Tissue-Specific Incorporation of Azadirachtin in the Malpighian Tubules of *Locusta migratoria*

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The retention, tissue distribution, and localization of [22,23-$^3H_2$]dihydroazadirachtin A was investigated in the female locust, *Locusta migratoria*. A constant quantity of 0.4 to 0.5 μg of dihydroazadirachtin A per gram body weight is recovered in the unchanged form 5 days after injection of any of the physiologically effective doses in the range of 1.5 to 3.0 μg/g. Malpighian tubules account for 73.7% of the amount of dihydroazadirachtin A which is retained in the whole body. In the Malpighian tubules it is localized in the basal and inner regions. It is inferred from these results, that dihydroazadirachtin A acts in its unchanged form through a high-affinity binding to organ-specific membrane receptors.

Introduction

Out of the biologically active compounds which have been isolated from seed of the neem tree, *Azadirachta indica,* the tetranortriterpenoid azadirachtin is the most active insect growth inhibiting compound. It elicits strong antifeedant and growth disrupting properties to insects of several orders [1–5]. More recently, four azadirachtins (A–D) were isolated [6] out of which azadirachtin A and B are the major components with similar biological activity. The chemical structures of both these compounds are established [7–10]. Reports published during the recent years [4, 11–15] suggest that azadirachtin disturbs moulting and egg maturation processes in insects when injected at microgram levels, primarily caused by changes in the hormone pool. However, not much is known about the direct mode of action, biodegradation and the nature of binding to the tissue. Hydrogenation of azadirachtin A [16] facilitates tritium labelling [6, 17] of the molecule. [22,23]Dihydroazadirachtin has the same biological activity as azadirachtin A which opens the possibility of studies on the biochemical mode of azadirachtin action by use of its [22,23-$^3H_2$]dihydro derivative. In an earlier study, both the excretion pattern and the biological half life of this compound were reported [6]. We now present further information on storage, organ distribution and histological localization of [22,23-$^3H_2$]dihydroazadirachtin A in the African migratory locust, *Locusta migratoria.* These studies enlighten the organ specific concentration of this compound in Malpighian tubules which are the most important organs involved in excretion and in maintenance of a homeostatic internal environment in the insects.

Materials and Methods

Insects

Gregarious colonies of *Locusta migratoria* were maintained at 40% RH and 34 °C (12 h light), and 24 °C (12 h dark). The insects were fed on wheat seedlings and bran.

Tritiation of azadirachtin A

In a 2 ml round flask, azadirachtin A (7.6 mg; 10.6 μmol) was dissolved in 1 ml dry ethyl acetate. As catalyst, 3.5 mg 10% palladium on charcoal were added. In a vacuum of 10^{-5} Torr, the suspension was hydrogenated with 1 Ci tritium gas (22 μmol) for 4 h. The reaction mixture was filtered, and the filter-paper rinsed five times with 5 ml portions of methanol. Filtrate and wash solutions were combined and evaporated to dryness. Purification of [22,23-$^3H_2$]dihydroazadirachtin followed the procedure as already described [6, 17]. Yield was 3.2 mg (42% of theory), with a specific radioactivity of 18.7 Ci/mmol.

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Application of [22,23-$^3H_2$]dihydroazadirachtin

The compound was dissolved in 10% ethanol/water to a concentration of 1 mg/ml (25.75 mCi/ml). Injections were made through the third abdominal inter-segmental membrane, using a Hamilton microliter syringe. Whole insects or organs, dissected in locust saline [13], were oxidized in a sample oxidizer (Packard), and tritiated water was measured in Monophase-plus (Packard), using a Kontron Analytical Scintillation Counter (BetaMatic BASIC). Quench corrections were made by internal standardization.

