14-Deoxyecdysteroids in an Insect (Gryllus bimaculatus)

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Following injection or ingestion of [\(^{3}H\)]ecdysone and [\(^{3}H\)]20-hydroxyecdysone, respectively, considerable quantities of 14-deoxyecdysteroids could be extracted from gut and faeces of female adult crickets. The compounds were identified as 14-deoxyecdysone and 14-deoxy-20-hydroxyecdysone on the basis of comigration with reference substances in two HPLC systems and by its mass spectrum (only 14-deoxy-20-hydroxyecdysone). The 14-dehydroxylation is most probably caused by bacteria from the gut lumen. Feeding of [\(^{3}H\)]ecdysone resulted in the appearance of labelled 14-deoxyecdysone also in ovaries, fat body, muscles, and rest of the body.

Introduction

Ecdysteroids are common amongst invertebrates and plants. In insects it is well established that they function as hormones controlling development and reproduction [1]. Since efficient analytical techniques have become available (HPLC, mass spectrometry, nuclear magnetic resonance spectrometry, radioimmunoassay [2, 3]), more than 60 ecdysteroids have been detected in insects as endogenous compounds, or have been isolated from metabolic studies in vivo or in vitro [4]. The metabolites are essentially issued from the modification of the five characteristic hydroxyl groups of the ecdysone molecule (three secondary alcoholic functions at C2, C3 and C22, and two less reactive tertiary alcoholic functions at C14 and C25) and from hydroxylation at C20 or C26 [5].

Several compounds lacking one or more hydroxyl groups (deoxyecdysteroids) have been isolated as potential precursors of ecdysone and 20-hydroxyecdysone, respectively. The very early steps in ecdysone biosynthesis, supposed to lead to 2,22,25-trideoxyecdysone (5β-ketodiol), are not well understood, but in several cases the final steps of hydroxylation are known [4]. There are strong indications that hydroxylation at C25 preceeds that at C22 and that introduction of the C2-hydroxyl group could be the last step in ecdysone biosynthesis. Results from recent experiments on prothoracic glands and follicle cells of Locusta migratoria support the operation of this route via 2-deoxyecdysteroids. The C14-hydroxylation occurs at a very early step of ecdysone synthesis, presumably before introduction of the 6-oxo group and/or the A/B cis ring junction [4, 6]. 14-Deoxyecdysone could not be found as an intermediate in ecdysone biosynthesis. The C25-deoxyecdysteroids ponasterone A (25-deoxy-20-hydroxyecdysone) and inokosterone A (25-deoxy-20,26-dihydroxyecdysone) have been detected only in Crustacea as yet.

Radiotracer experiments were frequently used as an experimental approach to identify ecdysone metabolites in insects. Dehydroxylation, however, has never been observed [7]. In contrast, ecdysone metabolism in mice essentially proceeds through 14-dehydroxylation [8, 9]. Following injection (i.p.) of labelled ecdysone, 55% of ecdysteroids were metabolized and 14-deoxyecdysone was identified as a major metabolite. Since most of the radiolabelled metabolite was found in intestinal and faecal extracts, it could well be formed by the intestinal flora.

In the present study we describe the occurrence of radiolabelled 14-deoxyecdysteroids in an insect, the Mediterranean field cricket (Gryllus bimacula-
Following injection or ingestion of [3H]ecdysone and [3H]20-hydroxyecdysone, respectively, and employing the techniques normally used in identification of ecdysone metabolites.

Materials and Methods

Animals and hormone application

Female adult crickets were reared as described previously [10]. [23,24-3H2(N)]ecdysone, spec. activity 60 to 80 Ci/mmol (2.27 to 3.02 TBq/mmol) was purchased from New England Nuclear, Dreieich. The radiochemical purity was >98% by thin-layer chromatography. [3H]20-hydroxyecdysone was prepared from labelled ecdysone by in vitro incubation with Gryllus bimaculatus midgut microsomes (W. Liebrich, unpublished). Reference 14-deoxyecdysone and 14-deoxy-20-hydroxyecdysone were generously provided by Dr. U. Kerb (Berlin) and Dr. B. Danieli (Milan), respectively. The latter compound was a mixture of 14α- and 14β-isomers from chemical synthesis [11] which were separated by HPLC and characterized by spectrometric procedures. Before injection, animals were immobilized by a short treatment with CO2. Females of six days old (five animals per experimental group) each received an injection of 1.85·104 Bq [3H]ecdysone or [3H]20-hydroxyecdysone, dissolved in 5 μl cricket saline [10]. They were sacrificed 0.75, 1.5, 3, 6, and 24 h after injection of hormone. For administration per os, labelled ecdysteroids (1.85·104 Bq per animal) were deposited on the cricket diet in methanol and the solvent was evaporated under a stream of nitrogen. The diet was presented to starving, six days old females which were maintained in individual plastic boxes. They were sacrificed 1.5, 3, 6, and 24 h after ingestion of hormone. The faeces were collected over a 24 h period following hormone application.

