Azadirachtin, a Chemical Probe for the Study of Moulting Processes in *Rhodnius prolixus*

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Z. Naturforsch. **41c**, 771–775 (1986); received November 22, 1985/April 1, 1986

Azadirachtin A, *Rhodnius prolixus,* Moulting Inhibition, Mortality, Blood Feeding

The effects of azadirachtin A after injection into timed 4th-instar nymphs of *Rhodnius prolixus* were studied. A dose-response relationship of Aza A was established using moulting inhibition and mortality as effective parameters. Aza A, if injected 1 to 3 days after blood feeding, inhibited the moulting process. However, if injected during and after the onset of epidermal mitosis, ecdysis was not affected. A single dose of Aza A was able to block the onset of mitosis in the epidermis, which is associated with the moulting cycle and is triggered by moulting hormone. Ecdysteroid titres, determined by radioimmunoassay, were too low for an induction of ecdysis in the Aza A treated nymphs. During the peak maximum, the predominant ecdysteroid was 20-hydroxyecdysone. The conjugated ecdysteroids were based on 20-hydroxyecdysone and ecdysone in the same ratio. The significance of these findings in relation to the possibility of Aza A to act either directly on the prothoracic glands or indirectly through releasing sites in the brain or through *Corpus allatum* is discussed.

Azadirachtin (Aza) is a feeding inhibitor and growth disrupting compound for most insect orders. It is present in seeds of the neem tree, *Azadirachta indica* A. Juss [1–5]. The compound disrupts the ecdysteroid-induced moulting processes in holometabolous insects such as *Epilachna varivestis* [6]. In hemimetabolous insects like *Locusta migratoria,* Aza modifies and suppresses the ecdysteroid titre in nymphs [7] and the juvenile hormone (JH) and ecdysteroid titres in adults [8, 9]. In *Rhodnius prolixus,* low doses of Aza, given orally, cause inhibition of both feeding and moulting [10, 11]. The latter effect could be partially or completely reversed by administration of JH or ecdysone, respectively [10]. The former effect could only be counteracted by ATP [11], a phagostimulant for *R. prolixus* [12]. It was then concluded that Aza is effective in regulating the growth of *R. prolixus* by direct interference with its endocrine system and not by an indirect effect through feeding inhibition and starvation [10, 11]. Furthermore, *R. prolixus* nymphs after application of a single dose of Aza were unable to moult for several months [10] or even for more than one year (Garcia & Rembold, unpublished data), although they continued to feed on blood normally. We now injected Aza A [13, 14] into timed 4th-instar nymphs of *R. prolixus* and present evidence that this substance interferes with the moulting process by inhibiting both the onset of epidermal mitosis and the ecdysteroid production in this bloodsucking bug.

Materials and Methods

Cultural conditions

Fourth-instar nymphs of *Rhodnius prolixus* were used throughout this study. Insects were reared and maintained as previously described [15, 16].

Groups of 18–20 4th-instar nymphs, weighing 13.8 ± 0.7 mg each, were starved following ecdysis for 25–30 days. They were then allowed to feed for 30 min on defibrinated rabbit blood by use of a special membrane feeding apparatus [16].

Rabbit blood

Defibrinated rabbit blood, stored at 4 °C for a maximum of 24 h, was used.

Azadirachtin A injection

The 4th-instar nymphs were injected, after a blood meal, 0.5 µl of an Aza A solution in 10 mM phosphate buffered saline (PBS), corresponding to 100–200 ng/nymph. For control, 10 mM PBS,

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0700–0771 $ 01.30/0

pH 7.2, was used. The injection was done with a tapered calibrated glass capillary using a microapplicator (Model 234, Instrumentation Specialities Co, Nebraska, USA) through the membrane joining the metathoracic coxa to the sternite.

Epidermal mitosis

Samples from the dorsal epidermis of the abdominal segments were fixed in Carnoy solution for 2 h, hematoxilin stained, followed by dehydration in ethanol, clearing in xylene and mounting on microscopic slides. Mitoses were counted in 4 separate regions of each epidermal sample. Each region comprised approximately 600 to 800 cells. Four replicate samples were observed for each day and the mitotic index was calculated.

