Corpus cardiacum – a target for azadirachtin

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Summary. Azadirachtin A, an insect growth inhibitor derived from neem seed, when injected at a physiological dose, inhibits the hormonally controlled ovarian development in *Locusta migratoria*. Its tritiated dihydro derivative concentrates more in the corpus cardiacum than in the brain. Translocation and release of the neurosecretory proteins labeled with L-[^{35}S]-cysteine in the corpus cardiacum is very poor in locusts under azadirachtin stress. It is concluded that azadirachtin may influence the release of trophic hormones from the corpus cardiacum leading to alterations in timing and titer of morphogenetic hormone pools.
Key words. Locusta migratoria; azadirachtin A; corpus cardiacum; neurosecretory protein.

Azadirachtin is a tetranortriterpenoid whose structure has been unambiguously determined recently[1]. At least four isomers (A – D) are known to occur in neem seed kernels[2, 3]. Azadirachtins A and B have identical biological activity[4]. This group of isomeric compounds has attracted attention mainly because of its antifeedant and growth disrupting properties for a wide variety of insect species[5–10]. Administration of this drug to locusts through their food is not possible, since it is a strong antifeedant. However, the growth-inhibiting effects of injected azadirachtin are well documented[11–14]. In insects like *Rhodnius prolixus* and *Epilachna varivestis*, azadirachtin is not an antifeedant and hence can be administered through food. In these cases, very low doses are sufficient for growth disruption compared to those needed for feeding inhibition[15, 16]. So far it is not clear whether azadirachtin, when injected into locusts, also causes feeding inhibition. The present data clarify that injection of azadirachtin does not lead to starvation. Not much is known about its exact mode of action though physiological studies show alterations in morphogenetic hormone pools[11–13, 17]. However, tritiated dihydroazadirachtin A, which induces the same biological effects as azadirachtin A[2], mainly concentrates in the Malpighian tubules[18]. Influence of azadirachtin A on endocrine centers is yet to be investigated. In this communication we show the recovery of free dihydroazadirachtin A from the endocrine centers of *Locusta migratoria*, and correlate this with the influence of azadirachtin A on neurohormone release, following the incorporation of L-[^{35}S]-cysteine into neurosecretory protein.

Methods. The experimental insects were derived from gregarious colonies of the migratory locust, *L. migratoria*, maintained under laboratory conditions as already described[11]. For the feeding experiment, individual insects were transferred on day 3 to plastic containers (18 × 13 × 6 cm) covered by a mesh. The control and the treated insects were offered *ad libitum* tender wheat seedlings and bran, whereas only tap water was provided to insects under starvation conditions. Data on body weight and feces produced were collected up to day 10.

Azadirachtin A was purified from neem seed kernels following standard procedures[2]. It was dissolved in 10% ethanol-water and injected under CO_2 anesthesia at a dose of 2.5 µg/g into female adults on the third day after emergence. Control insects were injected with an equal volume of 10% ethanol-water.

[22,23-$^3H_2$]Dihydroazadirachtin A (sp. act. 18.7 Ci/mmol) was prepared[4, 18] and dissolved in 10% ethanol-water. Day-3 adult females were given a dose of 2.5 µg/g b. wt. Dissected tissues were oxidized in a sample oxidizer (Packard) 5 days after injection and the tritiated water was counted in Oxisolve-T (Zinsser, FRG) using a Kontron Analytical Scintillation Counter (BetaMatic Plus).

Incorporation of L-[^{35}S]cysteine into neurosecretory protein of the corpus cardiacum, translocated from the neurosecretory cells of the brain, was investigated in azadirachtin A treated (3 µg/g on day 3) and control females of the same age (day 10). The labeled amino acid (sp. act. 80–120 mCi/mmol, Amersham) was diluted in locust saline containing unlabeled cysteine and injected at a dose of 0.31 mg/g (3 µCi). The storage lobes of the corpus cardiacum were dissected out, washed in locust saline, dissolved in Biolute-S (Zinsser) and counted in Quickszint 501 (Zinsser).