Sample processing

The radiolabelled metabolites were extracted from tissues (ovary, fat body, muscles, gut, haemolymph and rest of the body) and faeces using a SEP-PAK purification procedure as described recently [10]. The ecdysteroid fractions were eluted from the C18 SEP-PAK cartridges (Waters) as follows: 5 ml 25% (v/v) methanol in water-polar ecdysteroid conjugates 5 ml 60% (v/v) methanol in water-free ecdysteroids 5 ml 100% methanol-apolar ecdysteroid conjugates.

Chromatographic analyses

Both normal phase (NP) and reversed phase (RP) high performance liquid chromatography (HPLC) were used. RP analyses used a Nova-Pak C18 column (150 mm long, 3.9 mm i.d.; Waters) eluted with a linear gradient (8–30% in 60 min) of acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) (solvent system 1). NP analyses used a Zorbax-SIL column (250 mm long, 4.6 mm i.d.; DuPont) eluted with either dichlormethane:isopropanol:water, 125:35:2 (v/v/v) (solvent system 2) or dichlormethane:isopropanol:water, 125:20:1.5 (v/v/v) (solvent system 3). The flow rates were 1.0 ml per min. Radioactivity was determined either by a Berthold 506 D HPLC radioactivity monitor or by liquid scintillation counting of the HPLC fractions (0.4 ml) using Rotiszint 2211 (Roth) and a Packard TriCarb 460 C LSC system.

Mass spectrometry

Mass spectrum was obtained using the chemical ionization/desorption mode (CI/D) (Riber 10-10 B apparatus; Nermag S.A.) with ammonia as a reagent gas [2].

Results

Production of 14-deoxyecdysone following [3H]ecdysone injection

Six hours after injection of labelled ecdysone the radioactive metabolites were extracted from gut tissue and separated as described in Methods. Besides polar (P1 to P5 in Fig. 1a) and apolar ecdysteroid conjugates (not shown in the figure; [3, 10]) up to six peaks of radioactivity were detected in the 60% methanol fraction of the free ecdysteroids when analyzed by RP-HPLC (Fig. 1a). These substances were denoted as compounds 1 to 6 in order of the increasing elution time. Compounds 1 and 4 cochromatographed with 20-hydroxyecdysone and ecdysone, respectively. Similarly, compound 2 cochromatographed with 26-hydroxy-
14-deoxyecdysone located in gut and faeces at 0.75 to 24 h after [3H]ecdysone injection (2.0 to 8.0% of total recovered radioactivity).

Six hours after injection of [3H]14-deoxyecdysone into an adult female cricket, the faeces of the animal contained a labelled ecdysteroid with a slightly shorter retention time than 20-hydroxyecdysone in RP-HPLC (solvent system 1), but which has not yet been identified. Another peak comigrated with 14-deoxy-20-hydroxyecdysone (see below).

Production of 14-deoxyecdysone following [3H]ecdysone ingestion

Following feeding of [3H]ecdysone, considerable amounts of 14-deoxyecdysone were found in gut and faeces already after 1.5 h (Table I). Within faeces, 14-deoxyecdysone contributed up to 70% of total recovered radioactivity (6–24 h after ingestion). In contrast to the injection experiments, feeding of [3H]ecdysone resulted in the appearance of labelled 14-deoxyecdysone also in other tissues than the gut. 1.5 h after feeding 14-deoxyecdysone could be detected in ovaries (6% of total recovered radioactivity), fat body (12%), and muscles (5%), and with a delay of 90 min also in the “rest body” (12%).

Production of 14-deoxy-20-hydroxyecdysone following [3H]20-hydroxyecdysone injection and ingestion

After injection or ingestion of labelled 20-hydroxyecdysone, a peak of radioactivity was detected in the SEP-PAK 60% methanol fraction from faeces extracts which comigrated with authentic 14-deoxy-20-hydroxyecdysone in both a

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<th>Time after ingestion [h]</th>
<th>Gut E</th>
<th>14dE</th>
<th>Faeces E</th>
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<tr>
<td>1.5</td>
<td>72</td>
<td>8</td>
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Fig. 2. a. RP-HPLC analysis of free ecdysteroids (SEP-PAK 60% methanol fraction) from faecal extracts. Faeces were collected during a 24 h period following ingestion of [3H]20-hydroxyecdysone. Solvent system 1 was used. Arrows indicate retention times for reference substances 20-hydroxyecdysone (20-E), 14-deoxy-20-hydroxyecdysone (14-d20E), and ecdysone (E). b. Rechromatography of free ecdysteroids from Fig. 2 a on NP-HPLC. Solvent system 2 was used. Arrows indicate retention times for reference substances 14-deoxy-20-hydroxyecdysone (14-d20E), ecdysone (E), and 20-hydroxyecdysone (20-E).