Extraction of [22,23-$^3H_2$]dihydroazadirachtin from tissues

Malpighian tubules and fat bodies, respectively, from adult females ($n=35$) which were injected 2.5 μg [22,23-$^3H_2$]dihydroazadirachtin/g (53.15 μCi/g), were dissected 5 days later. The pooled tissues were homogenized in 5 ml ice-cold buffer (50 mM Tris-HCl, pH 6.8, 2 mM EDTA), using a Potter-Elvehjem homogenizer with Teflon pestle (20 passes at 1000 rpm). The suspension was centrifuged at $10000 \times g$ (0 °C) for 30 min and the pellet resuspended in 250 μl distilled water. This procedure was repeated twice. To remove the lipids, the pooled supernatants were extracted with 3 ml i-octane. The aqueous layer was dialyzed in an Amicon cell equipped with a DIAFLO-YM5 membrane (excluding M_r 5000), against 50 mM Tris-HCl buffer. The low molecular fraction was freeze-dried and the residue taken up in 5 ml distilled water. This solution was desalted by passing it through a LiChroprep RP8-column (6×0.5 cm; Merck), equilibrated with distilled water. The column was rinsed with 5 ml water and the radioactive material eluted with 2.5 ml methanol. This fraction was evaporated to dryness and the residue dissolved in 2 ml ethyl acetate. An aliquot of this solution, containing about 84000 dpm, was subjected to thin-layer chromatography in chloroform–methanol (4:1, v/v) as solvent. The plate with the labelled compounds was scanned in a Thin-Layer-Scanner II (Berthold, Wildbad, F.R.G.). The high molecular fraction was concentrated to 2.6 ml by means of the Amicon cell and the radioactivity measured directly.

Autoradiography

Attempts to localize azadirachtin by conventional histological techniques [18], involving liquid fixation and dehydration in solvents, lead to almost complete loss of radioactivity from the tissues. Hence the technique which retains the *in situ* conditions and prevents diffusion namely, the dry-mount autoradiography [19–21], most widely adopted to localize diffusible compounds and steroid hormones, was followed.

Female locusts were injected on the third day after emergence a dose of 2.5 μg [22,23-$^3H_2$]dihydroazadirachtin/g (spec. activity 18.7 Ci/mM, unless otherwise specified). Malpighian tubules were dissected out on the fifth day after injection, mounted on specimen holders and quenched in liquified propane cooled to approximately −180 °C. Three to four micron sections were prepared in a cryostat (Frigocut 2700, Reichert Jung) at −35 °C and freeze-dried for 18 h at 10^{-5} Torr, using a cryosorption pump. In an atmosphere of low relative humidity (25–35%), the sections were separated on a piece of Teflon and firmly apposed to desiccated emulsion (Kodak NTB2) coated slides. The slides with mounted sections were stored in light proof exposure boxes, containing Drierite (Aldrich), at −18 °C for 15 to 25 days. Autoradiographs were developed in Kodak D19 and stained in methyl green pyronin. Tissue sections from locusts, injected with non-radioactive azadirachtin, were processed similarly to examine the possible chemographic artifacts.

Results

The more or less constant radioactivity which had been retained in living insects five days after injection of physiologically effective doses of [22,23-$^3H_2$]-dihydroazadirachtin (0.5 to 3.0 μg/g), suggests an almost constant retention of the labelled compound irrespective of the doses applied. At 1.5 to 3.0 μg/g doses the incorporation was in the range of 0.4 to 0.5 μg/g (Table I). Injection of lower doses (0.5 and

Table I. Retention of [22,23-$^3H_2$]dihydroazadirachtin in *L. migratoria*, 5 days after injection of 0.5–3.0 μg/g.

Injection [μg/g]	Retention [μg/g]
0.5	0.17 ± 0.015
1.0	0.15 ± 0.027
1.5	0.41 ± 0.063
2.0	0.43 ± 0.088
2.5	0.56 ± 0.045
3.0	0.49 ± 0.105

Values are means (± S.E.) of 5 determinations.

Table II. Distribution of [22,23-$^3H_2$]dihydroazadirachtin in the tissues of *L. migratoria* 5 days after injection of 2.5 µg/g.