Results. The food consumption and excretion of insects injected with azadirachtin A were compared with those of control and starved insects, during days 3 to 10 after adult emergence. It is clear from table 1 that locusts injected with azadirachtin did not undergo starvation. While the starved insects significantly lost body weight between days 7 and 10, azadirachtin-treated insects continued to feed and produce feces, though there was no significant increase in body weight. The body weight of the control insects increased markedly in this time period. Concomitantly they excreted more feces. Hence on day 10, the azadirachtin-injected insects had a body weight of 1.34 ± 0.18 g as compared to 1.85 ± 0.13 g of normally maturing and of 0.86 g of the starved insects. The mortality due to starvation was as high as 80% on day 10. Examination of the state of ovarian development indicates that while control insects have maturing oocytes (4–5 mm) on day 10, the ovary remains imma-

Table 1. Effect of azadirachtin A on the body weight and fecal production of *Locusta migratoria* females

Days after eclosion	Azadirachtin A group* Body weight (g)	Feces (mg)	Control group** Body weight (g)	Feces (mg)	Starvation group*** Body weight (g)	Feces (mg)
3	1.31 ± 0.10	–	1.27 ± 0.08	–	1.12 ± 0.16	–
6	1.38 ± 0.11	154.3 ± 29.1	1.45 ± 0.08	246.1 ± 34.0	1.06 ± 0.13	–
10	1.34 ± 0.18	135.2 ± 89.9	1.85 ± 0.13	524.1 ± 71.5	0.86[a]	–

The data represent the mean values ± SD. Data on feces are on dry weight bases. * n=15, ** n=8, *** n=10, [a] n=2.

Table 2. Specific incorporation of [22,23-$^3H_2$]dihydroazadirachtin A into tissues of female *Locusta migratoria*

Tissue	pmol dihydroazadirachtin A/mg
Malpighian tubules*	29.60 ± 5.8
Corpus cardiacum**	6.42 ± 3.2
Brain***	2.57 ± 1.6

The values represent the mean (± SD) on the basis of the freeze-dried weight of tissue. * n=5, ** n=4, *** n=15.

Table 3. Translocation and release of L-[^{35}S]cysteine labeled neurosecretory protein by corpus cardiacum of 10-day-old female *Locusta migratoria*. Azadirachtin A: 3 µg/g body weight at day 3; control: same volume of 10% ethanol-water.

Time after injection of the label (h)	pmol L-cysteine/4 storage lobes of corpus cardiacum	
	Azadirachtin	Control
1.5	80.2 ± 16.2	49.5 ± 46.3
2.0	159.7 ± 61.8	20.0 ± 15.2
2.5	79.7 ± 28.7	160.2 ± 34.0
3.0	62.8 ± 21.2	325.9 ± 10.8
3.5	98.4 ± 14.2	335.4 ± 19.9
4.0	106.3 ± 44.2	429.6 ± 7.1
6.0	90.4 ± 24.4	161.3 ± 53.8
8.0	122.0 ± 67.0	158.1 ± 14.7

The values represent the mean of 3 replicates (+ SE).

ture as at day 3 in the case of azadirachtin-treated insects. Hence, the difference in body weight is attributed mainly to ovarian development.

Five days after injection of a physiological dose of 2.5 µg [22,23-$^3H_2$]dihydroazadirachtin A/g (3000 pmol/g) into 3-day-old females, only a minor fraction could be recovered from the tissues (table 2). Most of the radioactivity was identical with the unchanged injected compound as shown by HPTLC. Based on dry weight, Malpighian tubules retained 29.6 pmol/mg. Very minute quantities were recovered from the endocrine centers, namely brain and corpus cardiacum. Though the dry weight of the corpus cardiacum is ten times lower than that of the brain, recovery of this drug from it was twice as high as that from the brain.

Keeping the specific storage of dihydroazadirachtin A in the corpus cardiacum in mind, the turnover of neurosecretory protein labeled with L-[^{35}S]cysteine was investigated (table 3). Azadirachtin A (3 µg/g) was injected at day 3 after the adult molt and L-cysteine (0.31 mg/g) at day 10. The amount of L-cysteine incorporated into the neurosecretory proteins transported from the neurosecretory cells of the brain to the corpus cardiacum increased significantly in control insects 2.5 h after injection of the label. In the next 1.5 h, incorporation of the amino acid reached a maximum of 429.6 pmol/4 storage lobes, followed by a sharp decrease. The amount of L-cysteine in the neurosecretory proteins dropped to 158.1 pmol storage lobes at 8 h after injection. In the course of 2.5–4 h, azadirachtin A-treated insects incorporated 79.7–106.3 pmol L-cysteine/4 storage lobes into the neurosecretory proteins. At 4 h after injection of the label, the control insects had incorporated four times as much of L-cysteine into the neurosecretory proteins, in comparison to the azadirachtin A-treated insects. In contrast to the control, there was only an insignificant clearance of labeled neurosecretory protein from the storage lobes of the corpus cardiacum in the azadirachtin A-treated insects.