Fig. 3. Mass spectrum (CI/D) of 14-deoxy-20-hydroxyecdysone. RP-HPLC (Fig. 2a) and a NP-HPLC (Fig. 2b) system. Further evidence for its identity has been obtained from its Cl/D mass spectrum (Fig. 3). Cl/D mass spectrometry gave prominent ions at \( m/z \) 465 (\( M + H^+ \)), and at 447, 429 and 411, representing the loss of three successive water molecules, then at 347 (nuclear fragment resulting from C20–C22 cleavage), which indicate a molecular mass of 464 and the loss of one OH-group on the steroid nucleus when compared with 20-hydroxyecdysone, which gives the corresponding fragment at \( m/z \) 363.

14-Deoxy-20-hydroxyecdysone could be detected in gut and faeces, but not before 3 h after injection of 20-hydroxyecdysone. The amount of radiolabelled 14-deoxy-20-hydroxyecdysone was low (≥4% of total recovered radioactivity). No other tissues contained measurable amounts of the metabolite. Significantly higher amounts of the steroid were found in the faeces 24 h after feeding of the animals with 20-hydroxyecdysone (48% of total recovered radioactivity).

Discussion

In the Mediterranean field cricket, *Gryllus bimaculatus*, ecdysteroids have been found in several tissues of both female and male adults [10, 12, 13]. In females, endogenous ecdysteroid concentrations correlated with changes in ovary fresh weight and oviposition rate. Subsequent studies on the fate of injected [3H]ecdysone revealed several classes of apolar metabolites (ecdysone fatty acid es-
Ecdysone metabolism through 14-dehydroxylation has recently been demonstrated for mammals [9], but has not yet been proven to exist in arthropods [5]. In mice, ecdysone catabolism seems to proceed through the three sequential reactions 1) 14-dehydroxylation, 2) complete reduction of the 7-ene-6-one, and 3) epimerization at C3 [8]. Dehydroxylation of steroids is well known in bacteria metabolism [16]. The occurrence of 14-deoxyecdysone in intestine and faeces of mice supports the idea that 14-dehydroxylation of ecdysone in mammals will be caused by bacteria which live in the gut lumen.

High amounts of 14-deoxyecdysone and 14-deoxy-20-hydroxyecdysone in gut and faeces of crickets, especially after feeding of the animals with ecdysteroids, suggest that also in crickets the removal of the hydroxyl group at C14 may be caused by the intestinal flora. The mechanism for the 14-dehydroxylation is not known.

Björkhem and coworkers [17] have recently demonstrated a mechanism for 7α-dehydroxylation of cholic acid in the human intestinal Eubacterium sp. VPI 12708 that involves a 3-oxo-Δ4-intermediate. In an earlier in vivo study in the rat, Samuelsson [18] showed that the mechanism for 7α-dehydroxylation of cholic acid also involves a diaxial trans elimination of water followed by a trans hydrogenation in the 6α- and 7β-positions. Corresponding to these results, the mechanism for 14-dehydroxylation in the cricket intestinal tract might proceed through a 14(15)-ene compound. Low quantities of 14-deoxyecdysteroids that were found outside the intestinal tract (e.g., in the fat body and muscles after feeding of [3H]ecdysone) may be either of gut origin or synthesized at a yet unknown dehydroxylation site.

Generally, the presence of a free 14-hydroxyl group in ecdysteroids is essential for high activity in vivo as well as in vitro [11]. Certain phytophagous insects, but also the omnivorous, cannibalistic crickets can ingest huge quantities of ecdysteroids and, therefore, 14-dehydroxylation may represent an inactivation mechanism in these arthropods. 14-Dehydroxylation is not reversible, thus 14-deoxy-compounds cannot be converted back to 14-OH-compounds. However, Cherbas and co-workers [19] have found that 14-deoxy-muristerone A is unexpectedly 70–80 times as active as 20-hydroxyecdysone in the Kc cell system derived from Drosophila melanogaster. Nothing is known about the activity of 14-deoxycompounds in Gryllus.

Insects contain more than a million different species, and any generalization from one example would be highly questionable. Expansion of the present experiments to other species have to show, whether 14-dehydroxylation is a more common mechanism in the insect intestinal tract. Work is also in progress to define the detailed mechanism of 14-dehydroxylation in insects.

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