Ecdysteroids

For quantification of total and of conjugated ecdysteroids, haemolymph was taken from a puncture in the legs with a 5 µl micropipette. Protein was precipitated by injection of this haemolymph into 1 ml methanol. From the supernatant 0.5 ml were used for total ecdysteroid measurement and 0.5 ml for analysis of highly polar (conjugated) ecdysteroids. Ecdysone antiserum ICT-1 was a gift from K. D. Spindler (University of Düsseldorf), (23,24-^{3}H)ecdysone purchased from NEN/Dreieichenhain and used as standard for the RIA [17, 18]. The total ecdysteroids were quantified in the methanol extract directly. For measurement of conjugated ecdysteroids, 0.5 ml water was added to the methanol extract which was extracted with 1 ml methylene chloride. The aqueous phase was then diluted to 30% methanol with water, filtered through 0.5 g LiChroprep RP-8 (Merck), which was twice washed with 2 ml 30% methanol in water for complete elution of the highly polar ecdysteroids. Ecdysone and 20-hydroxyecdysone were eluted with 3 ml methanol. The fraction containing the conjugated ecdysteroids was brought to dryness in vacuo, redissolved in 2 ml 0.05 M citrate buffer, pH 5.25, and incubated with 10^3 units of *H. pomatia* esterase (37 °C, 17 h). For separation of ecdysone and 20-hydroxyecdysone the method as described by Sieber & Rembold [7] was used.

Results and Discussion

Single injection of Aza A into 4th-instar nymphs 2 days after feeding induced irreversible effects. Whereas 100% of the control nymphs moulted between 13–15 days after feeding, the groups of those insects having received 5 and 10 ng Aza A/nymph showed an extended growth period and only few of them underwent ecdysis (Table I and Fig. 1). With Aza A doses exceeding 50 ng/nymph, mortality was high and thus indicating the border of acute toxicity.

Azadirachtin A induced a drastic moulting inhibition when administered on days 1, 2 or 3 after feeding (Fig. 2). The intermoult period was extended to more than 30 days, always related to the moulting inhibition, and no more than 30% of the injected nymphs died. Controls moulted between days 13 and 15 after feeding by 100%. The nymphs injected with Aza A between days 7 and 13 after feeding moulted, and the adults did not differ morphologically from the controls. Nymphs treated on days 7 (30%), 8 (15%) and 9 (15%), died during the ecdysis process which was initiated but those animals were unable to shed off the old cuticle successfully. Ecdysis of the

Table I. Effects of different doses of azadirachtin A injected 2 days after a blood meal into 4th-instar nymphs of *Rhodnius prolixus*. The period between moulting of control and treated nymphs is indicated.

Doses [ng Aza A/nymph]	Days from feeding to moult (range)	Percentage of ecdysis during first 3 days after beginning of moulting in controls	Total of moulted nymphs [%]
Control	13–15	100	100
1	13–18	40	65
5	14–20	25	30
10	16–24	0	0
25	0	0	0
50	0	0	0

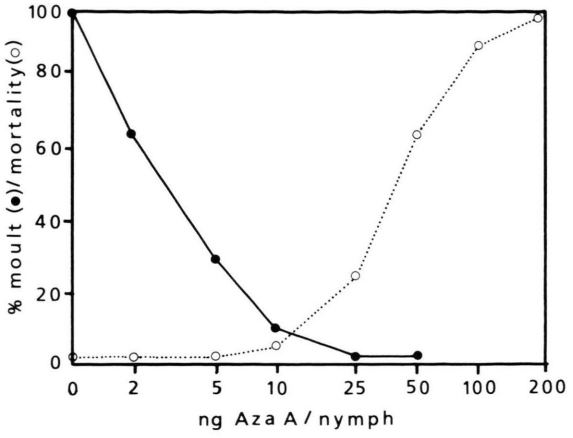

Fig. 1. Moult inhibition (●) and mortality (○) of *Rhodnius prolixus* 4th-instar nymphs after injection of azadirachtin A 2 days after bloodfeeding. Each point represents data from an experimental group of 18–20 insects. For more details see under Materials and Methods.

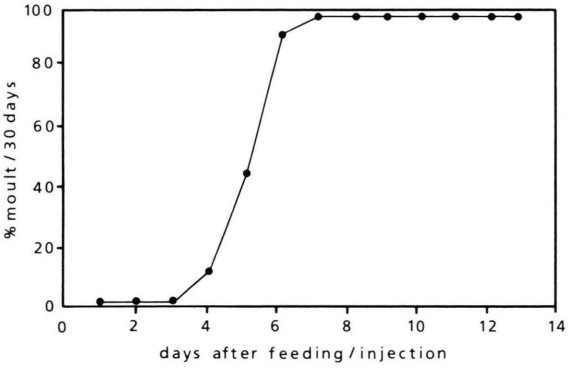

Fig. 2. Effect of azadirachtin A (15 ng/nymph) on moulting inhibition of *R. prolixus* 4th-instar nymphs. Controls moulted between days 13 to 15 after the last blood meal. Percentage of moults was calculated 30 days after feeding. Experimental details as described in Fig. 1.