Discussion. Azadirachtin is a strong antifeedant to locusts. However, injection of a physiological dose (2.5–3.0 µg/g) does not induce starvation. Such insects feed less and maintain their initial body weight but fail to undergo normal ovarian development. Since in these insects the hemolymph titer of the morphogenetic hormone pools and vitellogenesis are disturbed [13], it is reasonable to assume that azadirachtin interferes with the endocrine control of vitellogenesis. It was also shown for fifth instar *L. migratoria* that feeding inhibition is not the primary cause of growth disruption [11].

In this study, distribution of tritiated dihydroazadirachtin A in certain tissues is studied with a view to understanding its functional significance. When azadirachtin A was administered at a physiological dose (2.5 µg/g), only 0.5 µg/g was stored in the whole body, the rest being eliminated rapidly [18]. Hemolymph is free from azadirachtin A within 24 h after injection. Malpighian tubules account for a major fraction of azadirachtin, the functional significance of which is not fully understood. It is unlikely that it would be released into the ovary where it may have a direct effect on oocyte development, since azadirachtin is stored unchanged (quantitatively and qualitatively) even 15 days after injection, in several tissues including Malpighian tubules [18].

The observation that dihydroazadirachtin A is stored quantitatively more in corpus cardiacum than in brain, brings out its functional significance in the control of endocrine events. The corpus cardiacum in locusts is divided into storage lobes that receive the neurosecretory

axons from brain and glandular lobes whose intrinsic cells produce adipokinetic hormones[19-21]. The corpus cardiacum plays a vital role as a neurohemal organ from which trophic hormones like allatotropin originating in the neurosecretory cells of the brain are released.

The role of allatotropin in activating juvenile hormone synthesis by corpora allata[22], and the gonadotropic activity of juvenile hormone[23, 24] are well documented. Though these trophic hormones have not been chemically identified, there is ample proof that the neurosecretory proteins (carrier/precursor proteins) are rich in cysteine[25, 26]. The study on the turnover of L-[^{35}S]cysteine labeled secretory proteins demonstrates a remarkable difference between the maturing control females and the experimental females, whose ovarian development was inhibited by a single dose of azadirachtin A. In the experimental group, poor turnover is attributed not only to reduced transport from neurosecretory cells but also to its marginal release. Difference in uptake of cysteine could also contribute to poor turnover. However, such a difference in uptake of cysteine into the cerebral neurosecretory cells alone cannot be measured, owing to the impossibility of removing the surrounding non-neurosecretory tissue[26].

Synthesis and release of neurosecretory material are at an equilibrium controlled by feedback regulation operating under normal conditions of development[27]. In addition to recovery of a larger quantity of [22,23-$^{3}H_2$]dihydroazadirachtin A from the corpus cardiacum, histological studies[28] indicate that while this drug does not penetrate the blood-brain barrier efficiently, it covers the entire corpus cardiacum and concentrates more in the neurosecretory axons located in the storage lobes. The poor turnover of the neurosecretory proteins could hence be a result of the interference of azadirachtin A with the mechanism of their release from the corpus cardiacum.

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Regular oscillations in suspensions of a putatively chaotic mutant of *Dictyostelium discoideum*

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Summary. We have tested the light-scattering properties of suspensions of the *Dictyostelium discoideum* mutant *HH201* derived from the mutant *Fr17*. Previous studies indicated that *HH201* and *Fr17* possess highly irregular rhythmic properties which might represent aperiodic oscillations, i.e. chaos. We report that the former mutant can display regular oscillatory behavior. Possible explanations for this result are discussed, including that of a transition from chaotic to periodic behavior resulting from some parameter change or from strong intercellular coupling in cell suspensions.
Key words. Dictyostelium discoideum; cAMP oscillations; biological rhythms; chaos.