	Dry wt. [mg]*	ng/mg dry wt.**
Malpighian tubules	1.6 ± 0.3	21.3 ± 4.2
Ovary	4.1 ± 0.5	1.9 ± 0.7
Gut	19.5 ± 1.3	2.5 ± 0.5
Flight muscles	32.3 ± 2.3	1.1 ± 0.3
Head	84.6 ± 2.3	0.6 ± 0.1
Rest of biomass	157.4 ± 12	1.5 ± 0.3

* $n = 5$; ** $n = 8$, ± S.D.

1.0 µg/g) of the labelled compound elicited a reduced retention of 0.15 µg/g. Besides the stored [22,23-$^3H_2$]dihydroazadirachtin in the whole insects, its distribution was measured in various tissues. Table II presents the salient feature that Malpighian tubules retained the great majority of the total amount. Although they constitute a very minor fraction on dry weight basis (0.0033%), 21.3 ng dihydroazadirachtin/mg, *i.e.*, 73.7% of the total radioactivity, was recovered from this organ. This amount was significantly higher than that of any other tissues, like ovary, gut, muscles, head or the rest of the body.

Is there any further elimination of [22,23-$^3H_2$]dihydroazadirachtin later than five days after injection? Dissected organs were oxidized 10 and 15 days later to recover the retained amount of dihydroazadirachtin. Fig. 1 shows that the amount retained on the fifth day after injection was levelled up during the next 10 days. No further loss could be observed in any of the tissues, namely, Malpighian tubules, ovary, gut, and the rest of the body.

Extracts from Malpighian tubules and fat bodies were purified to know the metabolic fate of [22,23-$^3H_2$]dihydroazadirachtin. The radiochromatoscan presented in Fig. 2 demonstrates that even 5 days after injection the main radioactive com-

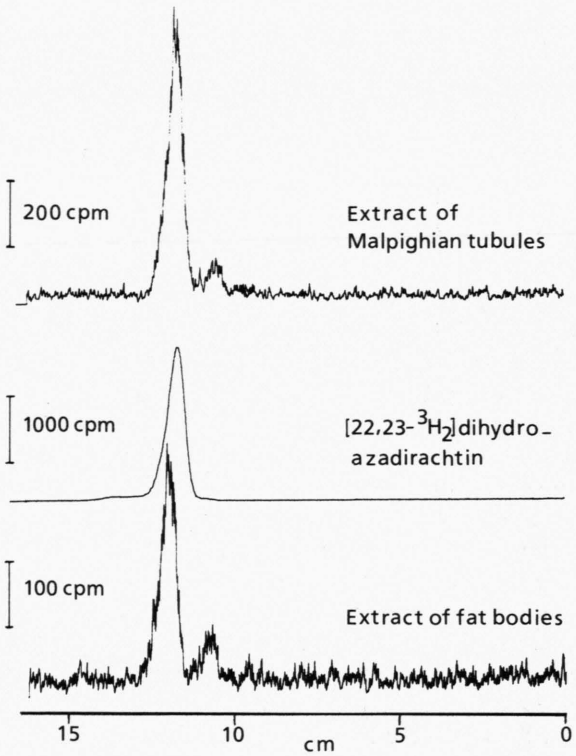

Fig. 2. Radiochromatoscan of the extracts of Malpighian tubules and fat bodies after separation on TLC plates (see Materials and Methods), showing the recovery of unchanged [22,23-$^3H_2$]dihydroazadirachtin.