groups which were injected on days 4 to 6 was partially inhibited. Moulting is related to the onset of epidermal cellular changes which end in ecdysis [19]. We followed the epidermal mitotic activity as an objective measure of the Aza A effect on the moulting process. Two groups of 120 nymphs each were injected, 1 day after feeding, with PBS (controls) or with 15 ng Aza A/nymph in PBS. A rapid increase in mitotic activity was initiated in the control after the 3rd day as shown after plotting the mitotic indices against days after feeding (Fig. 3). These indices remained elevated in the control between days 4 and 6 and then declined to low levels after day 7. In the Aza A-treated nymphs epidermal mitoses, after a minute increase during days 4 and 5, remained on background levels.

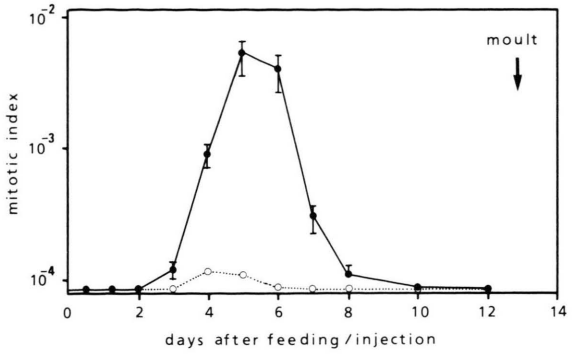

Fig. 3. Effect of azadirachtin A (15 ng/nymph) on mitotic activity of *R. prolixus* 4th-instar nymphs. Controls (●), Aza A injected (○). Each point represents the mean mitotic index of cuticles from 4 animals. The bars indicate standard deviation. For more details see under Materials and Methods.

Blockade of the epidermal mitosis by Aza A reflects an effect on the ecdysteroid titres (Fig. 4). The amount of ecdysteroids in control haemolymph was very low (below 50 pg/µl) during the first days. From day 5 onwards the titre steadily increased to a peak of about 800 pg/µl haemolymph, just after the peak of epidermal mitosis (Fig. 3). From day 6

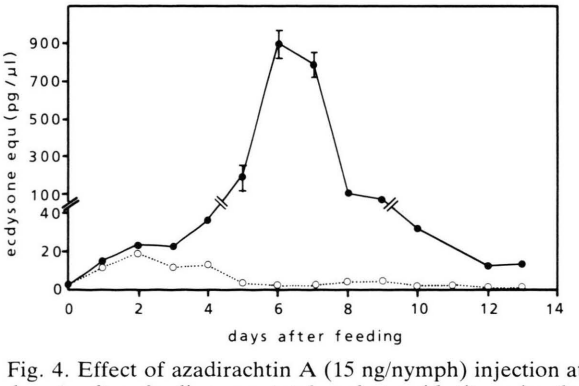

Fig. 4. Effect of azadirachtin A (15 ng/nymph) injection at day 1 after feeding on total ecdysteroid titres in the haemolymph of *R. prolixus* 4th-instar nymphs. Controls (●), Aza A injected (○). Each point represents the mean of 2 samples or mean standard deviation ($n = 4$). For more details see Fig. 5.

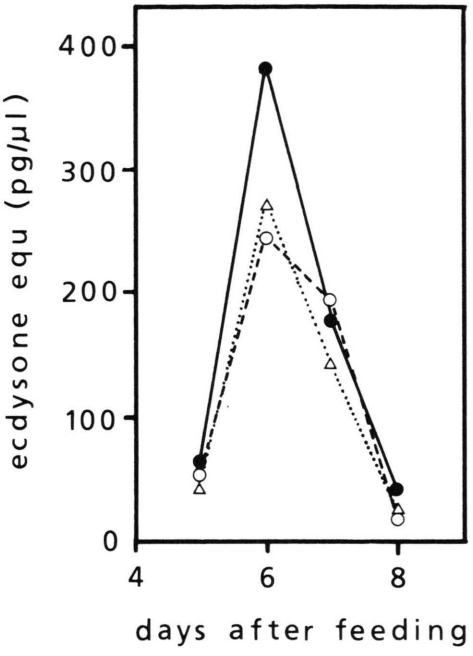

Fig. 5. Highly polar (conjugated) and free ecdysteroids in the control group of *R. prolixus* 4th-instar nymphs during peak of total ecdysteroids (see Fig. 4). Conjugated ecdysteroids (○), ecdysone (△) and 20-hydroxyecdysone (●). Each point is based on data from pooled haemolymph collected from 3–5 nymphs.

