high-activity glands.

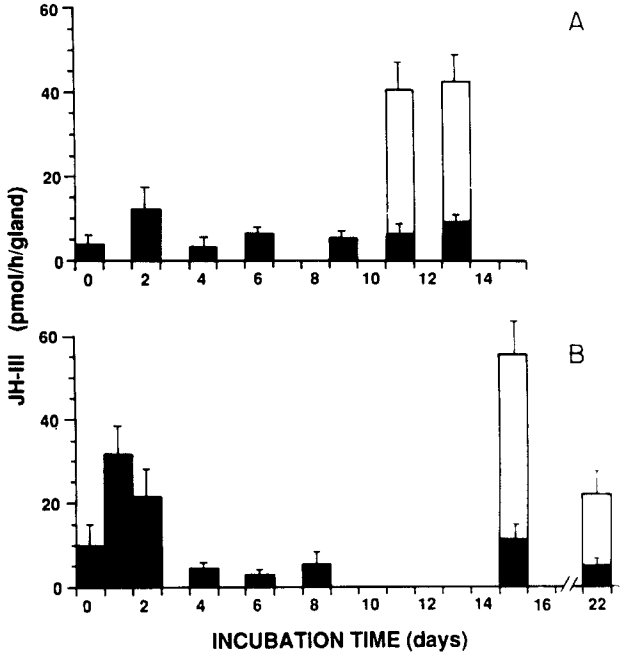

Fig. 3. Basal and farnesoic acid-stimulated rates of JH-III production by CA kept in long-term incubation and tested periodically by radiochemical assay; averages (columns) and SE (bars on top of columns) are shown. Black columns represent basal rates; white columns upon black ones represent farnesoic acid-stimulated increase in the rates. (A) A set of experiments in which farnesoic acid-stimulated rates were tested on day 11 and 13; $n = 8$. (B) Another set in which these rates were tested on day 15 and 22; $n = 8$.

Results

Corpora allata continued to produce JH-III for up to 30 days in long-term incubation (Fig. 1A). However, the rates of JH-III production were highly variable between glands and between sets of experiments (Figs 1 and 2) despite all CA being exposed to the same conditions *in vitro*. Analysis of variance showed that between-gland variability (for the eight glands in each set of experiments in Fig. 3) and within-gland variability (for the entire period) were both significant (Friedman two-way analysis of variance, $P < 0.001$). Thus the variation between glands and the variation in the activity pattern of each gland were independent of the external conditions. Usually both the right and left CA from the same female were used (occasionally one gland was lost), but they did not show similar patterns of activity, as demonstrated previously for freshly excised locust CA (Tobe, 1977). Juvenile Hormone-III production by freshly excised glands from 20–30-day-old females (day 0 of the experiment) was also highly variable, ranging from 0 to over 140 pmol/h/gland, and exhibited a skewed distribution, as also found by others (Pratt & Pener, 1983; Dale & Tobe, 1988). A clear pattern of CA activation was seen *in vitro* in some CA that had low activity on day 0, 20–30 days after the imaginal moult (see especially Fig. 1A). After 24–48 h these CA exhibited significantly higher activity than on day 0 (randomization test for matched pairs, $P < 0.01$). Subsequently, their activity fluctuated irregularly (Fig. 1A and B) with no discernible pattern (Friedman two-way analysis of variance, $P < 0.001$). In some glands, however, activation of the CA was not evident after 24 and/or 48 h in the long-term medium. Close examination of the data from other sets of experiments revealed that only glands that released JH at low rates on day 0 (less than 20 pmol/h/gland) had higher rates of JH production after a day or two in the long-term medium, whereas glands that were highly active on day 0

exhibited rapid declines in activity 24 h later (Fig. 2). Juvenile Hormone-III accounted for about half of the radioactivity extracted with hexane from the assay medium of all fresh glands and for 60–70% of the radioactivity for those kept in long-term incubation for various periods.

The rates of JH-III production by CA that are supplied with farnesoic acid are presumed to reflect the maximal synthetic capacity of the glands because the last two steps in JH-III production are not thought to be rate-limiting (Tobe & Pratt, 1976). We measured the farnesoic acid-stimulated rates of JH-III production at different times before and periodically during long-term incubation. In all cases the farnesoic acid-stimulated rates of JH-III production were higher than the basal rates (Figs 2B and 3). The farnesoic acid-stimulated rates also served as an assay for gland viability when basal activity was low. The progressive decrease in farnesoic acid-stimulated rates during incubation (Figs 2B and 3B) suggests that these conditions may not be ideal for maximal function of the CA.

Discussion

This paper describes specific conditions for long-term incubation of locust CA using an agar-solidified enriched medium. This medium may be more suitable than liquid medium because it provides a semi-solid matrix in which the gland is embedded and the gland has free access to oxygen from the enclosed humidified space above it (Lasnitzki, 1986). Enrichment of the incubation medium with fetal bovine serum and tryptose phosphate broth added many unknown factors and trace elements that may be necessary for long-term incubation, as in the maintenance of tick cells in culture (Munderloh & Kurtti, 1989). Although the long-term incubation medium is biochemically undefined, it does not contain any insect tissue or haemolymph except for the CA itself, which was carefully cleaned from all other tissue and nerve endings before incubation. Thus, regulatory humoral factors that exert control on CA activity *in vivo* or in haemolymph-containing media (Wilhelm *et al.*, 1987) are excluded from our long-term incubations, though we cannot eliminate the remote possibility that the non-defined components in the medium may contain similar essential factors to those in insect haemolymph. In these conditions, we were able to demonstrate that locust CA from sexually mature adult females can maintain their JH-III producing activity *in vitro* for up to 4 weeks. However, the progressive decline in basal activity and farnesoic acid-stimulated activity of the CA indicates that these incubation conditions are not optimal and should be improved in future work.