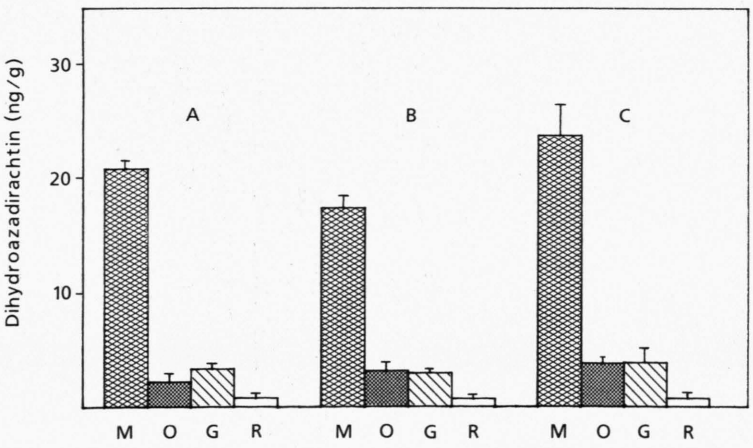

Fig. 1. Storage of [22,23-$^3H_2$]dihydroazadirachtin in whole *L. migratoria* females during 15 days after injection. [22,23-$^3H_2$]Dihydroazadirachtin was injected (2.5 µg/g, 52.15 µCi) on the third day after emergence into female *L. migratoria* and the samples were oxidized at 5-day (A = 5; B = 10; C = 15) intervals. The data represent the mean (± S.E.) of at least 4 samples. Malpighian tubules (M), ovary (O), gut (G) and the remaining body (R).

pound recovered from the tissue was the unchanged [22,23-$^3H_2$]dihydroazadirachtin(R_f 0.72). In addition, a minor labelled compound (R_f 0.65) was observed in extracts of both Malpighian tubules and fat bodies. Radioactivity in the high molecular fraction (M_r > 5000) was undetectable.

Autoradiographs of Malpighian tubules from insects that received a high specific activity of [22,23-$^3H_2$]dihydroazadirachtin (18.7 Ci/mmol) showed intense silver grains all along the basal region of the tubule, and the cytoplasm around the nucleus (Fig. 3a, b). Under low magnification (Fig. 3a), the intensely labelled structures form a radioactive trace along the tubule. The silver grain development extends to a width of approximately 8.5 μ into the tubule structure that constitutes the basal region. For further resolution, 3 μm sections were processed for autoradiography from locusts which had been injected a low specific activity (0.187 Ci/mmol). It can be seen that the apical region (microvilli), which is limiting the lumen of the tubule, is relatively unlabelled (Fig. 3c). The nucleus remained unlabelled, as in Fig. 3a, b, but the labelling of the nuclear membrane is clearly visible.

Discussion

The striking feature of dihydroazadirachtin incorporation is its storage in the whole body in an almost constant quantity, irrespective of the injected doses, which ranged between 1.5 to 3.0 μg/g. The compound elicits identical reduction in food intake, leading to reduced weight gain, in this dose range [11]. However, sub-effective doses (0.5 to 1.0 μg/g) do not influence feeding behavior. Similarly, the quantity of dihydroazadirachtin which is stored in the body is not dose-dependent but only differs between its effective (> 1 μg/g) and its sub-effective (< 1 μg/g) dose. Of the total quantity recovered from the insect, Malpighian tubules store the highest amount which is sig-