onwards it began to decrease reaching a background level before day 10 after feeding which was maintained until ecdysis on day 13. In those treated nymphs which were unable to initiate epidermal mitosis (Fig. 3) and moulting (Fig. 2), the ecdysteroid titres were negligible and remained at low level during the whole experimental period (Fig. 4). Qualitative and quantitative analyses of the haemolymph ecdysteroid RIA activity, during the peak maximum (days 5 to 8 after feeding), revealed the presence of highly polar conjugated ecdysteroids together with 20-hydroxyecdysone and ecdysone. Ratio analysis from day 6 nymphs showed that 20-hydroxyecdysone was the predominant ecdysteroid in the peak maximum of the control nymphs. On the other days, the ratio of conjugated ecdysteroids, 20-hydroxyecdysone and ecdysone was practically the same (Fig. 5). Identification of the conjugated ecdysteroid after β-glucuronidase hydrolysis revealed that 20-hydroxyecdysone and ecdysone were present as conjugates in a ratio of 1:1 during the peak of total ecdysteroid in control haemolymph.

The present data demonstrate the usefulness of azadirachtin as a chemical probe for the insect endocrinologist. A single injection of as little as 2 ng Aza A per nymph was enough for a 50% moult inhibition without any visible toxic effects even during an extended larval period of more than one year. The half effective dose of mortality (within 30 days) was with 40 ng/nymph 20-fold higher than the dose needed for 50% moult inhibition. Fig. 1 shows that even an ED-90 (10 ng/nymph) is accompanied by not more than 5% mortality and for a 95% inhibition of development a dose of 15 ng/nymph is accompanied by a mortality of only 10%. Using this 95% dose, a sharp time-dependent drop in the sensitivity of *R. prolixus* nymphs against Aza A can be titrated with a 50% effective time 5 days after injection (which was done short after feeding) and a 90% effective time 1 day later (Fig. 2). Between days 5 and 6, the regulatory program for controlling the next moult is switched on already, as demonstrated by the maximum of mitosis (Fig. 3) and of total ecdysteroids (Fig. 4) in the untreated control. Injection of Aza A (15 ng/nymph) on day 1 after feeding completely abolished mitosis and its control by a high ecdysteroid haemolymph titre. There is practically no moulting hormone present after the single Aza A injection except the low prepeak during days 3–4 (Fig. 4) which is possibly reflected by an extremely low raise of the mitotic index during days 4 and 5 (Fig. 3). Whereas the control nymphs moulted around days 13–15, with a dose of 10 ng Aza per nymph not a single one moulted during the normal period from 13–15 days and only 2 out of 20 within 30 days (Table I). All the others survived as 4th-instar nymphs without ecdysis. The close correlation of ecdysone titre and mitotic activity of the epidermal cells explains other observations [20] which correlate *R. prolixus* growth with the secretory activity of brain neurosecretory cells. Mitosis is not thought, therefore, to be a direct response to moulting hormone but much more to occur in such cells only which previously have been activated. Then, epidermal mitosis occurs at the end of a low but sustained level of ecdysteroids and the tissue must be continuously exposed to ecdysteroids [21, 22]. This is exactly proven by use of Aza A as a chemical probe. The fact of Aza to be effective as ecdysis inhibitor only before the onset of epidermal mitosis supports the view of ecdysone stimulating the deposition of a new cuticle [19, 21–24]. This indirect effect of Aza on moulting

can be reversed by ecdysone therapy [10]. Together with the present data our hypothesis is supported that Aza blocks ecdysone synthesis through the switching off neuroendocrine stimulation of prothoracic cells. It should also be emphasized that inhibition in such tissues of *L. migratoria* other than the prothoracic glands [8, 9] and of JH synthesis in adults [9] suggests an action of Aza on the neurosecretory centres. Furthermore, application of a JH analogue in Aza-treated *R. prolixus* nymphs also reverses the inhibition of ecdysis [10]. This effect remains unexplained since the prothoracicotropic effect of JH, as described in certain insects [25–27], has not yet been shown in *R. prolixus*.

Acknowledgements

E. S. G. is grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, and Max-Planck-Gesellschaft, FR Germany, for a fellowship.

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