Variations between CA in both the amplitude and pattern of JH-III production continue during long-term incubation, suggesting that each gland has an endogenous rhythm of activity that is only tuned or synchronized by external factors. However, non-active CA from newly emerged sexually immature adult females could not be activated in these conditions (Gadot, Pener and Schal, unpublished). It seems that in this case, a 'maturation factor', originating outside of the CA, is needed for the initial activation of the CA, which can later become partially autonomous. Glands that exhibited rather low initial rates of JH production were significantly activated after 1–2 days, whereas glands with initial high activity were not further activated and their activity declined. These findings indicate some removal of inhibition *in vivo* and/or some activation *in vitro* of those glands that showed low activity immediately after their excision. Dale & Tobe (1988) found a somewhat similar delayed and prolonged activation of locust CA *in vitro* in response to high potassium concentrations. They speculated that potassium induced the release of neurohormone or neurotransmitter from nerve endings within the CA or that it caused depolarization of CA cells which stimulated JH prouction. It is possible that the long-term medium contains a similar non-specific activating factor. The heterogeneity in the activity pattern of the CA and the fact that highly active glands are not activated further in long-term incubation support the hypothesis that the CA are producing JH in a pulsatile manner. This may result from both endogenous cyclic changes in the capacity of the glands to respond to stimulation and/or periodic release of allatotropin from neurosecretory endings within the glands (Dale & Tobe, 1988).

The pattern of farnesoic acid-stimulated JH-III production suggests that the glands are not maximally stimulated at any stage during the long-term incubation, since these rates were always higher than the basal rates of JH-III production. However, the very low basal rates of some freshly excised glands which exhibited high farnesoic acid-stimulated rates, may indicate that these glands were either inhibited *in vivo* or were temporarily refractive to stimulation. In either case, the inhibition/stimulation of JH-III production mainly affected rate-limiting step(s) preceding the synthesis of farnesoic acid, although some fluctuations in the maximal capacity of the CA were also evident.

Long-term incubation may provide a promising assay system for identifying long-term regulatory factors of the CA, especially those responsible for the initial activation of the inactive CA of newly fledged adults. If established for such insects which show markedly lower individual variations in the *in vitro* JH biosynthetic activity of the CA than locusts, it may also serve as an assay system for allatotropins and allatostatins exerting medium- or short-term effects on the CA.

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Appendix A. Media for long-term incubation

Stock solutions for media 1 and 2
(modified from Munderloh & Kurtti, 1989)

Stock solution A	
Double distilled water	100 ml
$CoCl \cdot 6H_2O$	20 mg
$CuSO_4 \cdot 5H_2O$	20 mg
$MnCl_2 \cdot 4H_2O$ (substitute for $MnSO_4 \cdot H_2O$)	160 mg
$ZnSO_4 \cdot 7H_2O$	200 mg
Stock solution B	
Double distilled water	100 ml
$Na_2MoO_4 \cdot 2H_2O$ (Substitute for $NaMoO_4 \cdot 2H_2O$)	20 mg
Stock solution C	
Double distilled water	100 ml
Na_2Se (substitute for Na_2SeO_3)	20 mg
Stock solution D	
Double distilled water	20 ml
Reduced glutathione	200 mg
Vitamin C	200 mg
$FeSO_4 \cdot 7H_2O$	10 mg
Stock solution A	200 µl
Stock solution B	200 µl
Stock solution C	200 µl
Stock solution E	
Double distilled water	100 ml
p-Aminobenzoic acid	100 mg
Cyanocobalamine (B12)	50 mg
d-Biotin	10 mg

Solutions were prepared in the order listed above and stored in aliquots at −20°C.

Medium 1: L-15B	
L-15 liquid medium	100 ml

L-aspartic acid	30 mg
L-glutamic acid	50 mg
L-glutamine	29 mg
L-proline	30 mg
α-Ketoglutaric acid	30 mg
D-glucose	900 mg*
Stock solution D	100 µl
Stock soultion E	100 µl

* to give final concentration of 50 mM in medium

Medium 2: Minimal essential medium-B (2×)

Double distilled water	75.6 ml
10 × Minimal essential medium (w/o sodium bicarbonate or glutamine)	20.0 ml
Hepes buffer	4.0 ml

All other components (except for L-15 medium) as in medium 1, in double amounts.

Medium 3: Tryptose phosphate broth (from Difco)

Double distilled water	100 ml
Bacto-Tryptose	2 g
Bacto-Dextrose	200 mg
NaCl	500 mg
Na_2PO_4	250 mg

For all three media the pH is adjusted to 7.2–7.3 with NaOH, and media filter-sterilized and stored at 4°C in 10 ml aliquots.

Preparation of long-term incubation agar media (adapted from Lasnitzki, 1986), for eighteen sterilized small glass dishes (about 2 cm inner diameter): (A) In sterile conditions, mix: 0.8 ml Medium 1, 0.6 ml fetal bovine serum, 0.6 ml Medium 3, and 0.3 mg gentamicine. (B) 2 ml 2% agar is sterilized by autoclave and heated to 90°C prior preparation, kept at 40–50°C for a short time until used, cooled to 37°C and mixed quickly with 2 ml Medium 2 (minimal essential medium-B (2×)) and dispensed in 200 µl aliquots into dishes. Before solidifying, 100 µl of mixture (A) is added and mixed in each dish. (C) After cooling, the cleaned corpus allatum is placed upon the solidified medium and the dish is covered with an airtight glass cover and sealed with molten paraffin.