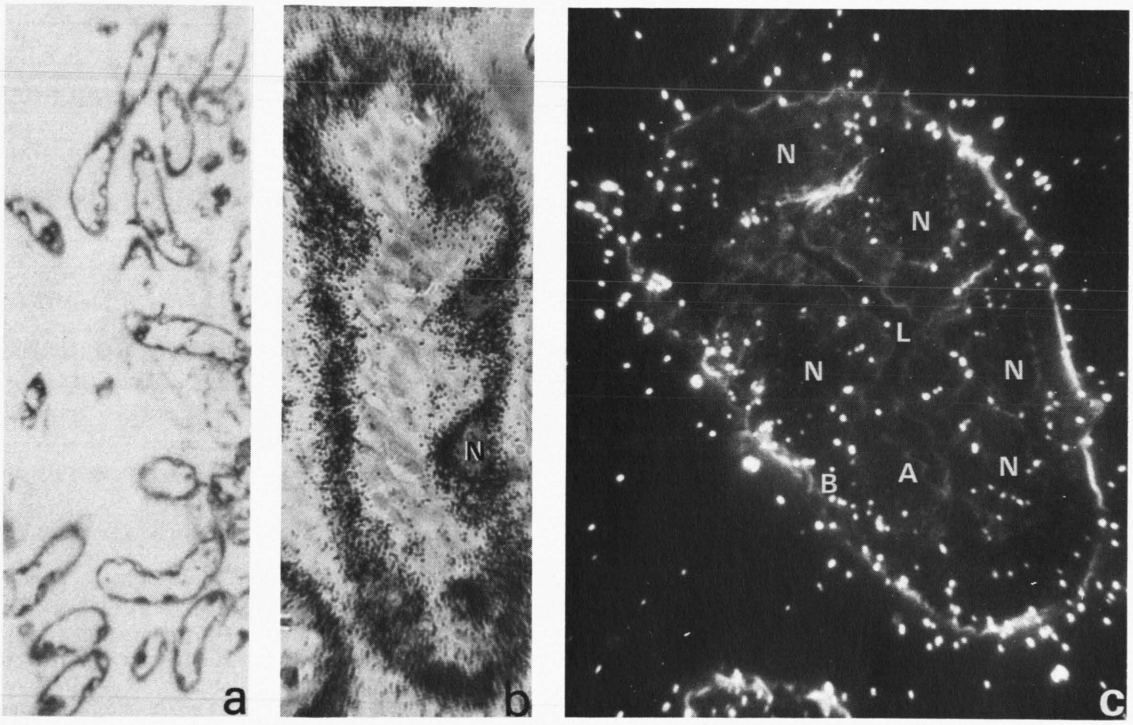

Fig. 3. Autoradiographs showing the localization of [22,23-$^3H_2$]dihydroazadirachtin in the Malpighian tubules of female *L. migratoria*. An intense labelling of the basal region and the cytoplasm around the nucleus is visible in a and b. Fig. 3c shows tangential section of Malpighian tubule from a locust injected with low specific activity (0.187 Ci/mM), with labelling of the basal region and also the nuclear membrane. Note that the apical region (microvilli), limiting the lumen of the tubule, is unlabelled. Magnification: a = ×90, b = ×710, c = ×1490. A = apical region, B = basal region, L = lumen, N = nucleus.

nificantly more than that in any other tissue. Malpighian tubules play an important role in excretion and clearance of drugs, toxins and other foreign compounds, and also maintain the hemolymph chemical composition [22]. No further loss of dihydroazadirachtin, later than 5 days after injection, from any of the tissues studied, and its recovery as unchanged molecule demonstrates that dihydroazadirachtin acts in its unchanged form and that its metabolic degradation is an insignificant process. Catabolites of dihydroazadirachtin could be detected in the feces, though it is excreted as unchanged molecule in the first 24 h [6]. The site of its metabolism is not yet known but our data suggest that at least fat bodies and Malpighian tubules are not the organs that degrade dihydroazadirachtin to a significant extent. Recovery of only a minor fraction of this compound from the gut, and detection of degradation products in the feces denote that it is probably degraded in the intestine. The influence on gut motility and passage of food [13] seems to be a temporary effect because of minute recovery of this molecule (2.5 ng/mg) 5 days after injection.

Our autoradiographic study of the Malpighian tubules provides evidence for the accumulation of dihydroazadirachtin A in the basal region and the cytoplasm around the nucleus. The Malpighian tubules of *L. migratoria* have a uniform histological structure over the length of the tubule, and it is similar to that of many other insect species [22–25]. At the ultra-structural level, the basal and apical regions reveal extensive infoldings of the surface membrane together with many mitochondria, whereas the inner region constitutes the cytoplasmic inclusions and the nucleus [26]. The basal region where dihydroazadirachtin is stored, includes the basal cell membrane and its infoldings into the cell. During the process of fluid secretion this compound would pass through the tubule and enter the lumen for excretion. However, recovery of most of the unexcreted dihydroazadirachtin, chemically unchanged, and its localization in the basal and inner regions of the tubulus cells, suggest a specific concentration at high-affinity binding sites.

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